UNIVERSITAT DE BARCELONA

Infección aguda/reciente por el VIH-1. Características clínicas, virológicas e inmunológicas y efectos del tratamiento inmunomediado

Omar Gustavo Sued

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Tesis Doctoral Presentada por

Omar Gustavo Sued

Para acceder al Grado de Doctor en Medicina

Enero de 2016

El doctor **José María Miró Meda**, Profesor Titular del Departamento de Medicina de la Universidad de Barcelona

CERTIFICA:

Que la presente Tesis Doctoral **"Infección aguda/reciente por el VIH-1. Características clínicas, virológicas e inmunológicas y efectos del tratamiento inmunomediado"** ha sido realizada por Omar Gustavo Sued bajo su dirección en el Departamento de Medicina de la Facultad de Medicina de la Universidad de Barcelona. El trabajo reúne las condiciones necesarias para optar al título de DOCTOR EN MEDICINA en el marco del programa de Doctorado de la Facultad de Medicina de la Universidad de Barcelona.

Barcelona, 11 de Enero de 2016.

El doctor **José María Miró Meda**, Profesor Titular del Departamento de Medicina de la Universidad de Barcelona

CERTIFICA:

Que la presente Tesis Doctoral **"Características clínicas, virológicas e inmunológicas y efectos del tratamiento inmunomediado en la infección aguda/reciente por el VIH-1"** presentada por el doctorando Omar Gustavo Sued se ha realizado siguiendo la normativa del Consejo del Departamento de Medicina aprobado el 9-5-06.

De acuerdo a dicha normativa, la presente Tesis Doctoral se ha realizado en la modalidad de presentación como compendio de publicaciones, que consiste en la agrupación de trabajos científicos originales de una misma línea de investigación. Los artículos se han publicado en revistas indexadas y en al menos uno el doctorando figura como primer autor.
El doctorando ha presentado 6 artículos, de los cuales solo los artículos 4, 5 y 6 son elegibles para estos criterios.

Barcelona, 11 de Enero de 2016

A Norma, mi guía y mi sostén.

A Santiago y Nacho, por la ilusión y la alegría.

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INDICE

ABREVIATURAS

ADN	Ácido desoxirribonucleico
ADCC	Citotoxicidad celular dependiente de anticuerpos (del inglés *antibody dependent cellular citotoxicity*)
ARN	Ácido Ribonucleico
ARNm:	ARN mensajero
ARNsc+:	ARN simple cadena con polaridad positiva
BLyS	Estimulador del linfocito B (del inglés *B lymphocyte stimulator*)
CD	Célula dendrítica
CMH	Complejo Mayor de Histocompatibilidad
CTL	Linfocitos citotóxicos (del inglés *cytotoxic T lymphocyte*)
CTLA-4	Antígeno 4 del linfocito T citotóxico (del inglés *Cytotoxic T-Lymphocyte Antigen 4*)
CV	Carga viral
CCR5	C-C quimiocina receptora de tipo 5 (del inglés *Chemokine C-C motif receptor 5*)
CDC	*Center for Disease Control and Prevention*
CXCR4	C-X-X quimiocina receptora de tipo 4 (del inglés *C-X-C chemokine receptor type 4*)
EC	Pacientes controladores elite (del inglés *elite controllers*)
Env	Envoltura
EEUU	Estados Unidos
GALT	Tejido linfático intestinal (del inglés *gut-lymphoid associated tissue)*
Gal-9	Galectina 9
gp41	Glicoproteína 41
gp120	Glicoproteína 120
gp160	Glicoproteína 160
HLA	Antígeno leucocitario humano

HDACi	Inhibidores de la histona desacetilasa
HR	Cociente de riesgo (del inglés *hazard ratio*)
HSH	Hombres que tienen sexo con hombres
IL	Interleucina
IFN- α	Interferón alfa
IFN- γ	Interferón gama
IN	Integrasa
INI	Inhibidores de la integrasa
INTR	Inhibidores nucléosidos de la transcriptasa reversa
INNTR	Inhibidores no-nucléosidos de la transcriptasa reversa
IP	Inhibidores de la proteasa
LCR	Líquido cefalorraquídeo
LPS	Lipopolisacárido
LTR	Repeticiones terminales largas (del inglés *Long Terminal Repeats*)
MIP-1β	Proteína inflamatoria de macrófagos 1β (del inglés *Macrophage Inflammatory Protein*)
NK	Células asesinas naturales (del inglés *Natural Killer*)
RT	Transcriptasa Reversa
SDF-1	Factor estromal 1 derivado de células
TARV	Tratamiento antirretroviral
TNF-α	Factor de necrosis tumoral alfa
TGF-b1	Factor de crecimiento transformante beta
Tregs	Células T regulatorias naturales
VEB	Virus Epstein-Barr
VIH	Virus de la inmunodeficiencia humana

ANTECEDENTES DEL TEMA: INFECCIÓN POR VIH

SIDA: LOS INICIOS DE LA EPIDEMIA

El síndrome de inmunodeficiencia adquirida (SIDA) representa la última etapa clínica de la infección por el virus de la inmunodeficiencia humana (VIH). Fue descrito por primera vez en Estados Unidos en 1981 a partir de la notificación de una agrupación de casos de neumonía por *Pneumocystis jiroveci* y un aumento inesperado de casos de Sarcoma de Kaposi en hombres que tenían sexo con otros hombres (HSH) [1,2].

El aumento exponencial de los casos desencadenó la búsqueda frenética de la etiología. En 1983, el equipo de los Drs. Françoise Barré-Sinoussi y Jean-Claude Chermann del Laboratorio de Retrovirus del Instituto Pasteur de Paris, dirigido por el Dr. Luc Montagnier, describió un nuevo retrovirus al que denominaron Virus Asociado a Linfadenopatías [3]. A este importante descubrimiento, pocos meses después se sumaron descripciones similares de los Dres. Robert Gallo y Jay Levy quienes denominaron a los virus aislados como Virus Linfotrópico Humano Tipo III [4] y Virus Relacionado al SIDA [5], respectivamente.

Después de una larga disputa sobre la propiedad intelectual del descubrimiento, que tenía importantes implicancias económicas y que alcanzó proporciones de escándalo diplomático internacional entre Francia y los Estados Unidos, en 1990 se reconoció como descubridores del VIH al equipo de investigadores franceses, hallazgo por el que los distinguieron en el año 2008 con el premio Nobel de Medicina.

El origen de la pandemia por VIH se remonta a inicios del siglo XX. Mediante estudios estadísticos de probabilidad de mutaciones en la secuencia viral de las primeras muestras identificadas se pudo localizar retrospectivamente el lugar de origen del

primer paso del virus desde los monos a los humanos, localizándolo en Kinshasa (actualmente la República Democrática del Congo) y datándolo aproximadamente hacia el año 1920 [6]. En esa investigación se sugiere que los importantes cambios socio-demográficos que se experimentaron en la región durante esa época pudieron haber facilitado la pandemia, entre los que se incluyen la expansión de las intervenciones médicas sin acceso a material descartable, el importante crecimiento poblacional, la apertura de nuevas rutas de comercio con las migraciones masivas asociadas y el aumento del trabajo sexual, que en conjunto facilitaron la diseminación al resto del África Subsahariana. A partir de los ´80, la generalización de los viajes intercontinentales, el turismo sexual y los cambios en los patrones de uso de drogas facilitaron la expansión global de la epidemia [6].

EPIDEMIOLOGÍA

Desde su descubrimiento se estima que el virus ha infectado a más de 75 millones de personas y causado más de 40 millones de muertes. A fines del año 2014 se estimaba que había 36,9 millones de personas con infección por VIH, con 2 millones de nuevos casos anuales y 1,2 millones de defunciones asociadas [7]. Un esfuerzo internacional sin precedentes en la historia mundial ha logrado que actualmente más de 15 millones de personas reciban tratamiento. Este logro, junto con las otras medidas de prevención ha permitido una reducción del 35% en las nuevas infecciones y salvar 19 millones de años de vida entre 1990 y 2013 [8].

La prevalencia mundial de infección por VIH se sitúa en 0,8% del total de la población, pero la cifra varía significativamente, desde valores extremadamente bajos a cifras de dos dígitos en algunos países de África Subsahariana. En 2014 en esta región se diagnosticaron 1,4 millones de personas con VIH, totalizando aproximadamente 26

millones de personas viviendo con VIH (lo que representa el 70% de la cifra global). Esta región es la más afectada pero también uno de los lugares donde mejor se logró acelerar el avance de los programas de tratamiento. La expansión de estos programas ha sido continuo y crecieron en forma exponencial desde el año 2000, aumentando la cobertura de las personas con VIH recibe el tratamiento desde menos del 1% al 40%, con un descenso en la cifra de nuevas infecciones del 41% con respecto al año 2000 [7]. Otro de los logros importantes es el control de la transmisión materno infantil, con una reducción del 58% de los nuevos casos de niños con VIH por transmisión materno infantil entre 2002 y 2012, aunque todavía se notifican 240.000 casos cada año [9]. En 2013 la OMS recomendó ofrecer TARV a todas las mujeres embarazadas, independientemente de la cifra de células CD4 y mantenerlo después del parto, como un esfuerzo para mejorar la cobertura de mujeres en tratamiento y reducir la transmisión [10]. En 2014 ONUSIDA propuso un plan aún más ambicioso en el cual se propone expandir la oferta de diagnóstico, promover el tratamiento universal y mejorar la retención de los pacientes para lograr que en el año 2020 el 90% de las personas con VIH se encuentren diagnosticadas, el 90% de estas estén recibiendo tratamiento y que el 90% logren tener una carga viral indetectable. Si se logran estos objetivos se podría reducir drásticamente la transmisión del VIH para el año 2030 [11].

En América Latina, a finales de 2013, se estimaban en 1,6 millones las personas viviendo con VIH. Esta cifra incluye a las 94,000 personas que se infectaron con VIH durante ese año [9]. En el continente la prevalencia de infección varía mucho de una subregión a otra, es más elevada en Centroamérica y el Caribe, donde la vía heterosexual es el principal mecanismo de trasmisión. En el Cono Sur la epidemia se asocia generalmente a comportamientos de alto riesgo y la prevalencia es mayor en hombres que tienen sexo con hombres (HSH), mujeres transgénero (personas que se

les asignó el sexo masculino al nacer pero que no se auto-identifican con ese género), trabajadoras y trabajadores sexuales y en usuarios de drogas inyectables (UDI) aunque la proporción de nuevas infecciones por vía heterosexual está aumentando con el consiguiente incremento de la prevalencia en mujeres. Al igual que en otras regiones, la proporción de personas con riesgo de exposición o con antecedentes de una infección de transmisión sexual que conoce su estatus serológico es baja. Estas personas se encuentran en alto riesgo de contraer el VIH y requieren programas específicos de prevención y cuidado que no siempre están disponibles [12].

Las mujeres también sufren un mayor impacto del VIH a nivel mundial, en particular las adolescentes. En África, las mujeres entre 15 y 24 años muestran una prevalencia de VIH del doble en comparación con los hombres de la misma edad [9]. Entre las múltiples causas que explican esta desproporción se incluyen las inequidades sociales y económicas, exacerbadas por la violencia política, el racismo, la pobreza y el machismo [13]. Las mujeres sufren en forma cotidiana situaciones que exacerban el riesgo de adquirir la infección y que incluyen la desigualdad de género, el sexo transaccional, las violaciones, la violencia de género, el sexo intergeneracional, la incapacidad para negociar el uso de preservativos, las barreras para acceder a los cuidados de salud sexual y reproductiva y el bajo acceso a la educación. Estos factores deben tenerse en cuenta, tanto a nivel individual, como comunitario. También es importante considerar la vulnerabilidad de las mujeres VIH negativas embarazadas que podrían estar en mayor riesgo de adquirir la infección durante, ya que la seroconversión al VIH durante el embarazo incrementa el riesgo de transmisión materno-infantil entre 5 y 15 veces en comparación con las mujeres que se embarazan estando infectadas previamente [14,15].

EL VIRUS DE LA INMUNODEFICIENCIA HUMANA

El VIH es un retrovirus perteneciente al género *Lentivirus* (virus con periodos de incubación largo), que se encuentra dentro de la familia *Retroviridae*, en la subfamilia *Orthoretrovirinae.* Esta familia incluye virus ARN con envoltura que se replican en la célula huésped mediante el proceso de retro-transcripción. El virión es una partícula esférica de aproximadamente 100 nm de diámetro formada por una envoltura lipídica externa proveniente de la membrana de la célula huésped en la que se encuentran dos glicoproteínas (gp41 de transmembrana y gp120 de superficie externa) que son imprescindibles para la entrada del virus a la célula huésped [16] (Figura 1).

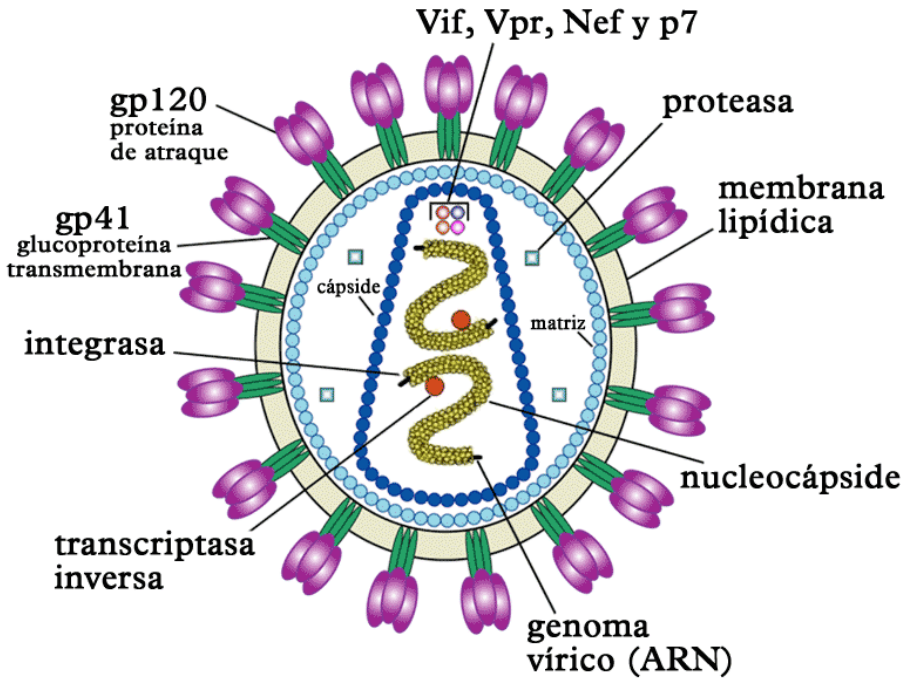


Figura 1: Estructura del VIH (Imagen de dominio público, NIAID/NIH)

Por dentro de la envoltura lipídica se encuentra la matriz formada por proteína MA (p17) y en el centro la cápside, una estructura cónica formada por 1500 monómeros de

proteína viral CA (p24) que se ensambla en hexámeros y pentámeros para darle la forma cónica y que contiene en su interior el genoma viral y las proteínas estructurales (p7 y p9) y no estructurales (integrasa, proteasa y transcriptasa reversa) [17].

El genoma del VIH-1 consta de dos moléculas casi idénticas de ácido ribonucleico de cadena simple con polaridad positiva (ARNsc+), cada una de 9,2 kilobases que codifican 3 genes que son comunes a todos los retrovirus (gag, pol y env) junto a otros 6 genes que codifican para proteínas regulatorias (tat y rev) y accesorias (nef, vif, vpr y vpu). La región gag codifica las proteínas estructurales internas del virión a través de dos precursores, Pr55Gag y Pr160Gag-Pol. Los mismos son procesados post-traducción mediante clivaje proteolítico produciendo las proteínas maduras MA, CA, NC, p1, p2 y p6 [18]. La región pol codifica las enzimas RT (que tiene las actividades ADN-polimerasa-ARN dependiente, ADN-polimerasa-ADN dependiente y ARNasa H), la IN y la PR. La región env codifica las glicoproteínas TM y SU, las cuales forman el complejo que interacciona específicamente con el receptor celular [19]. Una vez integrado al genoma celular, el ADN proviral se encuentra flanqueado por repeticiones terminales largas (LTR, del Inglés *Long Terminal Repeats*) generadas durante el proceso de transcripción reversa [20]. Los LTR están compuestos por las regiones U3, R y U5 y son los responsables de regular, al menos en parte, la expresión de los genes virales [21]. (Figura 2)

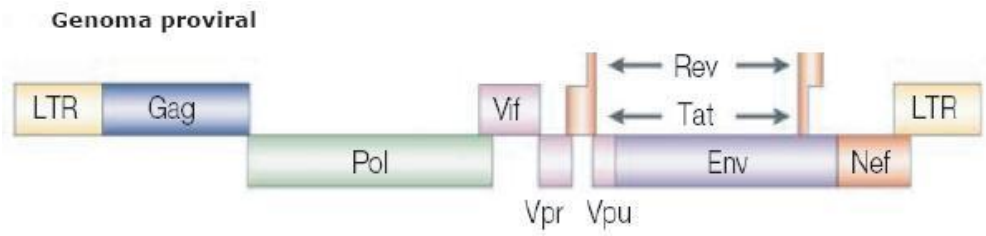


Figura 2: Genoma proviral del VIH (Modificado de Schwartz et al. [20]).

Se conocen dos tipos virales, el VIH-1, de distribución universal, y el VIH-2, aislado en África Occidental en 1985 donde es endémico. El VIH-1 tiene un alto grado de diversidad y variabilidad y se reconocen hasta el momento 4 grupos (M, N, O y P). El grupo M es el responsable de la pandemia global y se subdivide a su vez en 11 subtipos (del A al K) que se encuentran distribuidos en el mundo y que a su vez se pueden combinar entre sí para crear otras variantes virales. En América del Sur el subtipo B predomina en la mayoría de los países, salvo en Brasil, Uruguay y Argentina donde hay una proporción muy importante de formas recombinantes B-F y en menor medida de cepas subtipo F [22].

El virus también puede clasificarse de acuerdo al tipo de co-receptor que utiliza al unir la glicoproteína gp120 con el receptor CD4, ya sea el receptor C-C tipo 5 (CCR5) o el receptor C-X-C tipo 4 (CXCR4). Los virus R5 y los virus X4 se unen respectivamente al receptor C-C tipo 5 y al receptor C-X-C tipo 4, y algunas cepas tienen tropismo dual. Los receptores CCR5 se expresan en varias células como linfocitos T, células dendríticas y macrófagos y los virus R5 predominan durante las fases tempranas de infección. En los estadios avanzados emergen los virus X4 para lo cual se requiere un escaso número de mutaciones en la región altamente variable del dominio V3 [23,24].

TRANSMISION Y ESTABLECIMIENTO DE LA INFECCIÓN

La principal célula diana del VIH-1 en la que el virus puede reproducirse eficientemente está constituida por los linfocitos T CD4+. La secuencia de eventos que se producen desde la exposición de la mucosa al virus y la infección establecida con infección productiva en estos linfocitos implica una compleja serie de interacciones entre el virus y las células de la mucosa que todavía no se conoce completamente, pero se reconocen diferencias importantes en las diferentes mucosas en cuanto a la

susceptibilidad y la resistencia a la infección. Debido a que es muy difícil identificar y hacer estudios *in vivo* en personas recién infectadas, la mayor parte de la información sobre los eventos iniciales durante la transmisión sexual del VIH-1 proviene de la extrapolación de hallazgos de estudios *in vitro*, de estudios en explantes tisulares humanos y de estudios en modelos animales, en particular utilizando el modelo del macaco *rhesus* con infección por el Virus de la Inmunodeficiencia del Simio (VIS) [25–28]. Para poder iniciar una replicación sostenida, el VIH debe atravesar primero la barrera anatómica de la mucosa, que tiene características diferentes en la mucosa vaginal, endocervical, rectal, oral o del pene, evadir la inhibición del sistema de defensa innata y las respuestas adaptativas, y finalmente hacer contacto en forma masiva con las células que infectará para llevar a cabo la replicación [29]. Las erosiones, úlceras, cambios hormonales o embarazo pueden alterar la integridad de estas mucosas y facilitar la infección. La primera parte de este proceso, que implica infectar linfocitos CD4 intra-epiteliales, células de Langerhans o células dendríticas submucosas localizadas en la lámina propia puede tomar algunas horas (Figura 3). El tiempo que se demora en lograr una infección sostenida y la magnitud del pico de viremia dependen de la vía de infección y del inóculo. Es más rápido y con un pico más alto en infecciones por vía endovenosa, seguidas de la vía rectal y vaginal. En la mucosa rectal el epitelio columnar y la mayor presencia de tejido linfoide facilita la infección en comparación con la vagina. Cuanto mayor es el tamaño del inóculo mayor es la velocidad y el pico de viremia [30].

Las barreras mucosas también limitan el acceso a las diferentes variables virales creando un "cuello de botella" que favorece la infección viral por una población viral homogénea, lo que se observa en aproximadamente el 80% de los casos de transmisión

heterosexual y en menor proporción en el caso de las relaciones sexuales por relaciones sexuales entre hombres (60%) o por uso de drogas endovenosas (40%) [31].

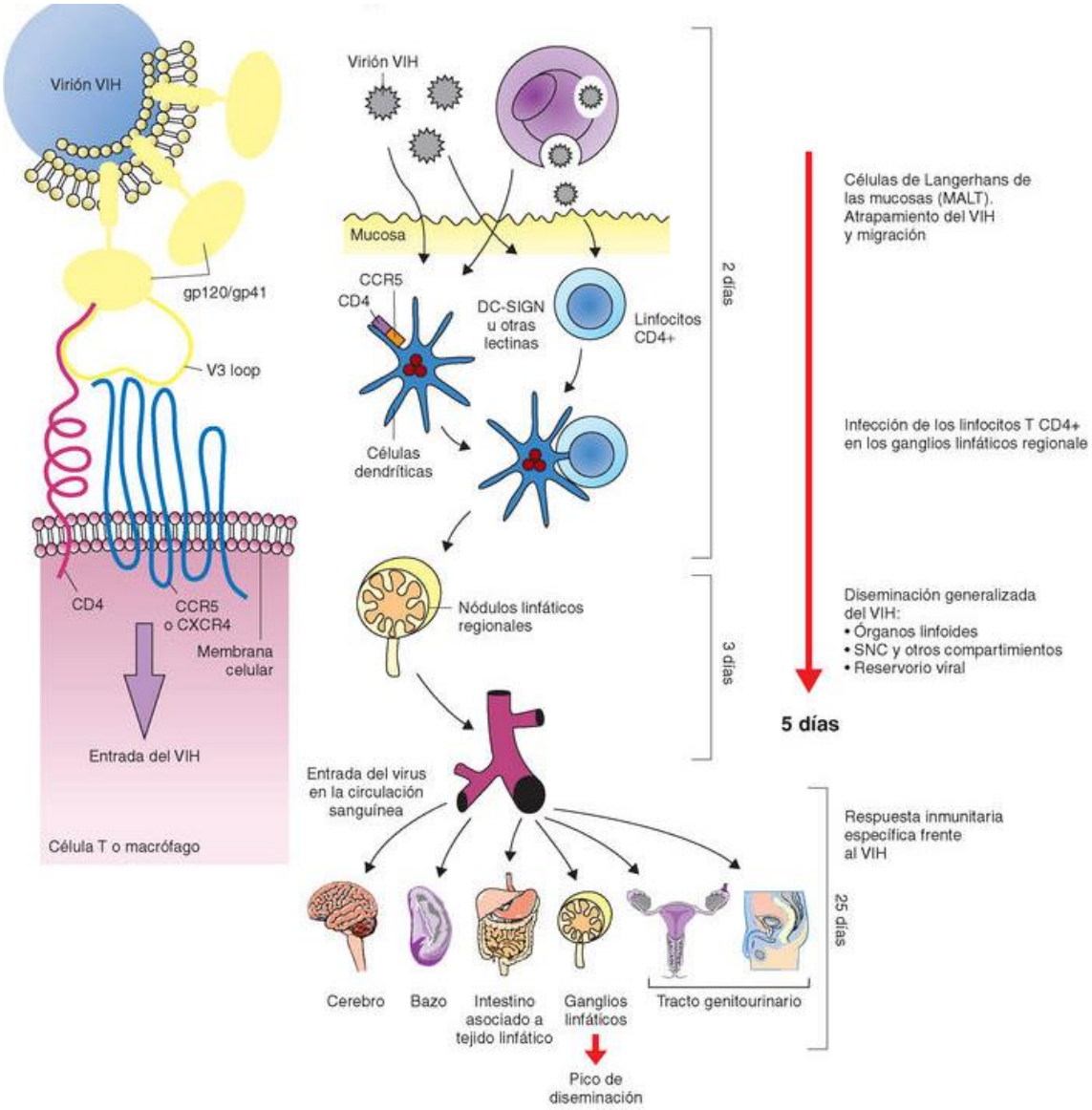


Figura 3: Eventos iniciales durante la transmisión del VIH-1 (Modificada de Pilcher et al. [32]).

Las células dendríticas (CD) son claves para facilitar la infección. Las CD mieloides inmaduras patrullan los tejidos mucosos detectando patógenos invasores y por lo tanto

son una de las primeras células en encontrarse con los viriones transmitidos durante contacto sexual. Las CD cargadas de virus migran desde el epitelio hacia los ganglios linfáticos regionales donde se encuentran con grandes cantidades de linfocitos T en el área parafolicular [33]. El VIH-1 toma la ventaja de las funciones de presentación de antígenos que tienen las CD para infectar las células CD4 mediante el mecanismo denominado sinsapsis virológica, un mecanismo de diseminación del virus en ausencia de infección productiva [34,35]. La gran concentración de células CD4 en los ganglios permite la infección masiva de las células diana y la consiguiente explosión de la replicación viral que se libera a la circulación sistémica [36], coloniza todos los tejidos y da lugar al establecimiento de los reservorios.

CICLO BIOLÓGICO DEL VIH

En el caso de la transmisión por vía sexual, que actualmente es la principal vía de transmisión, el virus tiene que sortear dos grandes limitaciones para lograr establecer la infección: la escasa cantidad de células diana (CD4+) en las mucosas y la protección que brinda el sistema de defensa local a través de las características de la mucosa y el sistema inmune innato [37]. El virus no infecta de forma productiva a las células dendríticas pero se une a las lectinas de su superficie (DC-SIGN y otras) para ser transportado, lo que favorecen la inflamación local y el reclutamiento de linfocitos [37]. La participación de las células dendríticas en las fases iniciales es una de las posibles explicaciones para la selección de virus R5 en las fases tempranas de infección que alcanza más del 90% de los individuos en algunas series [38,39]. Las CD producen factor derivado de células estromales-1 (SDF-1) que bloquea el uso del CXCRX4 por el VIH X4 y así selecciona negativamente las infecciones con este tipo de tropismo [40].

Para unirse a la célula, la glicoproteína viral gp120 debe entrar en contacto con el receptor celular CD4. Esta unión induce una serie de cambios conformacionales que exponen el dominio V3 y regiones adyacentes, que forman el dominio de unión de la gp120 a los receptores de quimiocinas. Esta segunda interacción induce cambios en la estructura de la gp41 que expone en la región N-terminal un dominio altamente hidrofóbico que se ancla en la membrana plasmática, lo que genera un movimiento de unión de los dominios heptaméricos de la gp41. Durante este proceso se produce el plegamiento de las membranas plasmática y viral que se aproximan y fusionan [41]. Posteriormente se internaliza la nucleocápside y se produce la decapsidación y liberación del genoma viral. Para poder llevar a cabo este proceso el virus evade la proteína celular TRIM5α, un factor de restricción que interfiere con los procesos de decapsidación de los retrovirus. La presencia del factor TRIM5α de monos rhesus y cynomolgus tiene una alta capacidad para inhibir al VIH-1 pero no al SIV, sin embargo el TRIM5α humano, que tiene algunas diferencias en la región variable 1 (V1) induce muy poca restricción del VIH-1 [42,43]. Para iniciar la síntesis de ADN a partir del ARN se debe realizar la retrotranscripción mediante la participación de la transcriptasa reversa. El virus puede infectar células activadas o en reposo aunque en linfocitos en reposo la retrotranscripción se produce de forma incompleta [44]. La falta de la actividad de control de lectura (*proof-reading*) de la transcriptasa reversa facilita la generación constante de mutantes o variantes denominadas cuasiespecies. El ADN proviral sintetizado se acopla a una serie de factores celulares y proteínas virales IN, RT, MA y Vpr [41] formando el complejo de preintegración que es transportado al núcleo, donde la integrasa viral lo integra en el genoma dando lugar a la forma proviral del VIH. Para iniciar la replicación se debe iniciar el proceso de transcripción del genoma viral [45], proceso que es influenciado por múltiples factores como las proteínas celulares NF-κB que regulan la expresión de múltiples genes celulares involucrados en los procesos

de reconocimiento y activación inmunitarios y por la proteína viral *Tat* que aumenta la transcripción o la proteína viral *Rev* que regula el procesamiento, transporte y acoplamiento del ARNm a los ribosomas. Una vez que se produce el transcripto se encapsida y se transporta a las regiones donde se produce la brotación.

Las proteínas virales *Vif* y *Vpu* intervienen durante el ensamblaje de las proteínas virales para formar las partículas maduras. *Vif* impide la función de la proteína APOBEC3G, uno de los mecanismos de inmunidad antiviral innata humana frente a retrovirus [46]. La proteasa viral procesa las poliproteinas *Gag* y *Gag-Pol* para formar partículas maduras que brotan a través de la membrana celular y se liberan al espacio extracelular para lo cual la proteína viral *Vpu* debe inhibir a la tetherina [47].

En ausencia de antirretrovirales y factores celulares que impidan la transcripción, la activación y proliferación celular de las células T CD4 infectadas determina un ciclo de replicación vírica incontrolada, que infectará nuevas células llevando a la infección diseminada y a la ocupación de lo que en el futuro será denominado reservorio viral. Cada uno de estos pasos son dianas para los antirretrovirales (Figura 4).

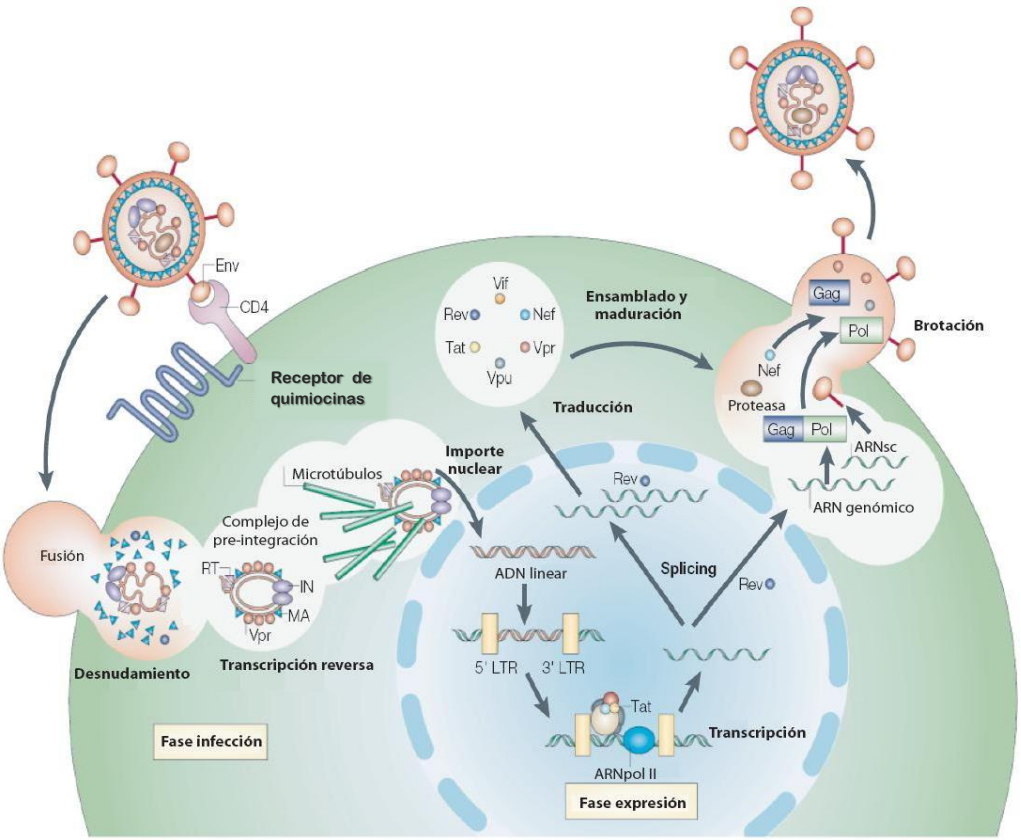


Figura 4: Esquema simplificado del ciclo de replicación del VIH-1.

FISIOPATOGENIA

Pérdida de linfocitos CD4

Los linfocitos CD4 son indispensables para mantener las respuestas adaptativas lo que otorga al VIH un carácter único ya que infecta las células que deberían controlar la infección, lo que impide su control y eliminación. La disminución progresiva de los linfocitos CD4 es la característica distintiva de la infección y que se produce por distintos mecanismos: piroptosis, apoptosis, redistribución, secuestro en ganglios periféricos y bloqueo en la regeneración linfocitaria por un mecanismo todavía no completamente aclarado [48–50]. En la infección aguda la pérdida inicial importante de linfocitos CD4 se produce principalmente por dos mecanismos, piroptosis y apoptosis.

La piroptosis es una forma programada de muerte celular asociada con respuestas antimicrobianas durante la inflamación. En este proceso las células inmunes que reconocen alteraciones en ellas mismas producen citocinas que las llevan a morir, lo que produce liberación de más citocinas y atracción de otras células. Estas reacciones están mediadas por la enzima caspasa 1[51]. La apoptosis se produce por dos vías, la extrínseca o externa y la intrínseca. En la vía extrínseca la exposición a las proteínas TAT, gp120 o Nef aumenta la unión del ligando Fas (FasL), TNF, y el ligando TRAIL/Apo-2 a sus receptores FAS/CD95, TNFR1, DR4, y DR5 lo que activa los mecanismos de muerte celular mediante la activación de las caspasas 8 y 10 que activan a su vez las caspasas efectoras 3 y 7. La vía intrínseca se inicia por alteración mitocondrial, que aumenta la liberación de citocromo C y activa la caspasa 9 que desencadena la formación de poros en la membrana mitocodrial y la muerte celular y que puede ser activada por la proteína viral Vpr [52]. Por lo tanto, en la infección por VIH ambos mecanismos contribuyen a la pérdida de linfocitos CD4 [53].

Los linfocitos activados y en estado de proliferación son especialmente susceptibles a la infección y a permitir la replicación viral debido a que presentan altos niveles del receptor CCR5 en la superficie, disponen de niveles elevados de nucleótidos y ATP y tienen activados los factores de transcripción que el VIH necesita para su replicación, por el contrario, las células en estado latente son resistentes a la infección productiva. Se propuso que en estas células se produce un bloqueo de la transcripción mediado por SAMHD1 después de la entrada del virus [54]. El virus infecta preferencialmente las células CD4 de memoria inmuno-específicas, lo que puede explicar la pérdida de estas respuestas y del control inmunológico durante la evolución de la infección [55,56].

Una vez que se produce la infección celular pueden producirse tres situaciones: a) en las células que no son permisivas a la infección (por ejemplo por la presencia de

factores de restricción) el ciclo viral se interrumpe antes de la integración en el genoma, b) en las células permisivas se completa el ciclo y la célula comienza a producir virus, c) algunas células que sostuvieron replicación pueden revertir al estado de latencia y pasan a formar parte del reservorio viral [57]. En estas tres situaciones se puede producir muerte celular debido a los mecanismos mencionados anteriormente.

Pérdida del tejido linfoide intestinal

Las CD y los linfocitos localizados en la lámina propia de la mucosa intestinal constituyen el sistema GALT *(gut-lymphoid associated tissue)* que representa desde el punto de vista cuantitativo el 50% del tejido linfático y conforma el principal mecanismo de defensa frente a los microbios que penetran en el tracto digestivo [58]. Debido a la mayor exposición a antígenos, la mayoría de los linfocitos tiene un fenotipo de memoria activado y una sobre regulación del receptor CCR5 lo que los hace extremadamente susceptibles a la infección por VIH produciéndose rápida pérdida de células CD4, en particular de las células CCR5+CCR6+Th17. Estas células son críticas para controlar la proliferación de bacterias y mantener la homeostasis de los enterocitos, secretando IL-17, IL-22, y reclutando células NK, y su déficit o su exceso pueden producir algunas patologías [59]. La pérdida de las mismas afecta la expresión de genes que regulan el mantenimiento de la barrera epitelial, aumenta la expresión de aquellos que favorecen la activación inmune y la apoptosis, induce el aumento concomitante de células T reguladoras y produce una disminución de la secreción de citocinas homeostáticas IL-17, IL-22 y defensinas [60].

El defecto en las uniones estrechas por la pérdida de enterocitos, la disminución de linfocitos Th17, la alteración de la arquitectura hepática y la pérdida de las células mielomonocíticas inducidas por el VIH son las causas de la *translocación bacteriana*; entendida como la diseminación de productos microbianos desde la luz intestinal a la

circulación sistémica [61]. Dentro de estos productos se incluyen lipopoliscaráridos (LPS), peptidoglicanos, ácido lipoteicoico, y ADN bacteriano. La afectación de los enterocitos puede ser de forma directa por las proteínas virales Tat y Gp120, que favorecen la apoptosis de los mismos, o por la alteración de la microbiota afectando la integridad de la membrana. El VIH interfiere mediante múltiples mecanismos el proceso habitual de aclaramiento de los productos microbianos que entran en la circulación y que viajan por la vena porta hasta ser reconocidos por los receptores *toll-like* en las células de Kupffer y en los hepatocitos [62,63]. El virus también induce un aumento de la fibrosis, probablemente porque estimula la secreción de TGF-b1 que favorecería la disposición de colágeno en los tejidos [64] que se observa tanto a nivel intestinal como en ganglios linfáticos [65]. Se establece así un círculo vicioso donde la infección provoca la pérdida de linfocitos del sistema GALT, favorece el daño tisular y la translocación bacteriana, la cual lleva a la activación inmune generalizada y persistente, afectando aún más al sistema inmune y al funcionamiento del tracto gastrointestinal. Por ello la disrupción de la integridad de la barrera intestinal y la translocación bacteriana son características fundamentales de la patogenia viral, asociadas a inflamación persistente e inmunoactivación sistémica sostenida que favorece el desarrollo de complicaciones no infecciosas [66–68].

Los marcadores más importantes de translocación incluyen la determinación directa de LPS y el aumento de un marcador soluble de activación de macrófagos inducida por LPS (sCD14) [69,70]. Este aumento se acompaña de aumento de IL-1β, IL-10, IFN-gama, y TNF-α, y se correlaciona con el aumento de CV. Algunos pacientes pueden presentar además aumentos importantes de IL-6, proteína C reactiva, neopterina y β2 microglobulina, y otros pueden mostrar patrones atípicos de inflamación [71].

El TARV ha demostrado reducir los niveles de sCD14 y el LPS pero los valores no se

llegan a normalizar. El tratamiento antirretroviral no logra reconstituir completamente los daños anatómicos ni revertir la fibrosis pero se asocia a mejor reconstitución cuando se inicia más temprano. La explicación más probable es la mejor restauración de las células Th17 y de las células de Kupffer, que suele ser más evidente si el TARV se inicia en etapas tempranas [72].

Inmunoactivación

La inflamación y la activación celular son eventos normales, propios de la interacción entre un patógeno y el sistema inmunitario. Si la infección o el daño no eliminan en forma completa persiste la inflamación, evolucionando inflamación crónica, con activación celular persistente, producción continua de citocinas, infiltrado celular mononuclear, formación de granulomas e intentos de reconstrucción tisular que llevan con el tiempo a la fibrosis [73]. Este es un proceso muy heterogéneo en cantidad y calidad, que se asocia a cambios en los niveles plasmáticos de diferentes citocinas y con activación de diferentes estirpes celulares y que se conoce de diversas maneras (inflamación de bajo grado, para-inflamación, inmunoactivación) [74]. En el caso de la infección por VIH, durante la fase aguda se observa una gran inflamación e inmunoactivación, un estado inflamatorio persistente durante la fase crónica que mejora pero no se resuelve completamente en los pacientes que reciben tratamiento generando un estado continuo de inflamación a bajo grado [75,76]. El mayor grado de activación inmune predice una mayor progresión de la enfermedad, incluso de una forma más precisa que el valor de la CV plasmática [77]. La persistencia de esta inmunoactivación, producida por la desregulación de un proceso normal de defensa, se ha relacionado con diversos cuadros clínicos como la aterosclerosis, el desarrollo de cáncer o los trastornos neurocognitivos, reconociéndose actualmente como uno de los factores más importantes que influyen en el proceso de envejecimiento y sus

enfermedades asociadas [78]. Por lo tanto, debemos considerar a la infección por VIH en sí misma como un factor de riesgo adicional a los factores de riesgo tradicionales asociados a enfermedad cardiovascular y otras comorbilidades [79,80] y establecer las intervenciones para reducir estos riesgos [81,82].

La activación inmune se caracteriza por un incremento en la tasa de activación y apoptosis de células T CD4+ y CD8+ y de células asesinas naturales (NK); activación de células B policlonales con un aumento en los niveles de inmunoglobulinas y una producción elevada de citocinas proinflamatorias [83]. Hay que resaltar que estas alteraciones solo describen lo que ocurre en sangre periférica, aunque hay cambios en todos los compartimentos. El tejido intestinal y los eventos que llevan a su alteración son cruciales para establecer la inmunoactivación generalizada. Dentro de este contexto, las células T citotóxicas, que deberían controlar la replicación viral, son afectadas por la inmunoactivación generalizada y por la carga antigénica que produce la estimulación crónica que altera sus funciones [84]. Una observación temprana que sintetiza el impacto de la inmunoactivación da cuenta de la asociación entre células CD4 activadas (CD4+CD38+HLA-DR+), valores altos de CV y caída rápida de las células CD4 [85].

Por lo tanto el control temprano de la inmunoactivación se ha convertido en uno de los objetivos del manejo de los pacientes con infección aguda. Las células que regulan la inmunoactivación incluyen las células T reguladoras naturales ($CD4^{+}CD25^{high}FoxP3^{+}$) (Tregs), las células CD8 regulatorias (CD8+FoxP3+) que se han estudiado extensamente y las células precursoras de células T o células doble negativas (CD3+CD4−CD8−) [86,87]. El rol de las Tregs y el potencial efecto de modular su función es objeto de muchos estudios [88,89]. Las células doble negativas son resistentes a la infección y muestran una relación inversa con la proporción de células CD8

activadas (CD38+HLA-DR+), la frecuencia de células CD8 en apoptosis (Ki-67+) y los valores de CV. Además los valores altos de células doble negativas predicen la posibilidad de mantener un bajo nivel de inmunoactivación a los 6 meses [87] lo que las posicionan como dianas terapéuticas de futuros enfoques terapéuticos [90]. Recientemente se ha comenzado a dar valor a otro parámetro como marcador de inmunoactivación, el receptor soluble específico de monocitos y macrófagos (sCD163). Su función es reciclar hierro extracelular, tiene actividad bactericida e inhibe la proliferación y activación de linfocitos T. Su aumento con respecto a personas seronegativas se asocia a la expansión y activación de monocitos, en particular de monocitos CD14+CD16+ lo que se correlaciona con la CV y progresión a SIDA [91]. El aumento de sCD163 en la fase aguda es moderado y es mucho más importante durante la fase crónica, el TARV durante la fase aguda puede normalizar los valores pero si se realiza durante la fase crónica los pacientes presentan una reducción de sus niveles pero persisten por encima de los valores normales en forma proporcional a la presencia de inmunoactivación demostrada por linfocitos CD8+HLA-DR+CD38+ [75].

La mayor inflamación se presenta con mayor producción de citocinas. La fase aguda de la infección se describió como una "tormenta de citocinas" [92] en la que se han descrito importantes aumentos de citocinas y quimiocinas proinflamatorias como IL-1β, IL-6, IL-18, TNF-α, IL-10 y la proteína 10 inducida por interferón [93,94]. Determinadas citocinas aumentan antes de que se produzca el pico viral (IFN-α, IP-10, IL-10) mientras que otras aparecen simultáneamente (IL-6, IL-12, IL-7). IL-1, IL-2 y GM-CSF pueden permanecer normales en algunos pacientes [92,95]. El valor de algunas citocinas puede predecir el valor de la CV a los 6 y 12 meses, entre esas se incluyen la IL-12. IFN gama, IL-7 e IL-15, aunque su determinación de rutina no se

recomienda.

En un subanálisis entre 75 pacientes que no recibían tratamiento en el estudio SPARTAC se pudo demostrar que el valor basal de IL-6 se asociaba a progresión. El valor medio fue de 1,45 pg/mL y se demostró que por cada pg/mL adicional aumentaba un 38% el riesgo de progresión a menos de 350 células CD4 durante el seguimiento [96]. El tratamiento antirretroviral tiende a normalizar todas las citocinas, aunque en ocasiones no se logra volver a los niveles basales, en particular con IL-18, IL-6, IL-10 e IL-12 [95,97].

Alteraciones metabólicas

La inflamación y la activación inmune tienen muchas ramas de conexión con el metabolismo, ya que establecer una respuesta inmune requiere de por sí un importante cambio en los procesos metabólicos, debido a la gran demanda de energía para la biosíntesis de moléculas que participan en la inmunidad innata (citocinas proinflamatorias, procesamiento de antígenos, fagocitosis, etc.) y adquirida (diferenciación y proliferación celular). Por otro lado, los pacientes con infección por VIH tienen cambios metabólicos secundarios a la inmunoactivación que a su vez empeora la situación metabólica [98]. Muchos de estos cambios no son evidentes desde el inicio de la infección. En un estudio, controlado por edad y factores de riesgo en 26 hombres en los cuales se evaluaron varios parámetros antes de la seroconversión y un año después, el único hallazgo significativo fue la disminución de HDL. Los valores de colesterol total, vitamina D, proteína C reactiva, dímero D y fibrinógeno no variaron significativamente después de la seroconversión [99].

Los radicales libres son moléculas altamente reactivas que se producen por el metabolismo del oxígeno, como el peróxido de hidrógeno (H2O2), anion superóxido,

o el radical hidroxilo. Tienen un rol en la señalización celular, la homeoestasis y la protección antimicrobiana y antitumoral y han sido implicados también en la inflamación crónica [100]. La producción aumentada de estos metabolitos aumenta el estrés oxidativo, lo que daña estructuras celulares y promueve la inflamación al aumentar la producción de citocinas inflamatorias (IL-1β, IL-6, INF, TNF-α) [101,102]. El organismo regula estos procesos mediante enzimas antioxidantes como la superóxidodismutasa, peroxidasa/reductasa catalasa glutatión, vitaminas A, C y E y pequeñas proteínas redox como el glutatión y tiorredoxina. Los pacientes con VIH presentan gran consumo de oxígeno, aumento de hidroxiperóxidos y reducción de las enzimas protectoras, probablemente por inducción directa de las proteínas virales Tat, Vpr, Nef y Gp120. Durante la infección, las células T aumentan la producción de radicales libres, lo que reduce la respuesta a las citocinas IL2, IL4, y promueve la disfunción celular y la apoptosis al inducir la expresión de PD-1 y reducir el receptor de vitamina D [103–106].

El triptófano es un aminoácido esencial cuyo catabolismo genera productos como la kineurina, un precursor de varias moléculas involucradas en la producción de energía. En las personas con infección por VIH el metabolismo del triptófano está aumentado, lo que se evidencia por un aumento de la actividad de IDO-1 [107,108]. Esto se correlaciona con la inmunoactivación y puede ser parcialmente revertido con TARV [109]. La inducción temprana de actividad IDO-1 en macrófagos y CD limita la respuesta antirretroviral y contribuye a la progresión de la enfermedad [110,111]

La inmunoactivación causada por VIH también resulta en un mayor consumo de glucosa para afrontar la proliferación, supervivencia y función inmune de las células [112] y la infección de las células T CD4 causa un aumento del flujo glucolítico a su máxima capacidad y facilita la apoptosis. De hecho, la expresión de GLUT-1 (el

transportador de glucosa en las células T) está aumentado en CD4 de pacientes con VIH, se asocia con inmunoactivación y con la pérdida celular que no mejora con el TARV [113]. Asimismo, los macrófagos infectados presentan una importante reducción en la captación de glucosa y menor generación de metabolitos intermedios [114]. Vpr inhibe la hexoquinasa 1 que es la enzima encargada de convertir la glucosa en glucosa 6 fosfato y favorece la integridad mitocondrial por vía no metabolica[115,116] lo que podría tener consecuencias en los esfuerzos de erradicación. Todos juntos, estos datos sostienen el impacto negativo de la infección por VIH en el metabolismo de la glucosa.

La función de los lípidos es importante. Aunque los triglicéridos solamente sirven para almacenar energía en adipocitos y células musculares, el colesterol es una parte fundamental de membranas celulares, esteroides, ácidos biliares y moléculas de señalización. El VIH aumenta la producción de ácidos grasos libres, LDL y de apolipoproteina A-1 [117]. Además la envoltura del virus se une a dominios ricos en colesterol, y la proteína viral Nef puede alterar la composición lipídica del virión y de los dominios de la membrana celular para mejorar la infectividad [118–120]. También se ha descrito un efecto directo de la proteína Nef en el aumento de las lesiones ateroscleróticas, el aumento del colesterol total y triglicéridos y la disminución del colesterol HDL, lo que proporciona evidencia de un factor adicional a los mecanismos multifactoriales que explican el mayor riesgo cardiovascular en estos pacientes [121].

Establecimiento del reservorio viral:

El reservorio está constituido por poblaciones celulares infectadas que persisten con la capacidad de sostener la replicación viral a pesar del TARV efectivo, lo que representa el mayor obstáculo para la erradicación viral [122]. El reservorio se establece tempranamente, aunque la evidencia de estudios en pacientes con infección aguda muestra que se trata de un proceso gradual [123]. Una de las mayores dificultades para

los estudios de reservorio viral es la falta de estandarización para identificar y medirlo [124,125].

Las células T de memoria y las células transicionales son capaces de persistir durante largos periodos de tiempo mediante proliferación homeostática [126], y se propuso que constituyen el principal reservorio [127], pero otras células y tejidos también juegan un papel importante, como las células T naive y células madre [128], los macrófagos y monocitos [116], el sistema nervioso central, en particular los astrocitos[129], el tejido intestinal [130], y las células estrelladas hepáticas. La composición del reservorio está inluenciada por el tipo de tejido que se estudia, el momento en el que se inició el tratamiento y factores genéticos y de respuesta inmune propios del individuo. El inicio temprano del TARV durante la infección aguda limita el tamaño del reservorio, la evolución viral, y puede mejorar la restauración inmune y la arquitectura del tejido intestinal con mucha mayor eficiencia que durante la infección crónica [131,132]. Estos hallazgos han estimulado la utilización de terapias combinadas dirigidas a eliminar estas células latentes infectadas con el objetivo final de lograr la erradicación viral [133].

RESPUESTA FRENTE A LA INFECCION POR EL VIH

En modelos animales, las interacciones entre el virus y la mucosa vaginal del organismo determinan si el virus se elimina en la mucosa o si se establece como agente infectante. La primera respuesta al virus es innata y se produce mediante el proceso de identificación del patógeno y la activación de las vías de señalización del sistema innato. La identificación se produce por el reconocimiento en los productos virales de patrones moleculares asociados a patógenos (PAMP, del inglés *pathogen-associated molecular patterns*). Este sistema induce los factores de restricción viral que van a suprimir o limitar la replicación viral, producir IFN y citocinas proinflamatorias y

quimiocinas que reclutarán y activarán células inmunes innatas como macrófagos y células NK [134]. La respuesta exagerada o la persistencia de la activación del sistema innato también pueden tener consecuencias deletéreas, ya que promueve la replicación viral y aumenta la pérdida de células CD4. Una gran activación de CD por ejemplo, puede exacerbar los efectos pro-apoptóticos en las células CD4 [37]. Actualmente, existe un gran interés en identificar los mecanismos reguladores de la respuesta innata en VIH para intentar identificar potenciales dianas terapéuticas. El Complejo Mayor de Histocompatibilidad (CMH) constituye el mayor determinante genético de respuesta inmune y sus polimorfismos pueden influenciar las respuestas innatas y adaptativas frente al VIH. Los individuos que presentan el alelo HLA-B57 presentan una menor progresión, presentan con menos frecuencia síntomas durante la infección aguda y logran un mejor control espontáneo de la viremia [135]. Se han descrito otros alelos con efectos beneficiosos (por ejemplo KIR3DS1/HLA-Bw480I) o con efectos negativos (HLA-B35-Px) en la progresión de la infección [136].

Las células dendríticas regulan el balance entre la tolerancia y la protección inmune, tanto en el sistema innato, como en el adaptativo. Una de las funciones más importantes de las CD es la regulación del desarrollo de las células B, la activación y la supervivencia mediante la producción del factor de crecimiento de células B (BLyS del inglés *B lymphocyte stimulator*). Durante la infección aguda la cantidad de CD mieloides maduras disminuyen y persisten por debajo de los niveles normales en las subsecuentes fases de la infección incluso después del tratamiento exitoso. Se observa también un aumento importante de la expresión de estimulador de linfocitos B (BLyS) en su superficie que se correlaciona con un aumento en sangre de los factores de crecimiento de células B, lo que podría ser responsable del aumento de las células B y de la hipergamaglobulinemia [137]. Estos hallazgos sugieren que los precursores

monocíticos se diferencian a CD con fenotipo inflamatorio y se convierten en una fuente de BLyS contribuyendo de esta forma también a la desregulación inmune [137].

Otra forma de regulación entre los sistemas innatos y adaptativos son las señales supresivas y estimulantes mediadas por las galectinas. Las galectinas son una familia de 15 lectinas, altamente conservadas durante la evolución que se unen a un beta galactósido, comparten una secuencia consenso en su dominio de reconocimiento de carbohidratos y tienen acción directa al unirse a membranas de bacterias o células activadas [138]. La galectina 1 promueve la infección celular por el VIH y favorece la replicación mediante la facilitación de la unión entre gp120 y la molécula de CD4. La galectina 9 (Gal-9) es una lectina, ligando de Tim-3, un receptor especifico de superficie de los linfocitos CD4, que funciona como un inmunomodulador negativo suprimiendo las respuestas Th1 y la generación de células CD4 generadoras de IL-17 e induciendo células T reguladoras (Treg). Sus niveles aumentan en forma muy importante a partir de los 5 días de detectarse la CV y en forma proporcional a la viremia y a los marcadores de inflamación como IL-10, TNF-α e IL-1 beta [139]. Los niveles disminuyen con el tratamiento [140], aunque persisten más altos que en la población sin VIH, incluso en aquellos con control virológico y en pacientes EC. Algunos expertos sugieren que la Gal-9 es responsable de la disfuncionalidad de los CD8 que se observa en la infección por VIH [139].

La respuesta adaptativa se genera aproximadamente a las 12 semanas de la infección y genera, tanto anticuerpos específicos como linfocitos CD8 con actividad citotóxica frente al VIH. La puesta en marcha de estos mecanismos de inmunidad específica consigue un control parcial de la replicación viral y provoca una caída brusca de la viremia que se mantiene posteriormente a un nivel relativamente estable y que varía en cada paciente (*set-point* viral). Esta viremia oscila entre niveles indetectables y

superiores a cientos de miles de copias, un parámetro que refleja el nuevo equilibrio producido entre la replicación viral y el control inmunitario y que representa un marcador pronóstico de la velocidad de evolución a SIDA [141]. Las respuestas inmunitarias específicas son, sin embargo, incapaces de erradicar el virus que se ha acantonado en distintos reservorios en los que replica de manera persistente. Durante todo el curso de la infección el sistema inmune mantiene una presión frente a la replicación, produciéndose paulatinamente respuestas humorales y celulares específicas para el virus circulante, y estimulando la generación continua de variantes de escape para evadir estas respuestas, sobre las cuales se volverán a inducir respuestas celulares y humorales específicas [142].

Después de varios años de infección el sistema inmunitario pierde progresivamente su capacidad de control. La alta tasa de recambio celular afecta la homeostasis, se produce el agotamiento clonal de células T asociado a la pérdida de células de memoria, la inflamación crónica y el depósito de colágeno producen daño permanente en los centros de producción de linfocitos y la mayor inmunoactivación proporciona más sustrato al virus, lo que lleva a la incapacidad del organismo para mantener los niveles de CD4, con la caída paulatina de su número y el desarrollo de la catástrofe inmune [84]. El agotamiento y la disfunción inmune se caracteriza por el aumento de la expresión de receptores inhibitorios de PD-1 [143] y de CTLA-4 [144].

Respuesta humoral

La infección por el VIH induce una respuesta humoral frente a prácticamente todas las proteínas reguladoras y estructurales del VIH, incluyendo anticuerpos frente a la envoltura, a las proteínas de la matriz, de la nucleocápside viral y a las proteínas reguladoras del virus. Los anticuerpos aparecen después de la reducción del pico inicial de replicación viral, por lo que se considera que tienen un papel limitado en el

control inicial y en el establecimiento del *set-point* viral. Los primeros anticuerpos se dirigen a epítopes no neutralizantes en la envoltura (Env). En los meses subsiguientes, una minoría de individuos tiene la capacidad de desarrollar anticuerpos neutralizantes [145] pero el virus escapa rápidamente a la inhibición por estos anticuerpos [146]. Algunos anticuerpos, como los dirigidos frente a gp41 y frente a determinados dominios de la gp120 tienen capacidad neutralizante *in vitro* [147].

Hasta el momento no se ha podido producir una respuesta inmune que permita crear una vacuna efectiva debido a varios factores, entre ellos, probablemente el más importante es la gran variabilidad de las partes expuestas de la proteína gp160 [148]. La envoltura es una estructura trimérica que oculta dominios conservados; a lo que se agrega la necesidad de interaccionar con el CD4 para poder exponer dominios de interacción [149]. En los últimos años algunos hallazgos han reactivado el interés en anticuerpos protectores neutralizantes con el objetivo de poder inducirlos con vacunas o como una estrategia de transferencia pasiva. En modelos animales la transferencia pasiva de anticuerpos previene la infección o suprime la CV [150], algo que también se ha observado en estudios iniciales con individuos infectados [151].

La vacuna con mayor efectividad desarrollada hasta la fecha se basa en un vector *Canaripox* y se dirige contra gp120 pero la eficacia en la prevención de la adquisición de la infección se limita al 31% [152]. Este estudio demostró la importancia del dominio variable V2 que interactúa con el receptor integrina α4β7 del organismo, y que puede ser importante para bloquear la infección, por lo que los anticuerpos contra esta región pueden ser importantes en el diseño de vacunas [153]. La presencia de estos anticuerpos con alta capacidad de neutralizar virus de varios subtipos se pudo demostrar en el 3% de los pacientes recientemente infectados [154]. Por ello, aunque la eficacia de esta vacuna es baja, su valor reside en que permitió abrir nuevas líneas de trabajo, ya que

fue la primera con la que se demostró que se podían generar respuestas protectoras. Idealmente, si fuera posible producir anticuerpos neutralizantes frente a la proteína de gp120, el primer componente que se pone en contacto con las células, estos anticuerpos podrían neutralizar la infección o estimular la citotoxicidad dependiente por anticuerpos. Sin embargo la producción de los mismos ha sido complicada por la hiper-variabilidad de esta región, el escudo de glicanos que la protege y los plegamientos de las proteínas sobre las regiones conservadas [155]. A esto se debe sumar la importante alteración en la producción de las células B productoras de anticuerpos, debido a la pérdida de más del 80% de los centros germinales del tejido linfoide gastrointestinal junto con afectación grave del ambiente que apoya la maduración de células B en el íleon terminal [156].

Recientemente se han identificado anticuerpos neutralizantes de amplio espectro en EC [157] cuyo suero puede neutralizar un amplio rango de variantes virales. Los anticuerpos dirigidos frente al dominio de interacción con CD4 –denominados VRC01 y VRC02–neutralizan más del 90% de los aislados del VIH-1 [158], aunque las complejidades y el costo impiden su uso como inmunoterapia pasiva. Actualmente la investigación avanza hacia la forma de identificar anticuerpos de gran amplitud y evaluar combinaciones de varios anticuerpos dirigidos contra diferentes sitios para evitar la emergencia de resistencia [159].

Respuesta celular

En la infección por el VIH la respuesta antiviral se produce por distintas poblaciones celulares: linfocitos CD4 colaboradores, linfocitos CD8 citotóxicos (CTL) y células NK [160]. La expansión de la respuesta celular de tipo citotóxico es un fenómeno muy importante en la modulación de la infección y en el establecimiento del *set-point* viral y riesgo de progresión. Mediante estudios en macacos infectados con SIV en los que

se eliminaron en forma temprana los linfocitos CD8 se demostró la importancia crítica de estas células para el control temprano de la replicación viral ya que en su ausencia la CV aumentaba hasta 10 veces [161]. Por eso, se ha sugerido que los linfocitos T citotóxicos CD8+ específicos de VIH y, en menor medida, la respuestas CD4+ y el agotamiento de células blanco son las causas del descenso del pico de viremia, de la desaparición de los síntomas agudos y del establecimiento del *set-point* viral [162]. Las células CD4 específicas, que también pueden reconocer y destruir células infectadas, podrían tener un efecto en el control viral. En un estudio longitudinal se observó que las personas que controlaban la infección espontáneamente tenían una mejor respuesta CD4+ Gag específica, incluso por sobre las respuestas CD8, proponiéndolas como un mejor predictor inmunológico de *set-point* y de evolución sin tratamiento y en una diana potencial para el desarrollo de vacunas [163].

Las primeras respuestas celulares frente al virus se dirigen a un espectro limitado de epítopes [164], y son capaces de frenar la replicación inicial. Sin embargo, el VIH escapa del control inmune por el CD8 mediante la selección de mutaciones. Esto explica por qué, a pesar de la importancia en la contención de la viremia inicial, ni la amplitud, ni la magnitud, ni la función CD8 pueden predecir el control sostenido de la replicación. Algunos pacientes con altos niveles de respuesta presentan un *set-point* alto sugiriendo además que estas respuestas reflejan de alguna manera el nivel de viremia [164].

Las funciones antivirales de las células T CD8+ se caracterizan por no tener una única función celular, sino que cada célula es capaz de producir un amplio espectro de moléculas efectoras. La polifuncionalidad celular se describe como la habilidad de una célula de producir por lo menos tres marcadores; entre los cuales se encuentran CD107A/B, MIP-1β, IFN-γ, IL-2 y TNF-α como los más típicos. La polifuncionalidad de la respuesta ha sido asociada con mejor control viral [165–167] y mayores niveles de

perforinas [168]. Los pacientes EC logran controlar la viremia sin tratamiento, no progresan a largo plazo y demuestran una mayor capacidad supresiva CD8 específica [169,170]. Sin embargo, la capacidad supresiva no se asocia a respuesta específica ni a polifuncionalidad de células CD8. En un estudio se demostró que todos los pacientes desarrollan respuestas inmuno-específicas CD8 durante la infección aguda, que se acompañan de una fuerte respuesta CD4, pero la mayoría carecía de una capacidad supresiva CD8, sugiriendo que la posibilidad de controlar la viremia que se observa en los EC no se asocia a amplitud o polifuncionalidad de respuesta sino a la selección natural o al desarrollo de capacidades supresivas, como mayor avideza o más rápida degranulación [171]. La disfunción de células CD8 y el agotamiento de la respuesta son características comunes de la respuesta a las infecciones virales crónicas como la hepatitis C. Los mecanismos son complejos, pero son mediados en parte por un conjunto de puntos de control inhibitorios (Tim-3, PD-1 y CD160). Un estudio ha demostrado la presencia de células doble positivas CD4+CD8+ específicas para VIH durante la fase aguda que serían las responsables del 16% de la respuesta proliferativa y más del 70% de la respuesta poli-funcional anti-VIH [172].

Mecanismo de escape viral

El mecanismo más importante de escape viral del VIH es la altísima tasa de variabilidad debida a la alta tasa de error de la transcriptasa reversa (una sustitución por cada 10.000 a 100.000 rondas de copia). Las mutaciones son responsables de una gran proporción de virus defectivos y de la gran variabilidad que favorecen el escape viral a la respuesta inmunitaria específica [147,148]. Las variantes virales aparecen a los pocos días por mutaciones en la región de la envoltura, lo que permite evadir la respuesta humoral establecida frente al virus a los pocos meses del desarrollo de los anticuerpos [173].

HISTORIA NATURAL DE LA INFECCIÓN POR VIH

La replicación viral y el daño producido por el VIH en las células inmunes son los determinantes más importantes de la progresión clínica en la infección por VIH no tratada. Podemos diferenciar tres estadios: la infección primaria o infección aguda, la fase asintomática (latencia clínica) y la fase clínica de SIDA (Figura 5).

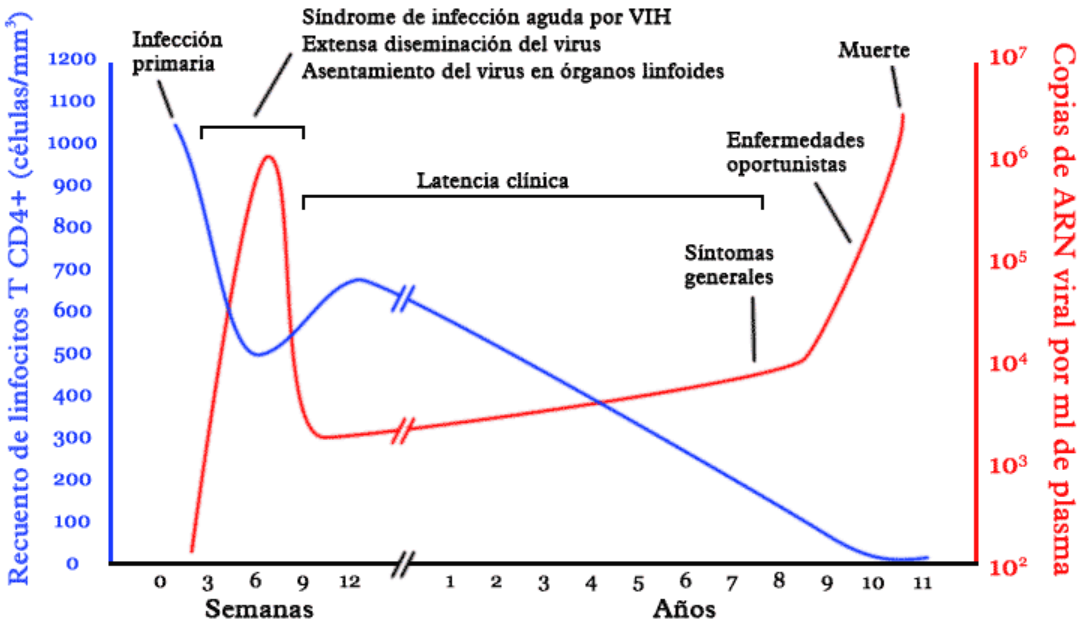


Figura 5: Historia natural de la infección por VIH desde la infección primaria a la muerte (Modificado de Pantaleo et al. [174])

La infección primaria, también llamada infección aguda o primoinfección por VIH se produce inmediatamente después de la penetración del virus al organismo. Una vez que el virus atraviesa la mucosa las células dendríticas lo captan y migran hacia los ganglios regionales donde llegan en 24 a 72 horas y lo presentan a los linfocitos. Dentro del ganglio se produce una explosión de replicación viral que produce altos niveles de CV y diseminación del virus a todos los futuros reservorios (mucosa gastrointestinal, sistema nervioso central, riñón, etc.) Este pico de viremia se asocia en

el 80% de los casos con algún síntoma, y en la mayoría da lugar a un síndrome denominado "síndrome retroviral agudo", consistente en fiebre faringitis adenopatías laterocervicales, cefalea, diarrea y sudoración nocturna que suele durar entre 2 y 3 semanas [175].

La mejoría de los síntomas se asocia en general a una disminución de la CV, debido a la generación de una respuesta inmune específica que controla parcialmente la infección y que logra un equilibrio de la CV aproximadamente a los 4-6 meses *(set-point)*. Los linfocitos CD4, que previamente a la infección suelen estar entre 500 y 1500 células por mm3, disminuyen durante la fase aguda, se recuperan a niveles cercanos a los normales durante los primeros meses post infección, y posteriormente disminuyen en promedio de 60 a 100 células/mm3/año, lo que constituye el patrón patognomónico de la infección por VIH y la principal explicación de la patogenia de las infecciones asociadas.

Una vez que se establece el *set-point* viral inicia la fase clínica estable, cuya duración dependerá del valor de CV que el individuo mantiene durante el seguimiento, de la velocidad de caída de linfocitos CD4 y de posibles intercurrencias que alteren este equilibrio.

La combinación de la CV y el tiempo de exposición es el factor más importante que predice mortalidad en personas con VIH [176]. Después de 8 a 10 años de esta fase clínica estable, el número de CD4 es tan bajo que comienzan a aparecer infecciones oportunistas graves (denominadas inicialmente enfermedades definitorias de SIDA) [177]. Si la persona no recibe tratamiento tanto para estas infecciones como para el VIH el riesgo de muerte es extremadamente alto.

VÍAS DE TRANSMISIÓN

El virus se transmite entre humanos por tres vías principales:

a) La vía sexual, a través de semen y secreciones cervicovaginales.

b) La vía parenteral, mediante sangre o productos sanguíneos (por transfusiones, por compartir agujas entre UDI, o también por contacto con algún elemento punzocortante contaminado).

c) La vía perinatal o transmisión de la madre infectada al hijo, ya sea intraútero o durante el paso por el canal del parto, y posteriormente a través de la leche materna.

Actualmente, más del 70% de los casos de infección por VIH se produce por transmisión sexual, debido a la exposición del virus a las superficies mucosas genitales, a pesar de que esta se considera una vía ineficiente ya que comparada con otras, tiene una baja tasa de transmisión [31]. El riesgo de transmisión sexual puede incrementarse por factores como úlceras genitales, o infección avanzada y disminuir en caso de circuncisión masculina.

Diferentes características sociales influyen en la transmisión y deben ser tenidas en cuenta a la hora de establecer o evaluar programas sanitarios de prevención, diagnóstico y tratamiento. En HSH, por ejemplo, la posibilidad de infectarse aumenta si las parejas tienen mayor edad [178]. Otros factores bien conocidos siguen siendo importantes y responsables de nuevas infecciones agudas, como por ejemplo, la concurrencia a sitios de encuentros sexuales anónimos y la falta de uso de preservativos [179,180]. Esto expresa la importancia de implementar estrategias innovadoras para reducir las nuevas infecciones entre personas a mayor riesgo, en particular aquellas intervenciones orientadas a reducir los factores sociales y las redes

de transmisión, así como otras intervenciones individuales de probada eficacia como la profilaxis pre-exposición [181,182].

El embarazo implica una mayor susceptibilidad para la infección no solo por los cambios biológicos que se producen, sino también por la baja frecuencia de realización del diagnóstico en las parejas masculinas. En Mozambique, en una cohorte de 1200 mujeres embarazadas se produjeron 14 seroconversiones pero solo el 19% conocía la situación de su pareja [183]. En lugares de alta incidencia hasta un 25% de las transmisiones pueden vincularse a seroconversión durante el embarazo [184]. Por estas razones cada vez son más los países que incorporan la solicitud de la prueba a la pareja y una repetición de la prueba en el tercer trimestre del embarazo como medidas para detectar las seroconversiones durante el embarazo [185,186].

La CV de la fuente es un factor de gran relevancia en determinar el riesgo de transmisión. La prueba más concreta de esta afirmación está dada por los resultados del estudio HTPN 052, en el cual las parejas negativas de personas con VIH que estaban bajo tratamiento tuvieron una reducción del 96% en el riesgo de adquirir la infección [187]. La misma estimación se obtuvo en el estudio PARTNER en el que se analizaron los eventos de transmisión entre 1.100 parejas serodiscordantes en las cuales la persona con VIH se encontraba indetectable bajo tratamiento durante 894 parejas-años de seguimiento [188].

Durante la infección aguda suelen seleccionarse virus con alta capacidad replicativa, lo que sumado a la ausencia de anticuerpos neutralizantes que puedan bloquear o inactivar los virus circulantes, y de respuestas celulares específicas explica valores extremadamente altos de cargas virales que se asocian a una mayor infectividad. Se calcula que por cada 10 veces que aumenta la CV, hay un aumento de 2,5 veces en el

riesgo de transmisión [189]. En modelos animales la infección con virus procedentes de otros animales en fase aguda de infección tienen una infectividad 750 veces mayor en comparación con infecciones con virus recolectados durante la fase crónica [190].

Varios meta-análisis revisaron las tasas estimadas de transmisión por cada evento de exposición entre parejas serodiscordantes, incluyendo meta-análisis para transmisión sexual [191,192], oral [193], y endovenosa [194]. (Tabla 1). Estas estimaciones pueden no reflejar la infectividad real ya que no distinguen la contribución de diferentes cofactores en el riesgo de transmisión que pueden tener un impacto significativo en la transmisión. Estos cofactores se detallan en la tabla 2.

Tabla 1: Riesgo por acto de exposición (compilado de referencias [191–194]).

Tipo de contacto	**Riesgo por acto (%)**	**IC95%**
Transfusión de sangre	92,50	89-96,10
Uso de drogas compartiendo jeringas	0,62	0,41-0,93
Accidente punzocortante	0,23	0-0,46
Sexo anal receptivo	1,38	1,02-1,86
Sexo anal insertivo	0,11	0,04-0,28
Sexo vaginal receptivo	0,08	0,06-0,11
Sexo vaginal insertivo	0,04	0,01-0,14
Sexo oral receptivo	ND	0-0,04
Sexo oral insertivo	ND	0-0,04
Trasmisión materno infantil	22,6	17-29

Tabla 2: Cofactores que aumentan el riesgo de transmisión (Referencias [191–194]).

Cofactor	**Riesgo Relativo**	**IC95%**
Infección aguda vs asintomática	7,25	3,05-17,3
Estadio final vs asintomática	5,81	3,00-11,4
CV alta (por log)	2,89	2,19-3,89
Úlcera genital	2,65	1,35-5,19

Las estimaciones pueden variar en función de la edad de la población estudiada, con valores un poco más altos para personas más jóvenes, así como valores mayores en personas con mayor actividad sexual [195]. Asimismo hay que mencionar que estos riesgos son substancialmente atenuados por el uso de preservativos y por el tratamiento antirretroviral [196].

INFECCION AGUDA POR VIH

DEFINICION

Se denomina **primoinfección** a la primera infección establecida del VIH.

Infección aguda es el período de tiempo que transcurre desde la entrada del virus al organismo y la seroconversión completa definida como la aparición de una prueba de *western blot* positivo para el VIH. La definición de aguda se estableció para diferenciar la etapa donde todavía no se han puesto en marcha los mecanismos inmunes de control [32,197].

A partir de la seroconversión confirmada hasta los primeros 180 días se denomina **infección reciente**, ya que los estudios de establecimiento del punto de equilibrio (*set-point*) se sitúan en este periodo. Esta definición se estableció por convención.

Se denomina **Síndrome Retroviral Agudo** a la sintomatología compatible con un cuadro de primoinfección.

MANIFESTACIONES CLINICAS

En 1985, Cooper et al. publicaron la primera descripción de los síntomas atribuibles a la infección aguda por el VIH en 12 HSH que presentaron un cuadro compatible con una "mononucleosis infecciosa" (fiebre, faringitis y exantema) que presentaban serología negativa para el virus Epstein-Barr (VEB) y en los que finalmente se confirmó la infección por VIH. Desde entonces este síndrome se conoce como "síndrome retroviral agudo" o "síndrome de seroconversión aguda por VIH".

El período de incubación de la infección por el VIH varía entre 1 y 3 semanas (típicamente 14 días) y la duración del período sintomático es de 7-14 días, raramente más de 2 semanas. En diferentes estudios, la prevalencia de síntomas durante la infección aguda por el VIH varía entre el 40 y el 90%. En estudios prospectivos de pacientes de alto riesgo y expuestos al VIH (p. ej., pacientes atendidos en centros de atención de enfermedades de transmisión sexual), comparados con un grupo control, entre el 53 y el 88% presentaron algún síntomas que se asociaba temporalmente a la infección. Sin embargo, hasta el 50% de los pacientes que no adquirieron la infección sufrieron síntomas similares, lo que demuestra su baja especificidad [198,199].

El espectro clínico de la infección aguda por el VIH (Tabla 3) varía desde cuadros banales, que se confunden con una virosis inespecífica, hasta cuadros graves con afectación neurológica. Los síntomas más frecuentes incluyen fiebre, exantema, úlceras orales y/o genitales, linfoadenopatías, astenia marcada, artromialgias y meningitis aséptica [200]

Tabla 3: Síntomas más frecuentes (tomado de Miró et al. [201])

Síntoma	**Frecuencia**
Fiebre	53 a 87,5%
Exantema	9 a 57,5%
Úlceras orales	7,5 a 37%
Atromialgias	24 a 54%
Faringitis	14 a 44%
Pérdida de peso	32
Astenia	68 a 72,5%
Cefalea	54 a 55%
Sudoración nocturna	50 a 51%
Adenopatías	7-37,5%
Diarrea	24%

Cuando se valora la gravedad de los síntomas, menos de la mitad de los pacientes consideran el cuadro suficientemente importante como para consultar a un médico. En otras series en cambio, hasta la mitad de los pacientes requieren ingreso hospitalario para su valoración y, en general, esto se produce por síntomas neurológicos o un síndrome febril prolongado [202]. La fiebre es el síntoma más frecuente (80-87% de los casos) y el primero en aparecer en todas las series realizadas. La duración de la misma es variable y oscila entre 3 días y 3 semanas. Hasta un 1% de los casos de adultos sexualmente activos con fiebre que consultan a urgencias están cursando una infección aguda [203],[204].

La temperatura suele ser muy elevada al inicio, desciende lentamente, y la febrícula puede persistir 2 semanas. Por lo general se asocia a sudoración nocturna y astenia importante, lo que obliga a un reposo prolongado [205]. El exantema suele aparecer en las 24-48 h posteriores al inicio de la fiebre, con una frecuencia que oscila entre el 21 y el 57% de los casos. Puede ser generalizado, o afectar sólo a la cara y el tronco, tiene características de máculas papulosas o eritematosas y en ocasiones se presenta como urticaria, descamación de palmas y plantas o alopecia. Se describe con mucha menor frecuencia en razas no caucásicas [206]. La afectación faríngea también se presenta con mayor frecuencia en las series de pacientes de raza blanca, en las que se ha descrito en el 60% de casos. Las lesiones más frecuentes son el dolor inespecífico, el edema, el enantema o las ulceraciones. Es poco frecuente el hallazgo de exudado [207].

Con menos frecuencia se han comunicado linfoadenopatías cervicales, axilares, occipitales o generalizadas, que en algunos casos pueden persistir durante varios meses, incluso años después de la seroconversión. Las manifestaciones gastrointestinales son en su mayoría diarrea, náuseas o vómitos. Se han descrito casos de infección aguda con ulceraciones crónicas genitales como el único síntoma [208]. Se

han comunicado casos infrecuentes de pancreatitis [199,209], fallo renal agudo [210], rabdomiólisis [211,212], retención urinaria aguda [213], anemia hemolítica autoinmune [214] rotura esplénica [215,216], pancreatitis aguda y trombocitopenia [217].

Las manifestaciones clínicas más graves de la infección aguda son los cuadros neurológicos. Globalmente estos cuadros afectan hasta al 20% de los pacientes y son la causa más frecuente de ingreso hospitalario. El VIH es un virus neurotrópico, y se ha demostrado que puede invadir las células gigantes multinucleadas, las células mononucleares, las endoteliales y la microglía. La infección de los macrófagos y la glía puede resultar en toxicidad y liberación de TNF-α, e IL-1β, que promueven la liberación de metabolitos de ácido araquidónico en los astrocitos. La infección por VIH promueve astrocitosis, disminución de la captación de glutamato y alteración de la homeostasis iónica causando secundariamente disfunción neuronal o lesión secundaria al aumento extracelular de K^+ y glutamato o al aumento intracelular de Ca^{2+} [218]. Además se produce disrupción de la barrera hemato-encefálica [219], que se correlaciona con un aumento de neopterina, mayor producción de glutamato [220], mayor activación de linfocitos CD4 y CD8 [221] así como una mayor activación de macrofágos [222]. La presencia de síntomas neurológicos se correlaciona con la CV en LCR, que suele ser menor que la plasmática [223] aunque se han descrito casos aislados con CV en LCR mucho más elevada que en plasma y que se asoció a un mal pronóstico [224]. Se han publicado casos graves de encefalitis aguda [225], particularmente en la región límbica [226,227]. Las alteraciones neurológicas suelen asociarse a cambios radiológicos, las alteraciones espectroscópicas en la resonancia magnética nuclear (RMN) sugieren un rápido recambio de lípidos de membrana, lo que se interpreta como inflamación glial sin que haya gran cambio de metabolitos indicativos de gliosis o daño neuronal, demostrado por una relación colina-creatina (CHO/CR) aumentada en los ganglios

basales y sustancia gris occipital[228]. Esto se interpreta como secundario a la importante infiltración celular y habitualmente mejora con el tratamiento [229]. Como los monocitos son células susceptibles a la infección por VIH y capaces de cruzar la barrera hemato-encefálica, podrían ser uno de los principales mecanismos de establecimiento de reservorio cerebral y de lesión neurológica asociada a VIH. Los niveles altos de CHO también correlacionan con activación macrofágica de los monocitos en la periferia (CD16+) [230].

La médula espinal puede estar comprometida. Se han descrito un caso de mielitis transversa que mejoró con TARV y corticoides [231]. La afectación del sistema nervioso periférico también es frecuente, presentándose hasta en un tercio de las personas con infección aguda [232]. El síndrome de Guillain-Barre es una complicación reconocida desde 1989, en ocasiones fatal [233–235]. Se han publicado también casos de síndrome de Parsonage-Turner [236] y neuritis del plexo braquial [237].

La depresión, medida por la escala de Depresión de Beck, puede afectar hasta a la mitad de las personas, en un tercio de forma grave [238], se han descrito cuadros de psicosis en los que por biopsia se demostró encefalitis por VIH con nódulos microgliales [239].

Además, algunos pacientes pueden presentar en forma simultánea infecciones relacionadas con su conducta de riesgo e incluso infecciones de las categorías B o C del *Center for Disease Control and Prevention* (CDC). En todos los pacientes hay que investigar otras enfermedades de transmisión sexual (p. ej., sífilis, hepatitis agudas o crónicas) y en los UDI hay que considerar infecciones relacionadas con esta práctica (p. ej., endocarditis infecciosa). Dependiendo del contexto pueden observarse otras infecciones. En Europa, en particular en Reino Unido se ha documentado una mayor

incidencia de hepatitis C aguda entre pacientes con infección aguda por VIH en HSH y UDI [240].

Con respecto a las infecciones relacionadas con el VIH durante la etapa aguda, en general se trata de episodios de herpes simple o zóster, candidiasis oral o esofágica [241,242] pero también se han descrito casos de tuberculosis [243,244], toxoplasmosis cerebral [245], neumonía por *Pneumocystis jiroveci* [246] o criptosporidiosis [247]. La mayoría de estos casos se observaron en pacientes que tuvieron una disminución significativa de los linfocitos CD4 [248].

Es muy importante conocer qué síntomas son los mejores indicadores de una infección aguda por el VIH en los pacientes expuestos, con el fin de sospechar rápidamente esta infección. En un estudio prospectivo se evaluaron los síntomas que presentaron 258 estudiantes universitarios que consultaron tras una exposición sexual de riesgo, 40 de ellos presentaron una infección aguda por el VIH confirmada. Analizados en forma individual, los síntomas más sensibles fueron la fiebre (80%) y la astenia (68%) mientras que los más específicos fueron la pérdida de peso de 2,5 kg (86%) y la presencia de úlceras orales (85%), pero la sensibilidad de estos dos últimos fue menor al 40%. La asociación de síntomas más específica fue la combinación de "fiebre con exantema" (especificidad, 91%). Sin embargo, fue poco sensible (sensibilidad, 46%) [198]. En otro estudio realizado en mujeres de Kenia que consultaban por infecciones de transmisión sexual, la presencia de dos o más síntomas o signos (el estudio consideraba fiebre, vómitos, diarrea, astenia, linfadenopatías inguinales y candidiasis vaginal) se correlacionó con la presencia de infección aguda por el VIH con valores similares de sensibilidad (51%) y especificidad (83%) [249]. La combinación de síntomas muy frecuentes pero poco específicos hace que no pueda formularse una definición de caso con un valor predictivo positivo alto y obliga a mantener un elevado nivel de sospecha,

especialmente en pacientes seronegativos con una potencial exposición reciente al VIH.

La mayoría de la sintomatología publicada corresponde a los subtipos B de VIH-1. Sin embargo, se ha demostrado que diferentes subtipos pueden tener mayor frecuencia y gravedad de síntomas, ya sea por tener mayor patogenicidad o mayor *fitness* viral. Este es el caso para los subtipos C y D [250].

Finalmente, se han propuesto puntajes para medir la gravedad y que podrían predecir el riesgo de progresión y permitir identificar pacientes que se beneficiarían de un tratamiento temprano. Sin embargo, la recomendación actual de iniciar tratamiento a todos los individuos infectados limita su utilidad [251].

Diagnóstico diferencial

El diagnóstico diferencial de la infección aguda por el VIH es amplio y en la mayoría de casos deben descartarse otros procesos infecciosos y febriles [252]. El cuadro clínico de fiebre asociado a faringitis y adenopatías (clásico de la mononucleosis infecciosa) se observa sólo en el 15% de los pacientes con infección aguda por el VIH, por lo que la sospecha no debe limitarse a esta forma de presentación [253]. En estos pacientes, sin embargo, la frecuencia de pruebas falsamente positivas para VEB es excepcional, lo que hace relativamente fácil el diagnóstico diferencial entre ambas [254] Por el otro lado, si consideramos los casos de sospecha de mononucleosis infecciosa que consultan a urgencias, la prevalencia de infección aguda por el VIH fue de alrededor del 1% [32]. En otro estudio retrospectivo de los casos de fiebre, o cuadro mononucleosiforme de los que se disponía de muestras de sangre, el 1,3% era reactivo al VIH, pero lo importante de este estudio es que en el 72.7% de los casos se perdió la oportunidad de diagnosticar

la infección por VIH oportunamente. El 44% de los casos eran muestras de infección reciente por VIH [255].

En África, en zonas endémicas, la malaria es la responsable de la mayoría de los síndromes febriles, pero hasta el 1% de los casos con fiebre atribuida a malaria presentan infección aguda por VIH [256]. A pesar de esta alta carga de enfermedad, la infección aguda por VIH es un evento poco reconocido en la mayoría de las guías de manejo de fiebre en África [257]. En esta región entre un 1,2 a 2,5% de los pacientes que consultaron a una clínica de enfermedades de transmisión sexual tiene una infección aguda por VIH [32].

La primoinfección por VIH debería considerarse también en el diagnóstico diferencial de fiebre en el viajero. La incidencia de alguna infección de transmisión sexual en este grupo es de 10/1000 viajeros, siendo el 27% de estos casos infecciones agudas por VIH [258]. Sin embargo, incluso en países de alta incidencia de VIH, la infección aguda no se incluye habitualmente en el diagnóstico diferencial de estos cuadros febriles [257].

Otras infecciones a incluir en el diagnóstico diferencial incluyen la sífilis [259], la gonococia, las infecciones por citomegalovirus [260], las infecciones por los virus de la gripe, herpesvirus tipo 6, rubéola o parvovirus B19, la toxoplasmosis, las hepatitis virales en la fase anictérica, la faringitis estreptocócica, las borreliosis y algunas rickettsiosis. En ocasiones, una toxicodermia en el contexto del tratamiento antibiótico de otro proceso puede simular una infección aguda por el VIH. Ante estos casos el médico siempre debe preguntar al paciente si ha tenido algún episodio de exposición sexual en el último mes, sobre todo con parejas ocasionales y sin protección.

DIAGNOSTICO DE LABORATORIO

El diagnóstico de infección aguda ha sido siempre dificultoso debido a varios factores:

1) Poca percepción de riesgo por parte de los pacientes
2) Falta de sospecha clínica
3) Presencia de un periodo de ventana para el diagnóstico serológico
4) Baja frecuencia de testeo periódico en poblaciones de riesgo

La sintomatología variada y la falta de conocimiento entre los pacientes y entre los trabajadores de salud hacen que el cuadro clínico, aunque característico, pase desapercibido. Incluso aunque el paciente consulte a emergencias no se suele jerarquizar el diagnóstico debido a la falta de experiencia o a la omisión de recabar información sobre posibles exposiciones sexuales en las últimas semanas. Por lo tanto la frecuencia de pacientes detectados en este estadio en relativamente baja, con menos del 3% de los pacientes actualmente en seguimiento diagnosticados durante esta fase [261].

Por eso, mejorar la capacidad de diagnosticar la infección aguda cobra fundamental importancia. Se requiere aumentar el nivel de alerta en las poblaciones expuestas, mayor formación del personal de salud para reconocer estos síntomas y también cambios a nivel nacional para modificar los algoritmos de diagnóstico. Para facilitar el entendimiento de las pruebas necesarias en cada momento, es útil utilizar los estadios de Fiebig (Figura 6), en el que se grafican claramente el tiempo en el que se van positivizando distintas técnicas. Fiebig realizó este trabajo estudiando paneles de plasma construidos con muestras recolectadas en forma seriada en personas con seroconversión en las que fue observando paulatinamente el momento de la positivización de cada prueba [262].

Las pruebas que se realizan para detectar la infección por VIH incluyen entonces (ordenados según el tiempo en que se positivizan):

a) Carga viral u otra prueba de ácidos nucleicos

b) Antígeno p24

c) Serología (ELISA de 4ª generación)

d) Serología de ELISA de 3ª o 2ª generación

e) Western blot

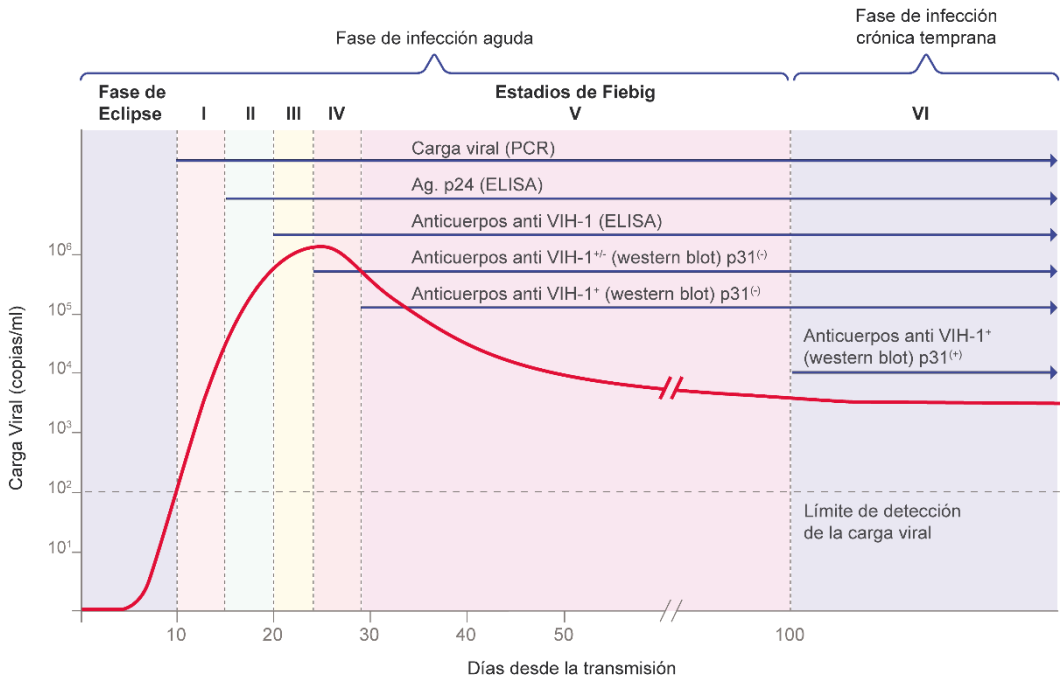


Figura 6: Estadíos de Fiebig para establecer el tiempo desde la infección al diagnóstico (Modificado de Fiebig et al. [263])

A partir de la entrada del virus al organismo se establece una fase inicial, o fase de eclipse, que dura aproximadamente 7 días y donde todas las pruebas de laboratorio, tanto para identificar anticuerpos como antígenos son negativas. A partir del séptimo día el virus ya puede detectarse en sangre por técnicas moleculares cualitativas y cuantitativas. El antígeno p24 aparece entre 4 a 5 días después de la CV. Entre 20 a 25

días de la infección la mayoría de las pruebas que detectan anticuerpos ya son positivas [264] por lo que para orientarnos si se trata de una infección aguda tendremos que buscar la documentación de una prueba negativa reciente o examinando el patrón de la prueba de western blot donde todavía puede obtenerse un resultado negativo o indeterminado. Con las pruebas de ELISA de primera y segunda generación se suele tardar al menos dos meses en detectar anticuerpos, ya que son menos sensibles que las de tercera y cuarta. Este fenómeno se utiliza para estudios de vigilancia epidemiológica, para conocer la incidencia de VIH en poblaciones recientemente diagnosticadas a las que se les realiza un ELISA de segunda y uno de tercera y se aplica un cociente entre ambos (detuned ELISA o ELISA dual) [265–268].

La sensibilidad y especificidad de las pruebas moleculares cuando el paciente tiene síntomas (lo que se estima a partir de los 14 días de la infección) es del 100% y 97% respectivamente[269]. Debido a su alto costo no se han incorporado en el diagnóstico individual en forma rutinaria, pero en muchos centros, en particular en bancos de sangre, laboratorios de centros de atención de enfermedades de transmisión sexual y en laboratorios públicos han demostrado ser costo-efectivas. En general, en estas situaciones se utilizan en forma de *pools* a fin de reducir el costo. Para esto se utiliza el material remanente de los individuos que tienen un resultado negativo por las pruebas convencionales (ELISA). Este material se divide en alícuotas y se agrupan en conjuntos de 8 a 10 muestras o grupos más grandes a los cuales se les realiza la prueba de CV. Si la CV del *pool* es positiva debe realizarse una carga viral individual a cada alícuota que formó parte de ese pool para identificar la muestra positiva [270–273].

La implementación de esta técnica resulta en un aumento importante en la detección de casos. Por ejemplo, en una experiencia limitada a HSH, se diagnosticó un 11,5% más de casos que con el ELISA de 3ª generación [274]. En otros estudios en individuos

de alto riesgo la tasa de detección fue de 39 casos cada 10.000 muestras procesadas con un costo de U$D 4.535 por caso detectado [275]. Sin embargo, su costo-efectividad en población general es discutible, ya que en esta situación la tasa de diagnóstico desciende a 6 casos cada 10.000 muestras procesadas con un costo estimado en EEUU de U$D 29.088 por caso detectado.

El costo puede ser significativamente diferentes entre países lo que puede afectar los estudios de costo-efectividad. En Tailandia en un proyecto de 4 años se realizaron 74.334 pruebas de VIH con ELISA de 4ª generación a los que se les agregó en forma rutinaria ELISA de 3ª y de 2ª generación a los casos positivos y carga viral por *pool* a los casos con serología negativa por ELISA de 4ª. Globalmente, el 10.9% de las muestras fueron positivas, se identificaron 81 casos de infección aguda mediante un test de ELISA de 4ª positivo y un test de 3ª o de 2ª generación negativo y la realización de la prueba de detección de ácidos nucleicos incrementó los diagnósticos en un 38% (31 casos adicionales), con solamente un aumento del costo del 20% [276]. En otros países, incluso de África, se encontraron tasas similares. En Nigeria entre 24.184 negativos se detectaron 9 casos positivos [277]. En Perú, en 1.191 muestras, la prevalencia de infección por VIH por Elisa de 4ª generación fue del 3,2% en población general y 10,5% en HSH y utilizando pruebas moleculares se incrementó la tasa de detección entre 5 y 8% [278]. El uso de métodos moleculares caseros puede abaratar el diagnóstico en estos lugares [279].

Cuando no hay acceso a *pool* de CV, puede utilizarse antígeno p24 en casos individuales. El antígeno se detectar en sangre también por métodos de ELISA (EIA), y se positiviza algunos días antes del inicio de los síntomas, desapareciendo con el aumento del nivel de anticuerpos en suero [280]. La sensibilidad y la especificidad de la antigenemia p24 en plasma son del 89 y del 100% respectivamente. El antígeno p24

se encuentra ahora incluido en las muestras de Elisa de 4ª generación, que se utilizan desde 2011 en Europa.

Los ELISA de cuarta generación se diseñaron para disminuir más esta ventana y combinan la detección de anticuerpos con la del antígeno. Aunque los primeros ensayos presentaban algunas dificultades ya que la sensibilidad del antígeno combinado en la prueba de cuarta generación era inferior que la sensibilidad del antígeno por separado [281,282], las presentaciones actuales muestran una sensibilidad del componente p24 comparable, y ahora se han recomendado como el estándar de prueba de cribado [283]. Su sensibilidad es alta si se realizan a partir de los 22 días de infección [284]. En un estudio de validación en Italia en más de 15.000 muestras, con ARCHITECT HIV Ag/Ab Combo (Abbott Diagnostics), y re-chequeando las muestras positivas con LIAISON-XL Murex HIV Ab/Ag (DiaSorin) que puede discriminar entre antígeno y anticuerpo todas las muestras fueron concordantes, y se pudieron identificar un 5,5% de las pruebas positivas como infecciones agudas, en las cuales en un 65% solo se detectó antígeno [285]. Su introducción se demoró en EEUU debido a que no estaba aprobado por la FDA, pero a partir de su aprobación, el CDC lo incorporó rápidamente y emitió recomendaciones para cambiar el algoritmo simplificando el proceso diagnóstico de VIH y facilitando la detección de infecciones agudas. El algoritmo actual se inicia con una prueba de 4ª generación, que en caso de ser reactiva debe ser seguida por una prueba basada en IgG capaz de discriminar VIH 1 o 2, lo que permite establecer la mayor parte de los diagnósticos. Las técnicas moleculares se reservan para los casos discordantes [286–288].

Las pruebas serológicas de cuarta generación son altamente sensibles y específicas para detectar infección general pero obviamente la sensibilidad en casos de infección aguda disminuye a medida que se acerca a la fecha de exposición. La prueba

ARCHITECT tiene una sensibilidad del 99.94% y una especificidad del 98.78% y en casos de personas con infección aguda la sensibilidad disminuye al 83% [289]. La mayoría de las pruebas de cuarta generación pueden fallar en identificar algunas cepas del grupo O y grupo P y de VIH-2. [290] A partir de la aprobación por la FDA casi todos los países están implementando ELISA de 4ª generación lo que ha permitido expandir el diagnóstico de VIH y aumentar los casos detectados en fase de infección aguda [271,291]. En Massachusetts la implementación del nuevo algoritmo basado en ELISA de 4ª aumentó el volumen de testeo, redujo la cantidad de nuevas consultas y diagnosticó un caso de infección aguda en cada mil pruebas realizadas [292]. Algunos especialistas sostienen que la realización periódica de pruebas de 4ª generación cada 6 meses podría ser más eficiente desde el punto de vista económico que la realización de pruebas moleculares [273].

Con respecto a las pruebas rápidas, cuando se basan solamente en la detección de anticuerpos no permiten identificar los casos de infección aguda. Recientemente se introdujeron pruebas rápidas capaces de diferenciar entre antígeno y anticuerpo. La más conocida es HIV-1/2 Ag/Ab Combo, una prueba que permite diferenciar la positividad por separado del antígeno y del anticuerpo y que permite diagnosticar los casos de infección aguda antes que el Determine-HIV 1/2. La sensibilidad y especificidad reportada en muestras de casos de infección aguda fue del 92 y 96%, respectivamente. Sin embargo, en varios estudios de campo la sensibilidad ha demostrado ser inaceptablemente baja por lo que se están desarrollando nuevas formulaciones de estas pruebas [293–296].

El western blot detecta anticuerpos contra proteínas específicas del VIH-1 y VIH-2 y, de acuerdo con el CDC, para ser considerado positivo se requiere la presencia de al menos dos de cuatro bandas (gp160, gp120, gp41, gp24). Los resultados con una sola

banda con o sin anticuerpos contra bandas adicionales se consideran indeterminados y requieren para ser positivos un estudio posterior con el mismo criterio. Hasta hace poco era considerado el método de elección para confirmar la serología de VIH. Durante la fase aguda es negativo o indeterminado y a partir de la cuarta semana de infección ya suele ser positivo y durante las semanas posteriores se irán añadiendo bandas adicionales. La banda gp31 suele aparecer a las 12 semanas y su ausencia hace sospechar una infección reciente [297].

Claramente se requiere innovación para expandir el diagnóstico de infección aguda y los avances recientes permiten mejorar la eficiencia del diagnóstico [298]. Se necesita implementar innovaciones, no solo tecnológicas, sino también en el enfoque, expandiendo masivamente el uso de pruebas rápidas, brindando la posibilidad de autotesteo, y utilizando enfoques complementarios [299,300]. Algunas experiencias positivas incluyen por ejemplo un estudio en Kenia donde el personal de las farmacias ofrecen a sus clientes que concurren sin prescripción o con enfermedades de transmisión sexual un cupón para diagnóstico de VIH, la mayoría de las personas lo aceptan y una cuarta parte lo realizan identificando una proporción significativa (4% en este estudio) de casos positivos [301]. Otra estrategia es la búsqueda activa en departamentos de urgencias, donde en algunos lugares hasta una cuarta parte de los nuevos diagnósticos de VIH eran casos agudos [302]. La gran dificultad para expandir estas estrategias es el número de personas que tienen que ser evaluadas para identificar casos positivos, lo que obliga a invertir tiempo y recursos en esta actividad [303].

Otro avance importante es la disponibilidad de métodos moleculares en el punto de atención para la detección del virus, de uso fácil, bajo costo y que se podrían utilizar para el diagnóstico de la infección aguda. El Alere q Analizer HIV1/2 y el SAMBA HIV son las dos plataformas más avanzadas que están siendo evaluadas para

diagnosticar la infección aguda, junto con otras metodologías como las técnicas de amplificación isotérmica [304].

Finalmente, se debe mencionar que existen herramientas que se utilizan en estudios epidemiológicos y que se basan en la madurez de los anticuerpos que se utilizan, como el algoritmo START, BED-CEIA o las pruebas de avidez. Estas pruebas no pueden utilizarse para casos individuales debido varias circunstancias facilitan que no se clasifiquen a los pacientes, como la infección avanzada, subtipos no-B, individuos EC, o supresión viral, etc.,[305–309]. Actualmente se desarrollaron los algoritmos multiensayos para realizar estudios transversales [310–313], en los que se aplican en forma secuencial diferentes ensayos para clasificar las muestras de acuerdo al tiempo probable de infección y que se están implementando en estudios clínicos como el HPTN-043.

En resumen, la clave del diagnóstico se basa en la sospecha clínica y en realizar las pruebas de laboratorio en el momento adecuado. La detección temprana es importante para el manejo del paciente ya que permite aprovechar los potenciales beneficios del tratamiento temprano y con fines epidemiológicos porque permite estimar mejor la incidencia, evitar transmisiones secundarias [314] y detectar tempranamente los cambios en la epidemia [315]. Algunas ciudades han incorporado la búsqueda activa de estos casos como una herramienta para el control de la epidemia de VIH [316,317]. Claramente para expandir el tratamiento se necesitan nuevos enfoques diagnósticos y el avance de la tecnología está creado oportunidades para lograr esta ampliación del testeo.

RESISTENCIA PRIMARIA EN PERSONAS CON INFECCION AGUDA

La prevalencia de la transmisión de resistencias a los fármacos antirretrovirales en una población determinada puede variar en función del tipo de prueba empleada (genotípica o fenotípica), de los valores de corte de los estudios fenotípicos, de las mutaciones utilizadas como indicadoras de resistencia en las pruebas genotípicas, de la utilización o no de métodos ultrasensibles [318], de las características epidemiológicas de la población estudiada y de la disponibilidad y las estrategias de implementación del TARV en un área o región determinada [319].

La primera evidencia de transmisión de una cepa de VIH resistente a la zidovudina se describió en 1993 en un caso de transmisión vertical [320]. En la actualidad se reconocen mutaciones específicas asociadas con resistencia al tratamiento para todos los tratamientos disponibles [321], lo que conlleva un riesgo potencial de transmisión.

En los EEUU, la frecuencia de transmisión de virus resistentes aumentó durante los primeros años de la terapia antirretroviral debido al uso de mono o biterapia con inhibidores de la transcriptasa reversa (INTR). En 1996 la frecuencia de resistencia a estos fármacos alcanzaba un 20% [322] y a partir del año 1998 la resistencia a los INTR disminuyó en forma constante, con un aumento simultáneo de hasta el 10% de la resistencia a los inhibidores de proteasa (IP) y en menor proporción para los inhibidores no nucléosidos de la transcriptasa reversa (INNTR) [322,323]. La expansión del tratamiento produjo una disminución de los pacientes virémicos y una estabilización de la resistencia transmitida a menos del 10% [324].

Las mutaciones asociadas a resistencia a las distintas familias de antirretrovirales pueden persistir en el tiempo y la posibilidad de detectarlas depende de la capacidad

replicativa *(fitness)* que le imponen al virus estas mutaciones [325,326]. Los virus con menor fitness que establecen una infección aguda suelen mantenerse en el tiempo, y se asocian con un mejor curso clínico, incluyendo una mayor capacidad para mantener los niveles de linfocitos CD4 [327].

TRATAMIENTO ANTIRRETROVIRAL

Desde el año 1987, cuando se utilizó por primera vez zidovudina para el tratamiento del VIH, hemos sido testigos de uno de los avances más rápidos en desarrollo de fármacos que resultó en la disponibilidad de más de 25 medicamentos diferentes. Los primeros esquemas basados en 2 INTR demostraron rápidamente los inconvenientes de las dosis, la toxicidad a largo plazo y la emergencia de resistencia asociada a la incapacidad de suprimir completamente la carga viral. En 1996 el manejo del tratamiento antirretroviral se revolucionó con la aparición de los IP y los INNTR. Por primera vez la combinación de una de estas drogas con 2 INTR permitió suprimir completamente la carga viral y reducir drásticamente la morbilidad y la mortalidad [328,329].

La carga de pastillas, la baja potencia de las primeras combinaciones, las dificultades farmacocinéticas de algunas drogas y la toxicidad aguda y a largo plazo y dificultaron la implementación de la recomendación inicial que se basaba en el aforismo *"hit hard, hit early"* [330]. A partir del año 2001 predominó el enfoque conservador e incluso se llevaron a cabo grandes estudios clínicos para evaluar el efecto de reducir la exposición a medicamentos mediante suspensiones periódicas o "vacaciones de tratamiento". El estudio más importante en esta línea fue el estudio SMART [331], que contrariamente a su hipótesis, sirvió para demostrar que la suspensión del tratamiento se asocia con un

mayor riesgo de desarrollar enfermedades asociadas a SIDA. De forma todavía más importante, este estudio puso en evidencia la estrecha asociación entre la replicación viral, la inflamación y el aumento de enfermedades no asociadas y co-morbilidades.

Actualmente disponemos de cinco clases de fármacos antirretrovirales: los inhibidores nucléosidos de la transcriptasa reversa (INTR), los inhibidores no nucléosidos de la transcriptasa reversa (INNTR), los inhibidores de la proteasa (IP), los inhibidores de la entrada [en este grupo se incluyen los antagonistas de los receptores de quimioquina CCR5 (maraviroc), el inhibidor de la fusión (enfuvirtide) y el inhibidor de la unión (fostemsavir) que se encuentra todavía en desarrollo], y finalmente los inhibidores de la integrasa (INI). Cada droga tiene su diana en un paso diferente del ciclo viral, desde la unión, el ingreso a la célula, la retro-transcripción, la integración y la liberación de partículas virales. Existen también en desarrollo avanzado un inhibidor de la maduración del virus (BMS-955176).

En los últimos años se produjeron avances muy importantes en el desarrollo de nuevos medicamentos incluyen la comprensión de la toxicidad mitocondrial que estimuló a abandonar los primeros INTR (zidovudina, estavudina, didanosina, zalcitabina), la identificación de nuevos inhibidores de proteasa con menor afectación metabólica y que pueden administrarse una vez al día [332,333], la disponibilidad de co-formulaciones de una sola toma diaria que facilitaron la expansión del tratamiento, el descubrimiento de los inhibidores de la integrasa (una familia con excelente potencia y perfil de seguridad) y el impacto de las comorbilidades, lo que re-direccionó la investigación hacia nuevas combinaciones más simples y con menor toxicidad. Ante la imposibilidad de lograr la erradicación, los objetivos del tratamiento siguen siendo los mismos que en las últimas décadas: suprimir la carga viral en forma sostenida para

mejorar las funciones inmunes y preservar las opciones futuras de tratamiento, mejorar la calidad de vida y reducir la morbimortalidad.

Pero sin duda uno de los avances más importante del último año ha sido poder cerrar la discusión de cuando comenzar el tratamiento debido a que contamos con la evidencia clínica necesaria para afirmar sin lugar a dudas que todas las personas con VIH necesitan iniciar tratamiento lo antes posible. Los estudios HTPN052 [187], START [334] y TEMPRANO [335] demuestran que incluso con valores altos de CD4, la infección por VIH no tratada se asocia con un mayor riesgo de morir, desarrollar SIDA o presentar enfermedades no asociadas a SIDA y que este riesgo se puede reducir con TARV, lo que ha influenciado todas las guías internacionales de tratamiento.

En este contexto, se refuerza todavía más la importancia de empezar tratamiento antirretroviral durante la infección aguda por VIH. Un importante número de razones teóricas apoyan el inicio inmediato de tratamiento en personas con síndrome retroviral agudo, incluyendo la posibilidad de disminuir la duración e intensidad de los síntomas agudos y mejorar la calidad de vida [119], limitar la pérdida brusca de células CD4 que ocurre en las etapas tempranas de la infección [336], evitar la diversificación viral [337,338], restaurar mejor las respuestas inmunes [339,340], disminuir la inmunoactivación, y eventualmente limitar el tamaño de los reservorios virales lo que podría eventualmente ayudar a controlar espontáneamente la infección en caso de suspensión [341,342].

La infección y la persistencia del VIH de las células CD4 de memoria que luego formarán parte del reservorio se inicia muy tempranamente. Se pudo demostrar que el inicio del tratamiento antirretroviral en fases muy tempranas (Fiebig I y II) puede reducir el porcentaje de estas células en el reservorio total, logrando un perfil similar al que presentan los individuos EC [127]. A nivel de mucosa intestinal los daños más

importantes se observan en el estadio III de Fiebig. El inicio de tratamiento en estadios Fiebig I y II previene la pérdida inmune de células Th17 en el intestino, mejora la función celular y resuelve la inmunoactivación local y sistémica [131]. Además, el tratamiento virtualmente "congela" la infección limitando la cantidad de virus en replicación activa y de esta forma frena la posibilidad de diversidad viral [343].

Otro beneficio que se observa casi exclusivamente en pacientes tratados en fase temprana es normalizar las cifras de linfocitos CD4. En una cohorte prospectiva de 353 pacientes con infección aguda, el 45% de los que iniciaron tratamiento dentro de los 6 meses de infección lograron normalizar la relación CD4/CD8 (>1), frente a un 11% que lo iniciaron después [344]. En otra cohorte seguida durante 48 meses que incluyó 597 pacientes con una mediana de CD4 de 495 células/mL el 64% de los individuos que iniciaron tratamiento dentro de los 4 primeros meses de infección alcanzaron una cifra de linfocitos CD4 mayor de 900 células/mL frente al 34% de los individuos que iniciaron el tratamiento después [345]. Normalizar los valores de CD4 puede tener implicancias clínicas, de acuerdo a un estudio que muestra que las personas que no logran alcanzar valores de CD4 mayores de 750 células/mL se encuentran en mayor riesgo de desarrollar infecciones oportunistas [346].

Durante la pasada década se diseñaron varios estudios (tabla 4) que evaluaron el impacto de un periodo limitado de TARV para determinar si era posible lograr controlar la replicación viral en ausencia de medicamentos antirretrovirales. El interés surgió a partir de la observación del primer "paciente de Berlín", publicado en 1999, un paciente tratado desde la infección aguda por el VIH que suspendió en forma intermitente el TARV en dos ocasiones por infecciones intercurrentes, seguidas de la suspensión definitiva del TARV y en el que no se observó rebote viral durante un seguimiento de 2 años [347].

TABLA 4: Estudios de cohorte de tratamiento antirretroviral en pacientes con infección aguda o reciente por el VIH-1 publicados desde el año 2010

Estudio	Pacientes	Diseño	Comentarios
Steingrover[348]	332 pacientes 64 comenzaron TARV 32 suspendieron	Cohorte	32 suspendieron el TARV y comparados con 250 sin tratamiento presentaron una reducción del *set-point* durante 83 semanas. El tratamiento fue más frecuente en los que tenían alta CV.
Koegl[349]	156 pacientes 100 recibieron TARV (9 meses)	Cohorte	TARV no bajo el *set-point* pero tuvo un impacto positivo en los CD4 y demoró la progresión a <350 cél/mm3.
Streeck[350]	20 pacientes 12 tratados por 6 meses	Cohorte	Aumento CD8 específicos anti VIH No diferencias en CV a 6 meses
Hecht[351]	45 pacientes con inf. reciente 13 pacientes inf aguda TARV 12 semanas 337 sin tratamiento	Cohorte	Disminución transitoria de la CV y mejor CD4 que el grupo sin tratamiento que se sostuvo 6 meses post-suspensión
Seng[352]	170 pacientes con TARV 123 pacientes sin TARV	Cohorte PRIMO y SEROCO	Rápida caída de CD4 hasta 5 meses post-suspensión y después lento. A 3 años no se observó beneficio clínico.
Fox[353]	28 pacientes tratados 3 meses	Prospectivo una rama	89% tenían respuestas específicas CD4 basal y 45% las mantuvo a los 3 años pero no tuvo relación con la respuesta virológica o clínica.
Pantazis[354]	348 tratados, 147 suspendieron TARV (38 6 meses, 40 de 6 a 12 y 69 mas de 12) 675 sin tratamiento	Cohorte	Pérdida de CD4 importante en los primeros 6 meses de la suspensión. Posteriormente similar a la rama sin tratamiento. No hubo diferencias en el *set-point* viral.
Zugna[355]	1627 tratados antes de 1 año y 2710 que iniciaron en infección crónica	Cohorte comparó TARV agudo con crónico	No hubo diferencias en la eficacia virológica o fallo entre iniciar en estadio agudo o crónico
Wyl [356]	33 tratados por media de 37 meses 79 sin tratamiento	Cohorte	Al año de suspensión en los tratados la CV era 0,8 log más baja pero desaparecía a los 3 años
Stekler [357]	40 tratados <30 días 82 tratados 31-180días 35 tratados >180 días 87 sin tratamiento	Cohorte	CD4 al año fue peor en los no tratados Tiempo a diagnóstico de evento asociado fue mayor (aHR 3,78) en los que no se trataron.
Hocqueloux [358]	35 infección aguda 207 infección crónica 4 años de seguimiento	Cohorte	ADN VIH < IA (2,15vs 2,84 log10c/mL) Recup. CD4 > IA (883 vs 619 cél/mm3) Relación CD4/CD8 > IA (1,31 vs 0,77)

Poco tiempo después Walker et al [341] describió 8 pacientes tratados desde la primera semana de los síntomas de seroconversión que suspendieron el TARV logrando cinco de ellos mantener una CV menor de 500 copias en los siguientes 6 meses y en los que el control se atribuyó a una mejora de las respuestas inmuno-específicas. La mayoría de estos estudios, que incluían periodos de 48 a 144 semanas de tratamiento seguidos de suspensión definitiva, fallaron en demostrar beneficio clínico. En algunos casos se pudo constatar una disminución del *set-point* viral [348], en otros no mejoró el *set-point* pero se demoró la pérdida de linfocitos CD4 [349]. La mejora de las respuestas inmuno-específicas fue variable.

Estos datos se confirmaron con estudios aleatorizados que exploraron un periodo limitado de TARV (tabla 5) en los cuales una pauta limitada de tratamiento retardó la progresión de la enfermedad durante pero al suspenderlo la infección progresaba [359]. En el estudio PRIMO-SHM en Holanda, se aleatorizaron pacientes a no recibir tratamiento o a tratarse durante 24 o 60 semanas, demostrando que el tratamiento podía bajar el *set-point* viral en 0,5–0,8 log10 copias/ml, aumentar las células CD4 y retardar la necesidad de reiniciar el tratamiento, aunque otra vez, estos beneficios se perdían después de 1 a 2 años [360]. La causa de la reducción del *set-point* no está clara y en este estudio no hubo diferencias en la respuestas inmuno-específicas entre ambas ramas, aunque se observó una discreta mejoría en la capacidad citolítica y en la producción de perforinas en los casos tratados [361]. Otro estudio destinado a evaluar el mismo objetivo debió suspenderse antes de tiempo debido a que la rama sin tratamiento presentaba una progresión clínica más alta que lo esperado [362]. El estudio OPTIPRIM, diseñado más recientemente proporcionó tratamiento en ambas ramas del estudio ya que su objetivo era evaluar si un tratamiento intensificado con 5 fármacos podía reducir el reservorio viral a los 2 años de tratamiento en comparación con el

tratamiento estándar. Aunque la tolerancia fue buena, los niveles de ADN proviral en ambas ramas fueron similares [363].

La información revisada (tabla 5) permite concluir que los tratamientos en la fase aguda se asocian con un impacto positivo significativo que se pierde cuando se suspende el mismo en un periodo variable de tiempo.

TABLA 5: Estudios aleatorizados de tratamiento antirretroviral en pacientes con infección aguda o reciente por el VIH-1 publicados desde el año 2010

Estudio	Pacientes	Diseño	Comentarios
SPARTAC [359]	366 pacientes analizados con infección < 6 meses. Seguimiento 4,2 años.	Estudio aleatorizado 1:1:1: a) TARV 48 sem b) TARV 12 sem c) No TARV Variable de resultado: <350 CD4 o TARV	HR progresión: a) 0,63 (IC95% 0,45 a 0,9) b) 0,93 (IC95% 0,67 a 1,29) c) Referencia La rama de 48 semanas de tratamiento redujo retardo la progresión durante aproximadamente un año. La rama de 12 semanas no tuvo beneficio. Hubo un aumento similar de las respuestas específicas en la rama a y b.
PRIMO SHM [360]	168 pacientes analizados	Estudio aleatorizado 1:1 a) 60 semanas de TARV b) 24 semanas de TARV c) No TARV Variable de resultado: CV a las 36 semanas sin tratamiento	*Set-point* CV a 36 semanas a) 4,3 log10 b) 4,0 log10 c) 4,8 log 10 Los pacientes aleatorizados a tratamiento tuvieron CD4 significativamente más altos.
SETPOINT [362]	130 analizados Pacientes con infección reciente (no aguda)	Estudio aleatorizado 1:1 a) 36 semanas de LPV/r-TDF-FTC b) No TARV Variable de resultado: CV a la semana 72-76 Seguimiento: 96 semanas	Cerrado antes de tiempo por una tasa inesperadamente alta de necesidad de tratamiento. Rama a) 10% requirió TARV Rama b) 50% requirió TARV
OPTIPRIM [363]	90 pacientes analizados con WB incompleto y síntomas o <500 CD4	Estudio aleatorizado 1:1 a) RAL-MVC-DRV/r-TDF-FTC b) DRV/r-TDF-FTC Seguimiento 24 sem. Variable de resultado: DNA proviral en PBMC.	DNA proviral: a) 2,35 [RIQ 2,05-2,50] $\log_{10}$ per 106 PBMC b) 2,25 [RIQ1,71-2,55] Un régimen de 5 drogas no fue más efectivo que uno de 3.

Aunque estos estudios no lograron estimular el control de la replicación viral, en la práctica se siguen encontrando pacientes que logran algún grado de control virológico en ausencia de tratamiento. En diversas cohortes hasta un 5 a 10% de los pacientes tratados desde la infección aguda pueden mantener la supresión viral posterior a la

suspensión durante algún tiempo, dependiendo de las definiciones utilizadas[364,365]. Las cohortes francesas de personas con infección aguda permitieron identificar pacientes que después de suspender el tratamiento eran capaces de mantener la CV baja (<50 copias/mL). Un estudio que incluyó 163 pacientes mostró que el 8,5% pudo mantener la CV indetectable al año de la suspensión del tratamiento, introduciendo el nuevo concepto "controladores post-tratamiento" [366]. En la cohorte CASCADE la frecuencia del control fue menor (4,3%) y se sostuvo por un tiempo más corto (1,7 meses) [367]. La duración del tratamiento, y otras variables pueden influir en las diferencias. En el estudio SPARTAC el 17% de los pacientes que recibieron tratamiento prolongado (> 12 semanas) logró mantener la CV indetectable después de suspender el tratamiento, frente a 9% de los que recibieron tratamiento corto [368] y este control se pudo relacionar con un menor reservorio viral [369]. En estos pacientes denominados "controladores post-tratamiento" se pudo establecer que el control no se correlaciona con una mejor respuesta citotóxica inmuno-específica ni con un perfil genético beneficioso lo que los diferencia de los pacientes EC. En 14 casos descritos en Francia en la cohorte Visconti, el único factor asociado a control virológico después de suspender el TARV fue tener un nivel muy bajo de virus residual al momento de la suspensión [370]. Casos similares se describen ocasionalmente. Recientemente, un bebé que inició tratamiento a las pocas horas del nacimiento y que lo recibió durante aproximadamente un año pudo controlar por un periodo limitado la replicación viral lo que se asoció también a reservorios limitados [371]. La CV permaneció indetectable durante 22 meses, y durante este periodo no se pudo detectar replicación competente ni respuestas inmuno-específicas frente al VIH. Los hallazgos hacen suponer que se trataba de un caso de control transitorio por un bajo reservorio sin embargo la infección reapareció durante el seguimiento [372].

Debido a que el reservorio bajo al momento de la suspensión es la única variable asociada cobra relevancia un estudio realizado en Tailandia donde se demostró que el inicio de tratamiento durante los estadíos Fiebig I y II puede lograr mantener valores extremadamente bajos de reservorios en sangre periférica (medido por ADN viral integrado) [132] y se refuerza la necesidad de iniciar el tratamiento lo antes posible a fin de limitar el reservorio viral y poder en el futuro beneficiarse de otras estrategias para lograr la erradicación viral [373].

EL TRATAMIENTO ANTIRRETROVIRAL COMO PREVENCION

El efecto beneficioso del TARV en reducir la transmisión del VIH está bien documentado a partir de la publicación del estudio ACTG 076 que demostró que un curso de zidovudina en mujeres VIH embarazadas reducía la transmisión materno infantil del 22.6% al 7,6% [374]. Durante las relaciones sexuales la transmisión se reduce a valores casi insignificantes cuando el individuo VIH se encuentra bajo un TARV efectivo [187,188,375]. A nivel comunitario varios estudios ecológicos realizados en Canadá [376], China [377] y Sudáfrica [378] muestran cómo la expansión de los programas de tratamiento se asocian a una reducción en la incidencia de nuevas infecciones por VIH.

Estos estudios proporcionan la base de evidencia para la estrategia de "tratamiento como prevención" donde la reducción de la CV comunitaria, mediante la expansión generalizada del diagnóstico y el tratamiento inmediato de los casos positivos podría reducir la transmisión del VIH en la comunidad a niveles que permitan la eliminación de la epidemia. La implementación de esta estrategia va a requerir una importante inversión de recursos y esfuerzos excepcionalmente grandes [379].

La fase aguda de la infección es un periodo de altísima transmisibilidad, además, se estima que entre 20 y 50% de las nuevas infecciones se adquieren de una persona con infección aguda o reciente por VIH [380,381]. Por lo tanto, una tasa alta de personas en fase de seroconversión dentro de una comunidad podría limitar la eficacia de estas intervenciones. En un modelo matemático en Malawi, donde se estima que un 38% de las nuevas infecciones se asocian a un caso de infección reciente, se estableció que si no se consideran los casos de infección aguda la eliminación al largo plazo del VIH es prácticamente imposible y requeriría el diagnóstico y tratamiento de la totalidad de casos con infección crónica de la comunidad. La adicción de intervenciones para identificar y tratar personas en estadios tempranos de infección mejora dramáticamente el modelo con una reducción importante de la incidencia de nuevos casos y la posibilidad de eliminación de la transmisión a los 30 años [382].

El principal desafío para identificar individuos en fases tempranas de infección es mejorar el diagnóstico. Se deben identificar y disminuir las barreras de acceso a la prueba, en particular para aquellas personas en mayor riesgo de infección. La falta de percepción de riesgo, la necesidad de concurrir varias veces para realizar la prueba y buscar los resultados y la ausencia de innovación como por ejemplo la promoción del autotesteo son las barreras más frecuentemente mencionadas [383]. Pero aunque los pacientes consulten a tiempo los médicos usualmente perdemos la oportunidad de diagnosticar al 70% de los casos, incluso en presencia de síntomas clásicos o reconociendo una exposición de riesgo [384]. Por ello es muy importante sensibilizar y mejorar el conocimiento sobre el síndrome retroviral agudo entre los médicos y también entre las poblaciones en mayor riesgo ya que diversos estudios muestran que más de la mitad de las personas con mayor exposición desconoce los síntomas de infección aguda [385,386].

Otra dificultad es la escasa prioridad que tiene para los sistemas de salud identificar los casos de infección aguda [387]. Esto se debe en parte a la necesidad de implementar tecnologías moleculares que permitan un diagnóstico temprano [287,388] o de realizar campañas para repetir las pruebas con más frecuencia en grupos afectados.

Estos conceptos justifican la necesidad de implementar a sistemas de diagnóstico integrados desde los laboratorios de referencia con los sistemas de vigilancia epidemiológica. Esto debería incluir la definición de caso, la identificación apropiada, la verificación de la información de laboratorio y la recolección de información clínica, la comunicación activa con el individuo para asegurar brindar la información necesaria y comenzar rápidamente el tratamiento y brindar otros servicios como notificación y manejo de contactos [317]. Al implementar estos programas se deberían considerar que existen experiencias exitosas en algunas ciudades que pueden servir de orientación para estos esfuerzos [389].

PERSPECTIVAS DE CURACIÓN DEL VIH-1

Podría decirse que el tratamiento antirretroviral iniciado en forma temprana permite reestablecer el daño ocasionado al sistema inmune, y en forma sostenida puede lograr una situación inmuno-virológica similar a la de los individuos EC, tanto en término de replicación residual, volumen de los reservorios y el perfil funcional de linfocitos CD4 y CD8 [390]. Por eso estos pacientes se encuentran en un lugar privilegiado a la hora de seleccionar pacientes para evaluar futuras estrategias de erradicación.

De hecho, en un estudio cualitativo en Londres en el que se entrevistó a personas con diagnóstico de infección aguda por VIH se evidenció la importancia que tiene para los pacientes iniciar el tratamiento en forma temprana como una estrategia "puente" hasta contar con un tratamiento curativo y la preocupación por la potencial toxicidad y la

necesidad de contar con mayor información sobre la eficacia de las distintas opciones [391]. La disponibilidad actual de fármacos con mejor perfil de seguridad y la evidencia incuestionable de los beneficios del TARV resuelven estas preocupaciones. Es muy importante brindar información adecuada tanto a los individuos de mayor riesgo, como a las organizaciones sociales que los nuclean, sobre la importancia del diagnóstico y el beneficio del tratamiento y las esperanzas futuras [392].

En los últimos años se ha renovado el interés por la agenda de la curación. Aun cuando las personas con VIH tienen una expectativa de vida cercana a la de la población general, la inflamación asociada al virus genera un exceso de mortalidad asociado a enfermedades no relacionadas al SIDA, problemas cardiovasculares y cáncer [393]. En la búsqueda de la cura se han producido avances muy importantes, como la identificación de las diferentes poblaciones celulares que componen el reservorio viral [394], una mejor la descripción de los mecanismos que regulan las respuestas inmunes [395], los mecanismos de eliminación de células CD4 infectadas [57], en los mecanismos de latencia y como evadirlos [396], estrategias para reducir los reservorios [397], entre muchos otros.

Uno de los mayores desafíos actuales es poder estandarizar los estudios de laboratorio para medir los reservorios [125]. Más del 90% del ADN viral que puede ser medible por los estudios estándar no replica y por lo tanto no debería ser considerado parte del reservorio. Se necesitan métodos que puedan describir y explicar mejor la discrepancia entre los métodos basados en cultivo viral y métodos moleculares. Eriksson comparó diferentes métodos frente al laborioso método de crecimiento viral de células CD4 infectadas en 30 personas bajo tratamiento en las que pudo evidenciar grandes diferencias en las frecuencias de células infectadas, debido a la mala sensibilidad de los estudios de PCR para detectar replicación activa y por la detección de células

infectadas con virus defectuosos [124]. Por eso la estandarización y la validación clínica de métodos para cuantificar el reservorio viral es una prioridad, siendo necesarios métodos que puedan medir no solo el virus dentro de células CD4 sino también en otras células y tejidos (macrófagos, monocitos, tejido gastrointestinal, etc).

El enfoque terapéutico requiere medidas adicionales al TARV. El tratamiento combinado [363], incluso iniciado en fases muy tempranas [132], no ha sido suficiente para lograr controlar la infección sin tratamiento. El virus integrado en células latentemente infectadas continúa siendo una barrera para la erradicación. Los inhibidores de la histona desacetilasa (HDACi) son capaces de reactivar la latencia en células infectadas pero no han podido reducir la cantidad total de ADN integrado [397]. Se han evaluado vorinostat, romdepsina y panobinostat con resultados positivos pero se deben identificar fármacos que puedan eliminar las células que reinician la transcripción viral.

Estas células podrían ser eliminadas por el sistema inmune, por lo que se siguen buscando formas de mejorar las respuestas específicas CD4, CD8, la inmunidad innata y los anticuerpos neutralizantes. Algunos autores proponen mejorar las respuestas citotóxicas antes de estimular la reactivación del virus latente basado en estudios *in vitro* que han demostrado ser efectivos [398]. En este sentido,

Se están ensayando enfoques combinados con HDACi más vacunas terapéuticas (basadas en vectores ADN, vectores virales o de células dendríticas), citocinas, o inmunomoduladores como inhibidores de PD-1 [399]. Las vacunas de células dendríticas autólogas demostraron generar una fuerte respuesta específica lo que las posiciona como un elemento importante a ser combinado con otras intervenciones en estudios de de erradicación [395,400]. Se han propuesto diferentes adyuvantes que puedan aumentar

la inmunogenicidad de estas vacunas como vectores virales [401], adenosín deamidasa [402] o nanopartículas cargadas con antígeno p24 [403]. Estas vacunas han podido demostrar una mejoría de la función inflamatoria de las células NK[404], una mejora en las respuestas inmunoespecíficas que en una proporción significativa se asoció a una reducción de más de 1 logartimo de CV [405] y aunque no disminuye el reservorio viral puede contener en forma transitoria el aumento importante en los reservorios en los pacientes que suspenden el tratamiento [406].

Los anticuerpos neutralizantes monoclonales son otros candidatos a ser utilizados en inmunoterapia y se han probado en modelos animales con buena respuesta. Los anticuerpos dirigidos a la unión de CD4 o a la región V3 redujeron la viremia y el ADN proviral aunque se detectaron situaciones de escape viral [407,408]. En la tabla 6 se mencionan algunas estrategias actualmente en estudio.

Tabla 6: Estrategias que exploran la curación en pacientes con infección aguda por el VIH-1 (Modificado de Thornhill [133])

Estudio	Lugar	Fase	Intervención.
NCT02231281	China	III	Linfocitos T autólogos VIH específicos + TARV vs TARV solo
NCT02018510	USA	I	Ac monoclonal neutralizante (3BNC117)
NCT01950325	USA	I	Ac monoclonal neutralizante (VRC601)
NCT02028403	USA	I	BMS936559 (anti-PD1) en pacientes suprimidos
CHERUB001	UK	I	Inmunoglobulina IV en pacientes agudos
NCT01365065	Australia	II	Vorinostat en pacientes suprimidos
NCT01319383	USA	I/II	Vorinostat en pacientes suprimidos
RIVER	UK	II	Vorinostat + vacuna (ChAdV63.HIV+MVA.HIVconsv)
NCT01933594	USA	I/II	Romdepsina en pacientes suprimidos
REDUC	Denmark	I/II	Romdepsina + vacuna (Vacc-4x)+GM-CSF
NCT01944371	USA	I/II	Disulfiram en pacientes suprimidos
NCT02071095	USA	I/II	Poli-ICLC (agonista TLR-3) + TARV en pac.agudos

En resumen, la búsqueda de las combinaciones que puedan posibilitar la cura funcional en personas con infección aguda es amplia. Con la disponibilidad de fármacos seguros y menos tóxicos, la evidencia de una larga expectativa de vida y los riesgos

desconocidos de algunas intervenciones propuestas, en particular de aquellas que requieren suspender el tratamiento, se impone la necesidad de discutir los límites éticos de la incorporación de humanos en algunas de estas estrategias [133]. El camino es difícil y seguramente todavía es largo, pero es importante mantener el ritmo y la esperanza.

JUSTIFICACIÓN

Existen muchos motivos por lo que se resulta de gran importancia profundizar el estudio de la infección aguda por el VIH y diseminar los resultados [30,32,324,409,410].

Desde el punto de vista epidemiológico: la infección aguda es el período con mayores tasas de transmisión de la infección [31,315,380,411,412]. Entre el 30 y el 50% de las nuevas infecciones están directamente relacionadas a una fuente con infección aguda por el VIH [413, 414]. Los valores muy elevados de CV, junto a la falta de uso de métodos de barrera adecuados, por el desconocimiento del diagnóstico, permiten explicar un riesgo de transmisión que se ha calculado en 500 veces superior al de la fase crónica [415].

Desde el punto de vista inmunopatológico: es una oportunidad única para estudiar los mecanismos inmunológicos, virológicos y genéticos implicados en la transmisión y la patogenia de la infección por el VIH y las interacciones iniciales entre el sistema inmunitario y el virus que determinarán la evolución posterior del individuo [416].

Desde el punto de vista terapéutico: el diagnóstico precoz de la infección aguda por el VIH puede permitir instaurar un TARV adecuado que, por un lado, puede acortar la duración de la enfermedad sintomática en los casos graves, generalmente asociada a una viremia muy elevada [417] y, por otro, puede evitar el daño del sistema inmunitario, reconstituirlo rápidamente y cambiar la historia natural de esta infección[10].

Desde el punto de vista sanitario: la identificación temprana facilita los estudios de incidencia [418], mejora la sensibilidad de los estudios de prevalencia de resistencia [419] y permite identificar y referir los contactos expuestos e iniciar tratamiento para reducir el riesgo de transmisión.

HIPOTESIS

1.- Es posible identificar y crear cohortes de pacientes con infección aguda por el VIH-1 en ciudades de países con diferentes niveles de renta que permitan describir las características y la evolución de estos pacientes

2.- Con la generalización y la mayor eficacia TARV combinado, la tasa de trasmisión de resistencia en los pacientes con infección aguda por el VIH-1 debe disminuir en relación con el periodo pre-TARV combinado

3.- Es posible identificar factores clínicos, virológicos e inmunológicos que identifiquen a los pacientes con infección aguda por el VIH-1 que tengan un mayor riesgo de progresión clínica y que por tanto se puedan beneficiar de un TARV precoz.

4.- El TARV por sí solo no puede erradicar la infección por el VIH. El TARV combinado con suspensiones estructuradas del tratamiento ("autovacunación") y terapias inmunomediadas (IL-2) podría preservar y aumentar la respuesta VIH específica (proliferativa y citotóxica) y posibilitar el control inmunológico de la infección por el VIH en ausencia de TARV, de forma similar a lo que ocurre con los individuos EC.

OBJETIVOS

OBJETIVO GENERAL

Mejorar el conocimiento sobre la infección aguda por VIH-1 y de los factores asociados con la respuesta al tratamiento.

OBJETIVOS ESPECÍFICOS

1. Describir las características clínicas de los pacientes con infección aguda por VIH-1 en Barcelona (España) y Buenos Aires (Argentina)
2. Describir la prevalencia de la trasmisión de resistencias y su evolución en el tiempo en pacientes con infección aguda por VIH-1 en Cataluña (España)
3. Identificar factores asociados a la progresión clínica e inmunológica en pacientes con infección aguda por VIH-1
4. Evaluar el impacto a corto y largo plazo de las interrupciones estructuradas del TARV combinadas con tratamiento inmunomediado en pacientes con infección aguda por el VIH-1

ARTICULOS SELECCIONADOS

ARTÍCULO I

Enferm Infecc Microbiol Clin. 2006 Apr; 24(4):238-44.
Primary human immunodeficiency virus type 1 infection: clinical, virological and immunological characteristics of 75 patients (1997-2003).
Sued O, Miró JM, Alquezar A, Claramonte X, García F, Plana M, Arnedo M, de Lazzari E, Gil C, Manzardo C, Blanco JL, Martínez E, Mallolas J, Joseph J, Pumarola T, Gallart T, Gatell JM.

ARTÍCULO II

Viral Immunol. 2011 Aug; 24(4):347-9.
Acute HIV seroconversion presenting with active tuberculosis and associated with high levels of T-regulatory cells.
Sued O, Quiroga MF, Socías ME, Turk G, Salomón H, Cahn P.

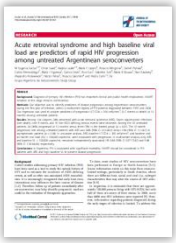
ARTÍCULO III

Enferm Infecc Microbiol Clin. 2011;29(7):482–489
Prevalence of transmitted antiretroviral resistance and distribution of HIV-1 subtypes among patients with recent infection in Catalonia (Spain) between 2003 and 2005.
Romero A, Sued O, Puig T, Esteve A, Pumarola T, Casabona J, González V, Matas L, Tural C, Rodrigo I, Margall N, Domingo P, Casanova A, et al.

ARTÍCULO IV

PLoS One. 2015 Jun 3; 10 (6):e0125837.
Trends in Transmission of Drug Resistance and Prevalence of Non-B Subtypes in Patients with Acute or Recent HIV-1 Infection in Barcelona in the Last 16 Years (1997-2012).
Ambrosioni J, Sued O, Nicolas D, Parera M, López-Diéguez M, Romero A, Agüero F, Marcos MÁ, Manzardo C, Zamora L, Gómez-Carrillo M, Gatell JM, Pumarola T, Miró JM.

ARTÍCULO V



J Int AIDS Soc. 2011 Aug 10;14:40.
Acute retroviral syndrome and high baseline viral load are predictors of rapid HIV progression among untreated Argentinean seroconverters
Socías M, Sued O, Laufer N, Lázaro M, Mingrone H, Pryluka D, Remondegui C, Figueroa MI, Cesar C, Gun A, Turk G, Bouzas M, Kavasery R, Krolewiecki A, Pérez H, Salomón H, Cahn P; Grupo Argentino de Seroconversión Study Group

ARTÍCULO VI

PLoS One. 2015 Jul 17;10 (7):e0131651.
Structured Treatment Interruptions and Low Doses of IL-2 in Patients with Primary HIV Infection. Inflammatory, Virological and Immunological Outcomes.
Sued O, Ambrosioni J, Nicolás D, Manzardo C, Agüero F, Claramonte X, Plana M, Tuset M, Pumarola T, Gallart T, Gatell JM, Miró JM.

MATERIAL Y METODOS

El propósito de este apartado no es describir la metodología utilizada para la realización de esta tesis doctoral, ya que estos se describen de forma detallada en cada uno de los trabajos publicados, sino exponer de forma breve y a modo de resumen el diseño escogido para la realización de los estudios para cada uno de los objetivos.

Los artículos I, II y III no cumplen con los criterios requeridos (menos de 5 años desde la publicación, un reporte de caso y un artículo utilizado para otra tesis) pero se mencionan porque siguen la línea de trabajo argumental de los objetivos planteados y fundaron las bases de los estudios posteriores.

Para el **Primer Objetivo** *"Describir las características clínicas de los pacientes con infección aguda"* se revisó retrospectivamente la información de la cohorte de 75 pacientes con diagnóstico de infección aguda atendidos en el Hospital Clínic de Barcelona durante el periodo 1997-2003 cuyos resultados se publicaron en el primer artículo. Como complemento, en 2011 se publicó un caso excepcional de infección aguda con una tuberculosis concomitante, que permitió describir el rol de las células Treg en la infección aguda y datos del artículo de la cohorte de Buenos Aires.

Artículo I: Sued O, Miró JM, Alquezar A, Claramonte X, García F, Plana M, Arnedo M, de Lazzari E, Gil C, Manzardo C, Blanco JL, Martínez E, Mallolas J, Joseph J, Pumarola T, Gallart T, Gatell JM. Primary human immunodeficiency virus type 1 infection: clinical, virological and immunological characteristics of 75 patients (1997-2003). Enferm Infecc Microbiol Clin. 2006 Apr; 24(4):238-44.

Artículo II: **Sued O**, Quiroga MF, Socías ME, Turk G, Salomón H, Cahn P. Acute HIV seroconversion presenting with active tuberculosis and associated with high levels of T-regulatory cells. Viral Immunol. 2011 Aug; 24(4):347-9.

Para el **Segundo Objetivo** *"Describir la prevalencia de resistencia en pacientes con infección aguda por VIH*" se llevaron a cabo otros dos estudios. En el primero (**Artículo III**), que se llevó a cabo en colaboración con el Centre d'Estudis Epidemiològics sobre les ITS i Sida de Catalunya (CEEISCAT), se evaluó la prevalencia de resistencia y el subtipo de los pacientes recientemente infectados en diferentes hospitales de Cataluña de 2003 a 2005, identificados mediante el algoritmo STARHS y posteriormente, en 2014 se revisó retrospectivamente la información sobre la prevalencia de resistencia y los subtipos de los pacientes con infección aguda identificados en el Hospital Clínic de Barcelona durante el periodo 1997-2012 (**Artículo IV**).

Artículo III: Romero A, **Sued O**, Puig T, Esteve A, Pumarola T, Casabona J, González V, Matas L, Tural C, Rodrigo I, Margall N, Domingo P, Casanova A, Ferrer E, Caballero E, Ribera E, Farré J, Puig T, Amengual MJ, Navarro G, Prat JM, Masabeu A, Simó JM, Villaverde CA, Barrufet P, Sauca MG, Ortin X, Ortí A, Navarro R, Euras JM, Vilaró J, Villà MC, Montull S, Vilanova C, Pujol F, Díaz O, Miró JM; AERI study Group. Prevalence of transmitted antiretroviral resistance and distribution of HIV-1 subtypes among patients with recent infection in Catalonia (Spain) between 2003 and 2005. Enferm Infecc Microbiol Clin. 2011 Aug-Sep; 29 (7):482-9.

Artículo IV: Ambrosioni J, **Sued O**, Nicolas D, Parera M, López-Diéguez M, Romero A, Agüero F, Marcos MÁ, Manzardo C, Zamora L, Gómez-Carrillo M, Gatell JM, Pumarola T, Miró JM. Trends in Transmission of Drug Resistance and Prevalence of

Non-B Subtypes in Patients with Acute or Recent HIV-1 Infection in Barcelona in the Last 16 Years (1997-2012). PLoS One. 2015 Jun 3; 10 (6):e0125837.

Para el **Tercer Objetivo** *"Identificar factores asociados a progresión"* se analizó la evolución de las personas con diagnóstico de infección aguda atendidas en la Fundación Huésped, Buenos Aires, Argentina, durante el periodo 1997-2008 y que no habían recibido tratamiento para identificar factores asociados con progresión clínica o caída de los CD4 a menos de 350 células/mm3. (**Artículo V**)

Artículo V: Socías ME, **Sued O**, Laufer N, Lázaro ME, Mingrone H, Pryluka D, Remondegui C, Figueroa MI, Cesar C, Gun A, Turk G, Bouzas MB, Kavasery R, Krolewiecki A, Pérez H, Salomón H, Cahn P; Grupo Argentino de Seroconversión Study Group. Acute retroviral syndrome and high baseline viral load are predictors of rapid HIV progression among untreated Argentinean seroconverters. J Int AIDS Soc. 2011 Aug 10;14:40.

Finalmente, el **Cuarto Objetivo** *"Evaluar el impacto a corto y largo plazo de tratamientos inmunomediados en la infección aguda"* se analizó la información recolectada mediante un estudio prospectivo, en el cual se incluyeron pacientes exitosamente tratados desde la infección aguda, con buena recuperación inmunológica y que iniciaron ciclos de interrupción estructurada del TARV o interrupción estructurada del TARV más IL-2.

Artículo VI: **Sued O**, Ambrosioni J, Nicolás D, Manzardo C, Agüero F, Claramonte X, Plana M, Tuset M, Pumarola T, Gallart T, Gatell JM, Miró JM. Structured Treatment Interruptions and Low Doses of IL-2 in Patients with Primary HIV Infection. Inflammatory, Virological and Immunological Outcomes. PLoS One. 2015 Jul 17; 10 (7):e0131651.

ARTÍCULO I

Primary human immunodeficiency virus type 1 infection: clinical, virological and immunological characteristics of 75 patients (1997-2003)

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OBJECTIVES. To describe the epidemiological and clinical characteristics and the evolution of a cohort of patients with primary HIV-1 infection from the Barcelona area.
METHODS. Prospective cohort study of HIV-infected patients diagnosed with primary HIV infection in a tertiary hospital in Barcelona (Spain) from 1997 through 2003. Descriptive analysis of epidemiological and clinical characteristics and effect of highly active antiretroviral treatment (HAART) on outcome.
RESULTS. A total of 75 patients were diagnosed, accounting for 2.9% of the total of newly diagnosed HIV patients during the same time period. Eighty-one percent of the patients were males and the median age was 30 years (IQR 26-38). The most frequent transmission route was homosexual (72%), followed by heterosexual (17%) and intravenous drug abuse (11%). Seventy-seven percent of patients presented symptoms, the most frequent being fever (98%), asthenia (86%), arthralgia-myalgia (65%), lymphadenopathy (55%), night sweats (48%) and rash. Sixty-five percent started HAART, although the proportion of patients that received HAART decreased from 79% during the period 1997-2000 to 49% during the period 2001-2003 (p < 0.01). After a median follow-up of 37 months (IQR 26-66), one patient died and eight cases were lost to follow-up. The patients who did not receive HAART had a higher probability of immunological or clinical deterioration during the follow-up when compared to the group that received HAART (42.3% versus 12.3%; p < 0.001). In treated patients, dyslipidemia and lipodystrophy were diagnosed in 58% and 37% of cases, respectively.
CONCLUSIONS. Primary HIV-1 infection was diagnosed more frequently in homosexual males, and its clinical characteristics were similar to those observed in previous studies. HAART given during primary HIV infection was effective, but was associated with a high percentage of adverse effects.

Key words: **HIV-1. Primary HIV infection. Acute retroviral syndrome. Antiretroviral treatment.**

Infección aguda por el virus de la inmunodeficiencia humana: características clínicas, virológicas e inmunológicas de 75 pacientes (1997-2003)

OBJETIVOS. Describir las características epidemiológicas, clínicas y evolutivas de una cohorte de pacientes con una infección aguda por el virus de la inmunodeficiencia humana (VIH) en el área de Barcelona.
MÉTODOS. Estudio prospectivo de pacientes diagnosticados de infección aguda por el VIH en un hospital terciario de Barcelona durante el período 1997-2003. Análisis descriptivo de las características epidemiológicas y clínicas e influencia del tratamiento antirretroviral (TARV) en la evolución.
RESULTADOS. Se diagnosticaron 75 pacientes, lo que representó el 2,9% del total de pacientes diagnosticados de infección por el VIH en el mismo período de tiempo. El 81% eran varones y la mediana de edad fue de 30 años (rango intercuartil [RIC], 26-38). Las vías de contagio fueron las relaciones homosexuales (72%), seguida de las heterosexuales (17%) y del uso de drogas intravenosas (11%). El 77% de los pacientes presentó síntomas, siendo los más frecuentes: fiebre (98%), astenia (86%), artromialgias (65%), linfoadenopatías (55%), sudoración nocturna (48%) y exantema (45%). El 65% comenzó TARV, disminuyendo el número de pacientes tratados del 79% en el período 1997-2000 al 49% en el período 2001-2003 (p < 0,01). Tras una mediana de seguimiento de 37 meses (RIC, 26-66), un paciente falleció y 8 casos se perdieron de seguimiento. Los pacientes que no recibieron TARV presentaron una mayor probabilidad de presentar

This study was presented in part at the XI Congress of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC). Bilbao, 16-19 May 2004. Abstract 255. This study has been funded in part by research grants FIPSE 3128/00 and FIS PI 04/0363. Drs. J.M. Miró and F. García received a research grant from IDIBAPS.

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deterioro inmunológico o clínico durante el seguimiento en comparación con el grupo que recibió TARV (42,3% frente a 12,3%; p < 0,001). La dislipemia y la lipodistrofia se diagnosticaron en el 58 y 37% de los pacientes tratados, respectivamente.

CONCLUSIONES. La infección aguda por VIH se diagnosticó con más frecuencia en los varones homosexuales, siendo sus características clínicas similares a las descritas previamente. El TARV instaurado en esta fase de la infección por VIH fue eficaz pero se asoció a una frecuencia elevada de efectos adversos.

Palabras clave: **VIH-1. Infección aguda por el VIH. Síndrome retroviral agudo. Tratamiento antirretroviral.**

Introduction

In 1985, Cooper et al published the first description of symptoms attributable to an episode of primary human immunodeficiency virus infection (PHI) in 12 homosexual males who presented a clinical picture similar to "infectious mononucleosis" (fever, pharyngitis and exanthema), but with negative Epstein-Barr serology. Seroconversion to HIV was confirmed in these patients and the clinical picture was named "acute retroviral syndrome"[1]. According to the World Health Organization, 14,000 new cases of HIV infection occur every day over the world. Most of these cases occur in Africa and Asia, but they are still quite frequent in Europe. Nevertheless, this entity is often underdiagnosed, since clinical symptoms at time of the seroconversion[2-4] are wrongly attributed to non-specific viral infections in most cases.

Early diagnosis of PHI is important both for the patient and to control the epidemic. Notifying the patient can help reduce the risk of transmitting the infection and highly active antiretroviral therapy (HAART) can be considered. In terms of public health, identification of these cases enables us to study contacts, evaluate the efficacy of preventive interventions, ascertain the growth pattern of the epidemic and determine the transmission of resistant viruses[5,6].

This article describes the epidemiological, clinical, immunological and virological characteristics, and the response to HAART of a 75-patient cohort with PHI diagnosed consecutively at a tertiary teaching hospital in the Barcelona area over a seven-year period (1997 to 2003).

Methods

In 1997, the Infectious Disease Department of the Hospital Clínic in Barcelona implemented a program for the diagnosis and follow-up of patients with PHI. The cohort included all patients presenting one of the following criteria during the first evaluation:

1. Negative or undetermined HIV-1 serology (negative enzyme immunoassay [EIA], or positive EIA with a negative or undetermined line immunoassay [LIA]) associated with the detection of virus in blood (HIV-RNA or p24 antigen).
2. Evidence of seroconversion during the last 6 months by EIA and LIA.
3. Syndrome suggestive of PHI during the last three months with a negative HIV-1 serology during the last 12 months.

In all cases, PHI-related epidemiological information, the presence and type of symptoms, and laboratory tests carried out during the first visit and follow-up visits were recorded using a standard computerized system. All patients gave their informed consent for the use of data according to the study protocol approved by the IRB of the Hospital Clínic, Barcelona.

At the first visit, the following were recorded: epidemiological history and clinical data, physical examination, routine blood and biochemistry analysis, HIV-1 RNA viral load in plasma (VL), genotypic resistance test, T-cell sub-populations, baseline serology studies (conventional serological test for hepatitis B [HBV], hepatitis C [HCV], cytomegalovirus [CMV] and *Toxoplasma gondii* and reagin test for syphilis [VDRL]); tuberculin skin test (PPD) and chest X-ray. The indication and type of HAART used were based on current recommendations, on the available drugs and on the availability of clinical research protocols for the treatment of PHI. Clinical and laboratory follow-up were carried out every three months. Lipodystrophy was considered as the loss or accumulation of body fat as observed by the patient and confirmed by the physician. Dyslipidemia was defined as two consecutive determinations of cholesterol or triglycerides above normal values.

Virological studies

HIV-1 serology was determined by microparticle EIA (MEIA) using the AxSYM system (Abbott Laboratories, North Chicago, IL) and confirmed by LIA (Inno-LIA HIV I/II Score. Innogenetics. Ghent, Belgium). Quantification of VL was by Cobas Amplicor Monitor (Roche Molecular Systems, Branchburg, NJ) with a sensitivity limit of 200 copies/mL. Genotyping of resistance mutations was done in genes coding for reverse transcriptase (RT) and protease enzymes using the ViroSeq HIV Genotyping System v.2 (Abbott Laboratories, North Chicago, IL) and the results were reported according to the consensus document of the *International AIDS Society Resistance Testing-USA Panel*, 2005[7]. Viral subtype was determined using the FASTA sequence from the Stanford database (http://hivdb.stanford.edu).

Immunological parameters

T cell subsets (CD4+, CD8+) were determined by cytofluorometry (FACScalibur, Becton Dickinson).

Statistical analysis

The study closed on 30 July 2004. The date of the infection was set at the day of exposure to HIV (unprotected relations, or syringe exchange with a source of positive or unknown serology). In the case of patients who presented several potential exposures, the date of infection was set at the closest exposure to the 14 days before the onset of symptoms. In the case of asymptomatic patients, this was defined as the halfway point between the last negative serology and the first positive serology. Loss to follow-up was defined as the loss of more than two consecutive visits. Progression events were defined as the appearance of a type C clinical diagnosis according to the 1993 revised CDC classification or a CD4 count of < 350 cells/mm^3 on two occasions after 6 months following exposure.

Descriptive statistics were expressed as proportions and percentages for qualitative variables and as medians and interquartile ranges (IQR) for quantitative variables. The T-test and ANOVA were used for comparisons between two and more than two groups, respectively, in the case of normally distributed continuous variables. In the case of non-normally distributed variables, the Mann-Whitney U and the Kruskal-Wallis tests were used for two and more than two groups, respectively. Categorical variables were compared using the Chi-squared test or Fisher exact test if the expected frequency in more than 25% of the squares was lower than 5. Survival functions were calculated with the Kaplan-Meier method and compared using the log-rank test. A two-sided p value < 0.05 was established as the level of statistical significance for all the tests. The statistical analysis was performed with SPSS version 10.

TABLE 1. **Baseline demographic, clinic and laboratory characteristics of the patients**

	Total	IVDU	Homosexual	Heterosexual	p
Total	75	8	54	13	
Age*	30 (26-38)	26 (23-34)	30 (26-36)	38 (24-48)	0.011
Gender**					
Female	14 (19)	3 (37)	–	11 (85)	0.041*
Male	71 (81)	5 (63)	54 (100)	2 (15)	
Days from infection to first evaluation*	64 (40-96)	44 (30-74)	60 (37-101)	82 (65-104)	0.216
Symptomatic PHI**	58 (77)	5 (62)	43 (79)	10 (76)	0.558
Duration of symptoms (days)*	14 (7-21)	15 (10-20)	13 (7-17)	18 (9-22)	0.041
CD4 absolute number*	576 (369-730)	401 (257-703)	538 (367-691)	756 (607-889)	0.026
CD4 percentage*	25.5 (18.4-32.2)	19.8 (12.2-29.5)	25 (18-31)	33 (23.5-40)	0.044
CD4/CD8 ratio*	0.52 (0.36-0.87)	0.48 (0.20-0.90)	0.52 (0.36-0.69)	0.88 (0.46-1.26)	0.100
Viral load* (log_{10})	5.0 (4.2-5.6)	5.8 (5.3-6)	5.0 (4.3-5.6)	4.4 (3.2-5.2)	0.004
HBV**	21 (28)	3 (37)	17 (31)	1 (8)	0.188
HCV**	7 (9)	7 (87.5)	0	0	< 0.001
*Toxoplasma gondii***	36 (48)	5 (62)	24 (44)	7 (54)	0.844
CMV**	60 (80)	7 (87.5)	44 (81)	9 (69)	0.745
PPD+**	5 (7)	1 (12.5)	4 (7)	0	0.493

*Expressed in medians and interquartile ranges 25-75.
**Expressed in whole numbers and percentages. Among homosexuals and IVDU.
IVDU: intravenous drug users; HBV: hepatitis B virus; HCV: hepatitis C virus; CMV: cytomegalovirus; PPD+: positive tuberculin skin test.

Results

Demographic characteristics

Demographic characteristics are presented in table 1. Between 1997 and 2003, our hospital attended 2,577 new HIV-infected patients, among whom 30% reported homosexual or bisexual (HMS) relationships as the probable route of transmission, 33% reported heterosexual (HTS) relationships and 35% the use of intravenous drugs (IVDU). During the same period, 75 cases of PHI (2.9%) were identified. Sixty-one patients were male (81%) and the median age was 30.5 years (IQR 26-38). The probable routes of transmission were HMS relations in 54 patients (72%), HTS relations in 13 (17%) and IVDU in 8 (11%). Women accounted for 85% of patients who contracted infection by HTS relations. Thirteen patients were immigrants (17%), eight from Europe, four from South America and one from North Africa. At the first evaluation, 39 patients (52%) presented HIV-negative serology with a positive VL, 27 (36%) presented documented seroconversion during the last six months and 9 (12%) presented evidence of seroconversion during the last year. Median time from the date of exposure to HIV until the first clinical visit was 64 days (IQR 40-96): HTS, 82 days; HMS, 60 days and IVDU, 44 days. Median overall follow-up was 37 months (IQR 26-66), which represents a total of 265 patients/year. Only 8 patients (11%) were lost to follow-up.

Baseline immunological, virological and serological parameters

Laboratory parameters are shown in table 1. The first evaluation was carried out a median of 64 days (IQR 40-96) after exposure to HIV-1. Median HIV-1 RNA VL was 5.0 (IQR 4.2-5.6) log_{10} copies/mL and the absolute and percentage CD4 lymphocyte counts were 576 (IQR 369-730) cells/mm^3 and 25.5% (IQR 18%-32%), respectively, with a CD4/CD8 ratio of 0.52 (IQR 0.36-0.87). Viral subtype and presence of mutations associated with drug resistance were determined in 61 patients (81%). All were subtype B and eight patients (13%) presented mutations associated with resistance to an antiretroviral drug. When VL and CD4 lymphocyte count were compared according to route of transmission, HTS patients presented lower HIV-1 RNA VL values ($p = 0.007$) and higher CD4 lymphocyte counts ($p = 0.026$). However, when these parameters were analyzed according to time to the first evaluation, the differences lost statistical value ($p = 0.09$). The percentage of patients who presented serological evidence of previous infection by *Toxoplasma gondii,* CMV, HBV, and HCV was 48%, 80%, 28% and 9%, respectively. The tuberculin skin test (PPD) was positive in 7% of cases. One patient had chronic hepatitis B (HbsAg-positive for more than 6 months) and another had positive serology for syphilis of indeterminate duration. The seven HCV-infected patients were IVDU.

Clinical manifestations

The clinical manifestations of the patients are shown in table 2. PHI was symptomatic in 58 cases (77%). Globally, the most frequent symptoms were fever (76%), asthenia (67%), arthralgia/myalgia (51%), lymphadenopathy (43%), and pharyngitis (41%), with no significant differences between the groups. Only 15 patients (20%) had symptoms consistent with infectious mononucleosis (fever, pharyngitis, and laterocervical adenopathy for more than seven days). A combination of fever and rash was present in 35% of cases. No patients presented C events during HIV-1 seroconversion, although two patients had B events (*Candida* angular chelosis and oral hairy leukoplakia).

Hospital admission

During the acute episode, 17 (23%) patients were hospitalized. In 11 cases, the reason for admission was fever of unknown origin. In five patients, admission was for neurological symptoms (three episodes of viral meningitis, one accompanied by facial paralysis, one episode of severe paresis of the lower limbs and one patient with convulsions). Another patient was admitted for a study of polyarthritis, which was considered reactive to HIV-1.

Associated infections

An acute infection was present in 13 patients at the same time as PHI. A total of nine patients, all HMS, presented a sexually transmitted disease. One of these patients presented acute HBV hepatitis, seven presented syphilis (one case of primary syphilis and the rest secondary), and another patient had both infections simultaneously.

In four IVDU, PHI was diagnosed during the course of a severe bacterial infection (two cases of tricuspid endocarditis due to *Staphylococcus aureus,* one case of severe cellulitis in the lower limbs and one episode of pneumococcal pneumonia). In these patients, PHI was suspected due to the persistence of fever despite correct antibiotic therapy.

Highly active antiretroviral therapy

A total of 48 patients (65%) started HAART during the first 180 days after infection, with a median of 69 days (IQR 45-102). The number of patients who received HAART fell over time: 79% in the group of patients evaluated during 1997-2000 and 49% during 2001-2003 (p = 0.01) (fig. 1).

Outcome of patients receiving HAART

Ninety percent of the patients treated (n = 43) received a combination of two nucleoside analog reverse transcriptase inhibitors (NRTI) and a protease inhibitor (PI), boosted with ritonavir in a third of the cases. The five remaining patients (10%) started therapy with two NRTI and a non-nucleoside analog reverse transcriptase inhibitor (NNRTI). At 12 months of follow-up, 98% had a non-detectable VL (NDVL) and an increase (median, IQR) in CD4 lymphocyte count of 324 (117-537) cells/mm^3.

In half the patients the first HAART regimen was changed due to toxicity (17 cases), simplification (4 cases), boosting with low-dose ritonavir to simplify posology (2 cases), and failure to suppress viral load (1 case). The median duration of the first regimen was 64 weeks (IQR 18-127). The most common toxicity was indinavir-induced renal lithiasis (7 cases), peripheral polyneuritis (3 cases), allergic reactions (2 cases), and gastrointestinal disorders (2 cases). A total of 58% of patients had dyslipidemia and 37.5% had lipodystrophy.

In total, 21 patients stopped HAART after a median of 37 weeks' treatment (IQR 21-46), 14 in the framework of structured treatment interruptions and seven for personal reasons. During follow-up, only six patients presented CD4 lymphocyte counts below 350 cells/mm^3, although none developed opportunistic infections. One patient with PHI-associated myelitis who required corticosteroid therapy died three months after being infected due to disseminated aspergillosis. She received HAART the last six weeks.

TABLE 2. **Clinical manifestations in 58 symptomatic patients**

	Global (%)	IVDU*	Homosexual*	Heterosexual*
Fever	98	100	97.7	100
Asthenia	86	100	86	80
Rash	45	20	46.5	50
Headache	34.5	40	33	40
Cervical lymphadenopathies	55	40	53.5	70
Pharyngitis	53	20	53.5	70
Arthralgia/myalgia	65.5	60	63	80
Gastrointestinal symptoms	24	40	23	20
Night sweats	48	60	44	60
Neurological symptoms	9	20	7	10
Oral ulcers	15.5	20	14	20
Genital ulcers	5	–	2	20
Mononucleosis syndrome	26	–	28	30
Fever + rash	35	20	46.5	50
Number of symptoms	5 ± 3	4 ± 2	5 ± 2	6 ± 2,5

*The comparisons between 3 groups did not present significant differences. IVDU: intravenous drug users.

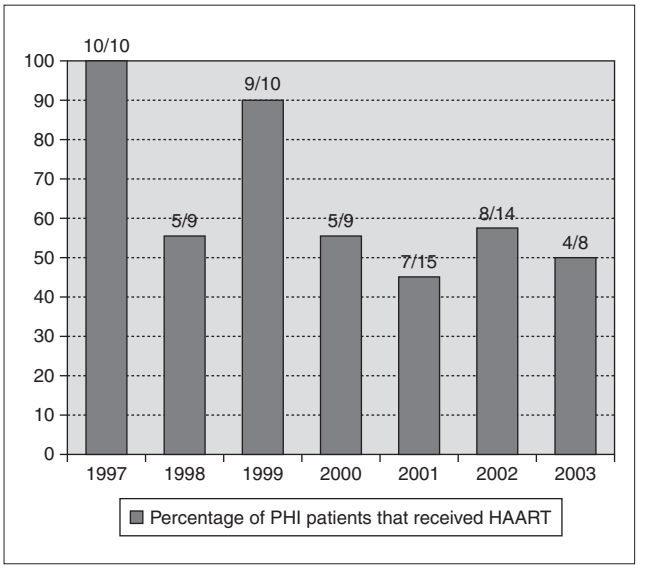

Figure 1. Annual percentage and number of cases of patients with acute HIV infection receiving HAART. Number of treated/diagnoses cases are on the top of boxes.

Figure 2 compares HIV-1 RNA VL and CD4 lymphocyte values during the follow-up of these patients with respect to those who did not receive HAART.

Outcome of patients who did not receive HAART

A total of 27 patients did not receive HAART during PHI. In 15 cases, the therapy was offered but the patients preferred not to take it. In the other 12 cases, the physician decided not to offer HAART, as it was impossible to include the patient in therapeutic research protocols (11 cases), or due to non-compensated psychiatric illness (one case). In one of these 12 cases, the patient was recei-

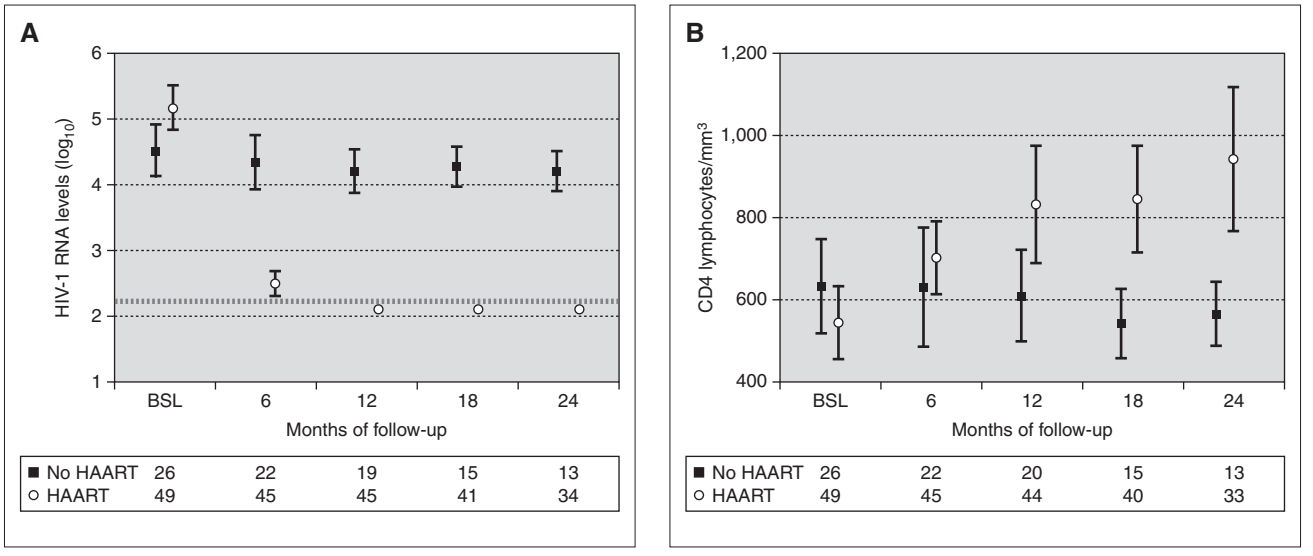

Figure 2. HIV-1 RNA levels (A) and CD4 lymphocytes count (B) in patients with and without HAART at baseline, and at 6, 12, 18 and 24 months of follow-up (median and IQR). ■, Patients without HAART; ○, patients with HAART; ---, limit of detection 200 copies HIV-1 RNA/mL. HAART: highly active antiretroviral treatment.

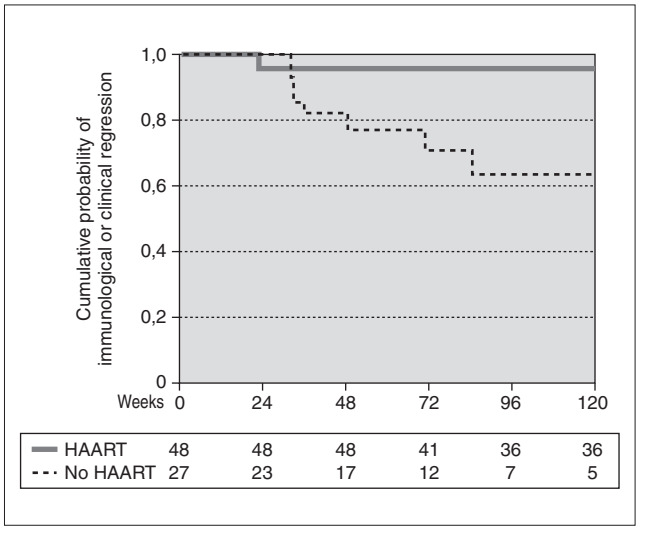

Figure 3. Accumulated probability of immunological (i.e. CD4 lymphocytes < 350 cells/mm^3) or clinical progression in patients with PHI according to receive or not HAART. The difference was statistically significant ($p < 0.001$).

ving lamivudine for chronic hepatitis B. In comparison with the patients who received HAART, this group had a greater interval between PHI and the first evaluation, a median of 91 days (IQR 64-131) *vs.* 46 (IQR 33-78), $p < 0.001$; a lower frequency of symptomatic seroconversion (54% *vs.* 92%, $p < 0.014$); and a lower median VL (4.6 [IQR 3.7-5.3] *vs.* 5.1 [IQR 4.5-5.9] log_{10} copies/mL; $p < 0.001$). However, this group presented faster immunological progression to CD4 counts below 350 cells/mm^3 (fig. 2). During follow-up, 11 patients had CD4 lymphocyte figures below 350 cells/mm^3. One patient developed Kaposi sarcoma and multidermatomal herpes zoster, and another recurrent oral candidiasis. This group of patients presented a greater likelihood of immune deterioration and/or clinical progression during follow-up than the HAART group (42.3% versus 12.3%; $p < 0.00001$) (fig. 3).

Discussion

PHI is defined as the 30- to 45-day period from the date of infection until the appearance of a full humoral anti-HIV response, generally detected by Western-Blot. The period from this point up to 180 days after the infection is known as *recent infection* and the next phase as *chronic infection*. The prevalence of PHI in countries with a high rate of infection is almost 1.8% in sexually transmitted disease clinics[8]. In the West, it is detected in up to 0.5/1000 serology tests[9] and, when the serology is performed in patients with consistent symptoms, in up to 1% of cases[10]. In our series, PHI represented 2.9% of all newly diagnosed cases of HIV infection attended in a Barcelona teaching hospital during the seven years of the study.

Most of the patients in the cohort were homosexual males. This positive selection may have been influenced by characteristics that are specific to this population (greater frequency of serological testing, greater access to information on HIV and, in particular, the symptoms of PHI). Moreover, this group was more frequently exposed to CMV, an indirect marker of sexual activity, and had a high frequency of sexually transmitted diseases (syphilis and acute HBV hepatitis), which may also have favored the consultation. The low number of patients infected after heterosexual relations, despite the fact that this is currently a common route of transmission[11], suggests that delays in the diagnosis of HIV infection are much more notable in this population, since PHI infection is not usually suspected. The group least represented in our series was IVDU. The diagnosis of PHI was made in half the cases during admission to hospital for severe bacterial infections, when the drug user continued with fever despite correct antimicrobial therapy. These data taken as a who-

le suggest that PHI should be actively investigated in patients with fever and a history of high-risk sexual exposure in the past month, especially if they have a sexually transmitted disease, and in all IVDU with a febrile illness.

As in previous series[2,12], most patients presented symptoms associated with seroconversion. It has been suggested that the progression to clinical or immunological deterioration is faster in patients with symptomatic primary infection, and even worse in those with a long symptomatic phase (more than 15 days) or with a short incubation time[13,14]. The presence of symptoms and faster progression may represent epiphenomena of a higher VL since the VL level is the most widely recognized risk factor for HIV progression[15]. This relationship has even been described as "dose-dependent", owing to the fact that, for each symptom present, there is an increase in the VL of 0.4 $\log_{10}$ copies/mL[16]. The most common symptoms during PHI include fever, rash, oral and/or genital ulcers, lymphadenopathy, marked asthenia, arthralgia/myalgia and aseptic meningitis[17,18]. The frequency of presentation of these symptoms in our series was no different from previously published series. The typical mononucleosis syndrome was present in only 20% of patients; one study has recommended that this combination of symptoms should not be used as a PHI case definition criterion due to its low frequency[19]. Historically, it has been suggested that the most severe manifestations are the neurological symptoms. There have been reports of patients with aseptic meningitis, meningoencephalitis, myelitis, peripheral neuropathy, Guillain Barré syndrome, facial paralysis, convulsions and psychotic disorders. The presence of neurological symptoms has also been associated with the level of viral load in cerebrospinal fluid and with a greater rate of clinical progression[20,21]. A quarter of the patients presented headache in our series, although aseptic meningitis was only diagnosed in three cases. The only patient with severe neurological manifestations (transverse myelitis) died after a few weeks from disseminated aspergillosis.

With regard to the differences in clinical presentation between patient groups with different types of exposure to HIV, we observed a lower frequency of symptomatic PHI in IVDU than in the other groups. Although one study had established a similar frequency of symptoms in all the risk groups[22], the same authors later reported a lower frequency of symptoms in IVDU[23]. From the practical viewpoint, it is very difficult to establish whether intravenous transmission leads to a different clinical presentation or whether active consumption of drugs can mask the symptoms. Conversely, heterosexual patients presented a higher number and longer duration of symptoms, and a greater frequency of pharyngitis, laterocervical lymphadenopathy, arthralgia/myalgia and genital ulcers. We cannot establish whether this clinical picture is representative of these patients or, and this seems more likely, whether it involves a bias in which the most highly affected patients consulted a physician. Some authors have reported the simultaneous appearance of opportunistic infections at the time of PHI, such as viral infections (e.g. herpes simplex or zoster virus), esophageal candidiasis[24] and, less frequently, other AIDS-defining events[25-27]. We did not observe any C event in our cohort during PHI.

With regard to HAART in PHI, no randomized studies have shown a clinical benefit from starting this therapy during acute or recent HIV infection as compared to treating patients during the chronic phase of infection[28]. Nevertheless, some studies assessing the repercussion of HAART on the immune system have shown that HAART may be beneficial during PHI as it preserves the immune system and maintains/restores the specific cell response against HIV[29,30]. Other studies have shown a greater rate of viral suppression and immune recovery than when HAART is started during chronic infection[31,32], or the possibility of transient control of HIV-1 RNA VL after stopping HAART[33,34]. Finally, some cohort studies have suggested that clinical progression and the development of AIDS is greater in patients who did not received HAART during the acute phase when compared with patients who received HAART[35,36]. Furthermore, the disadvantages of HAART (cost, adverse effects due to longer exposure to drugs, possible non-adherence and resistance), the lack of evidence of a clear clinical benefit, and the impossibility of eradicating HIV infection has meant that physicians are currently less likely to prescribe HAART. This fact has been observed in the French PRIMO cohort[37] and in our center since 2001. In our experience, HAART was effective, since almost all patients had an undetectable VL and, after a median follow-up of three years, the possibility of clinical or immunological progression fell in comparison with untreated patients. Nevertheless, the high frequency of adverse effects was a limiting factor. The small number of patients in this series and the descriptive nature of the study prevent definitive conclusions from being established. This question must be answered in the coming years. The availability of new diagnostic methods that will enable us to detect more cases of acute or recent infection and new HAART regimens that are more patient-friendly, more potent and less toxic, would justify multicenter and randomized clinical trials to ascertain whether HAART started during this phase of infection, either alone or in combination with immune-mediated therapies, is beneficial to PHI patients from a clinical viewpoint.

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ARTÍCULO II

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Acute HIV Seroconversion Presenting with Active Tuberculosis and Associated with High Levels of T-Regulatory Cells

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Abstract

A patient with well-defined acute HIV infection who developed concomitant pulmonary tuberculosis during the retroviral acute syndrome is reported here. In this patient high levels of T-regulatory cells (Tregs) and a low proliferation response to *M. tuberculosis* were initially detected, which normalized throughout follow-up. This case calls for the consideration of tuberculosis in patients in the early stages of HIV, and emphasizes the need for further study of the potential causal relationship between Treg cells and the risk of TB reactivation in HIV patients.

Introduction

Acute HIV infection is characterized by a highly immune-activated state and a massive depletion of CD4 cells due to multiple mechanisms (1). Most patients present with a febrile illness 2–3 weeks from HIV exposure, which usually resolves spontaneously, although some cases present with severe disease with neurological or systemic involvement (2), and/or development of opportunistic diseases. Esophageal candidiasis, toxoplasmic encephalitis, *Pneumocystis jirovecii* pneumonia, or cytomegaloviral disease have been reported and were attributed to CD4 depletion during acute HIV infection. However, as far as we know there are no reported cases of active *Mycobacterium tuberculosis* disease concomitantly with acute HIV infection. The only report of mycobacterial infection involved the presentation of *M. kansasii* in an HIV seroconverter (3).

Regulatory T cells (Tregs) have been implicated in the modulation of immune responses to avoid overactive immunity. In HIV infection, following the initial acute infection, Tregs are upregulated and may contribute to suppression of anti-HIV immunity, promoting acute viremia and facilitating persistent infection, while in chronic infection gradual depletion of these cells allows the development of immune activation (4,5). On the other hand, patients with tuberculosis (TB) present with an upregulation of Tregs, which contributes to decreased production of interferon-γ and IL-10 and suppresses immune responses to TB antigens (6,7). In addition, individuals latently infected by TB present with a rapid depletion of *M. tuberculosis*-specific Th-1 cells during the acute phase of HIV infection due to direct viral infection of these cells, mostly expressing the surface co-receptor CCR5 (8).

Case Report

In May of 2008 a 33-year-old man was examined because of high fever and night sweats of 5 days' duration, skin rash, and oral and perianal ulcers. He denied having cough or expectoration, or close contact with anyone with cough or confirmed TB. His medical history included frequent use of inhaled cocaine, risky sexual behavior with multiple unprotected sexual relationships with women and men in the past months, syphilis treated 4 y prior, and a positive hepatitis C serology in 2004. Abnormal laboratory values included WBC count 3400 cells/μL, hemoglobin 15.6 g/L, platelets 90,000/μL, AST 74 U/L, and ALT 108 U/L. A chest x-ray and serologic studies (including HIV) were ordered, antipyretics were prescribed, and a follow-up visit was scheduled for the next week. At the next visit the patient's fever and rash had gradually disappeared. The chest x-ray showed a small patchy infiltrate with micronodular shadows in the apical segment of the right upper lobe, but he denied having respiratory symptoms. A MEIA fourth-generation screening HIV test (to detect antigen and antibodies against HIV1/2) was reactive, with a negative Western blot (WB). Two weeks after initial presentation the patient developed a productive cough. The smear test was negative for acid-fast bacilli, and a sample was processed for tuberculosis culture. Twenty-five

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days later the patient was completely asymptomatic, but a chest computed tomography scan showed a patchy interstitial peribronchial shadow with calcified nodules in the upper right lobe and calcified lymph nodes in the pretracheal retrocaval space. At the same time, the tuberculosis skin test was negative, and a new WB showed gp160 and p24 bands. Baseline HIV-RNA viral load (bDNA) and CD4 count were 62,679 copies/mL (4.79 $\log_{10}$) and 419 (25%) cells/μL, respectively. At 38 days after the beginning of TB symptoms and sputum collection (53 days after the first evaluation at the emergency department), the sputum culture developed *Mycobacterium tuberculosis* that was sensitive to first-line drugs. That same day, the patient started 9 months of conventional treatment for TB, which was completed successfully. While on TB treatment, he maintained a consistently low viral load and high CD4 counts, and therefore antiretroviral therapy was not initiated. In June 2009, with a CD4 count of 357 cells/mm^3, the patient started HAART, which he continues today. He has remained asymptomatic since then, and his recent laboratory values are viral load <50 copies/mL, and CD4 count 481 cells/mm^3.

Percentages of Tregs were determined by peripheral blood flow cytometry using anti FoxP3, CD25, and CD4 antibodies as previously described (6). Initially (concomitantly with HIV seroconversion and the development of TB symptoms), the frequency of Tregs was higher and decreased over time. Concurrently, the proliferation *in vitro* of peripheral blood mononuclear cells to *M. tuberculosis* H37Rv strain [determined using the CFSE dilution method as described elsewhere (7)] was undetectable, although it showed a transitory increase after starting TB treatment (Fig. 1). The expression of CD38 and HLA-DR molecules on CD4 and CD8 cells remained high throughout follow-up, especially on CD8 T-lymphocytes (the median percentage of CD8+CD38+HLA-DR cells was 34%).

Our patient had a well-documented acute HIV infection. In addition, the clinical course of tuberculosis, the anatomic localization, and the radiology support the diagnosis of post-primary TB rather than a new infection.

Our patient is interesting for two reasons: (1) to the best of our knowledge, this patient is the first reported case of acute HIV infection and concomitant pulmonary TB; (2) in this patient we could detect initial high levels of Treg cells, which normalized after TB treatment. Although speculative, it seems plausible that immune dysregulation during acute HIV infection, in particular upregulation of Treg cells, may have contributed to a tolerant state (i.e., a negative TST and low proliferative responses to *M. tuberculosis* despite relatively preserved CD4 counts) that may have favored the reactivation of TB.

Conclusions

This case underlines the need to explore the role of Tregs, both in HIV-TB co-infected patients, and in the small subset

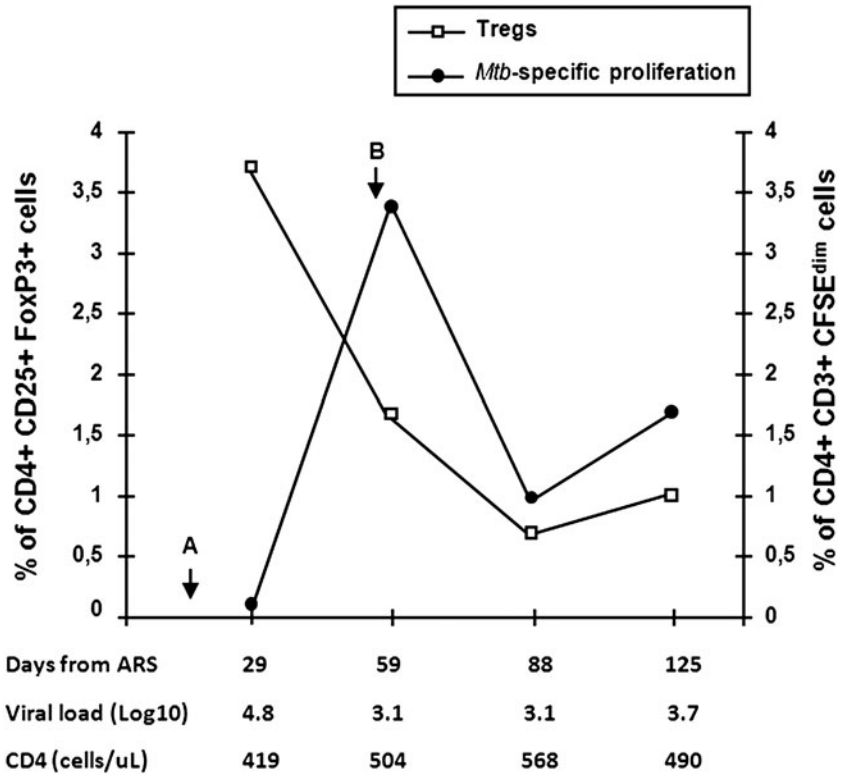

FIG. 1. Frequency of Treg lymphocytes and proliferation responses to *M. tuberculosis*. Percentages of Treg lymphocytes were determined in peripheral blood by flow cytometry using anti-FoxP3, CD25, and CD4 antibodies. The proliferation of peripheral blood mononuclear cells to the *M. tuberculosis* H37Rv strain was determined *in vitro* in CD3+CD4+ lymphocytes using the CFSE dilution method. Tregs, MTB-specific proliferation, viral load (b-DNA), and CD4 count (flow cytometry) are shown for each follow-up sample (ARS, acute antiretroviral syndrome; arrow A indicates sputum collection, and arrow B initiation of TB treatment).

of HIV patients who develop opportunistic infections during the acute phase of infection.

Patient Consent and Ethical Committee

This patient, who is participating in the Argentinean HIV seroconverters cohort "Grupo Argentino de Seroconversion (Site Fundación Huésped)," has read and signed the informed consent form for this cohort. In addition, the patient signed a specific consent form to publish this article.

Author Disclosure Statement

No competing financial interests exist.

Authors' Contributions

O.S. conceived of the study and drafted the manuscript, M.F.Q. participated in the study design and carried out immunological studies, M.E.S. followed the patient and collected samples, G.T. participated in the immunological studies, and H.S. and P.C. participated in the coordination, analysis, and interpretation of the data. All authors read and approved the final manuscript.

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Original article

Prevalence of Transmitted Antiretroviral Resistance and Distribution of HIV-1 Subtypes Among Patients with Recent Infection in Catalonia (Spain) between 2003 and 2005

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ABSTRACT

Objectives: The objectives of this study were to assess the prevalence of transmitted HIV-1 drug resistances (TDR) and HIV-1 subtypes in recently infected patients in Catalonia between 2003 and 2005 and to describe the characteristics of these patients according to the presence or absence of TDR and HIV-1 subtype.

Methods: After application of the Serological Testing Algorithm for Recent HIV Seroconversion (STARHS), residual aliquots of serum samples from recently infected antiretroviral-naïve individuals were genotyped. FASTA sequences were analyzed using the HIVDB Program. The World Health Organization 2009 List of Mutations for Surveillance of Transmitted HIV-1 Drug Resistant HIV Strains was used to estimate the prevalence of TDR.

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Results: Of 182 recently infected patients, 14 (7.7%) presented TDR. Seven (3.8%) had genotypic evidence of TDR against non-nucleoside reverse transcriptase inhibitors, 6 (3.3%) against nucleoside reverse transcriptase inhibitors, 3 (1.6%) against protease inhibitors (PIs), and only 2 individuals (1.1%) presented TDR against more than one class of drugs. Thirty-five (19.2%) patients were infected with a non-B HIV-1 subtype.
Conclusion: This is the first study to estimate the prevalence of TDR in recently infected patients in Catalonia. The results are similar to those of studies performed in other Spanish regions. Correct monitoring of these parameters requires systematic epidemiologic surveillance of transmitted resistance.

Prevalencia de resistencias primarias transmitidas y distribución de subtipos VIH-1 en pacientes con infección reciente en Cataluña (2003-2005)

RESUMEN

OBJETIVOS

Los objetivos de este estudio fueron evaluar la prevalencia de las resistencias primarias transmitidas (RPT) y de subtipos de VIH-1 en pacientes recientemente infectados en Cataluña entre 2003 y 2005, y describir las características de estos pacientes según la presencia o ausencia de RPT y el subtipo de VIH-1.
Métodos: Después de la aplicación del algoritmo de pruebas serológicas para la seroconversión reciente al VIH (STARHS), alícuotas residuales de las muestras de suero de individuos recientemente infectados no tratados previamente con antirretrovirales fueron genotipados. Las secuencias FASTA se analizaron con el programa HIVdb. Se utilizó el listado de mutaciones de la Organización Mundial de la Salud del 2009 para estimar la prevalencia de resistencias transmitidas.
Resultados: De 182 pacientes recientemente infectados, 14 (7,7%) presentaron RPT. Siete personas (3,8%) presentaban evidencias genotípica de RPT a los inhibidores de la transcriptasa inversa no análogos a nucleósidos, 6 (3,3%) frente a inhibidores de la transcriptasa inversa análogos de nucleósidos, 3 (1,6%) frente a los inhibidores de la proteasa, y solo 2 personas (1,1%) presentaron RPT a más de una familia de medicamentos. Treinta y cinco (19,2%) pacientes estaban infectados con un subtipo no-B del VIH-1.
Conclusión: Este es el primer estudio que estima la prevalencia de RPT en pacientes recientemente infectados en Cataluña, y los resultados son similares a los de estudios realizados en otras regiones españolas. Para el adecuado seguimiento de estos parámetros es necesaria la vigilancia epidemiológica sistemática de las RPT.

Palabras clave:
Resistencias transmitidas
Infecciones recientes
Subtipo de VIH-1

Introduction

Emergence of transmitted HIV-1 drug resistance is well documented almost everywhere combined antiretroviral treatment (cART) is available. Global resistance estimates vary between 5% and 25% in primary infection, depending on study population, definition of resistance, cART strategy, infection status (recent or chronic), and year of evaluation.[1,2] In Spain, the rate of transmitted HIV-1 drug resistance has been reported to be between 7.1 and 12.1%.[3,4]

Continued surveillance of transmitted HIV-1 drug resistance provides useful information on cART as a first-line regimen. Baseline resistance has been shown to impair the response to highly active antiretroviral therapy (HAART),[5] although a genotype-guided cART regimen can prove just as effective in patients with primary drug resistance as in patients with wild-type virus.[6]

In addition, increased population movements resulting from immigration, international travel, and sexual contact with individuals from countries where non-B subtypes are endemic have led to increased prevalence of these strains in developed countries.[7] The presence of circulating non-B subtypes complicates the interpretation of tests (viral load or resistance testing) that have been developed mainly for B subtypes. Many reported minor mutations associated with resistance by B subtypes are natural polymorphisms in some non-B subtypes, although their impact on the susceptibility of antiretroviral drugs has not been clarified to date.

The objectives of this study were to report the prevalence of transmitted HIV-1 drug resistance and HIV-1 subtypes in patients with recent HIV-1 infection in the area of Catalonia (Spain) between 2003 and 2005 and to describe the characteristics of these patients according to the presence or absence of transmitted HIV-1 drug resistance and HIV-1 subtype.

Patients and methods

Patients

The study population was composed of new HIV-1 diagnoses, antiretroviral-naïve individuals who were identified as having newly diagnosed with recent HIV-1 infection between 2003 and 2005 at 26 of the 28 laboratories participating in the AERI project.[8] We were only able to include samples from 17 of the 26 laboratories (1 each from Badalona, L'Hospitalet de Llobregat, Lleida, Sabadell, Palamòs, Reus, Tortosa, Cornellà, Granollers, and Vic, as well as 5 from Barcelona and 2 from Mataró).

The protocol was approved by the ethics committees of all the participating centers. An infection was considered recent if the sample reacted with the sensitive enzyme immunoassay (EIA) but not with the modified less sensitive EIA (LS-EIA (ie, had low HIV-1 antibody titers). Since antibody titers can fall in advanced stages of HIV-1 infection, patients whose samples did not react and who presented clinical criteria for AIDS were not considered recently infected. All patients who had initiated cART before sampling were excluded from this analysis.

Specimen collection

Residual aliquots of serum collected for diagnostic purposes were frozen and sent by the participating laboratories to the coordinating center (*Centre d'Estudis Epidemiològics sobre les ITS i la Sida de Catalunya*, CEEISCAT). After application of Serological Testing Algorithm for Recent HIV Seroconversion (STARHS) samples were

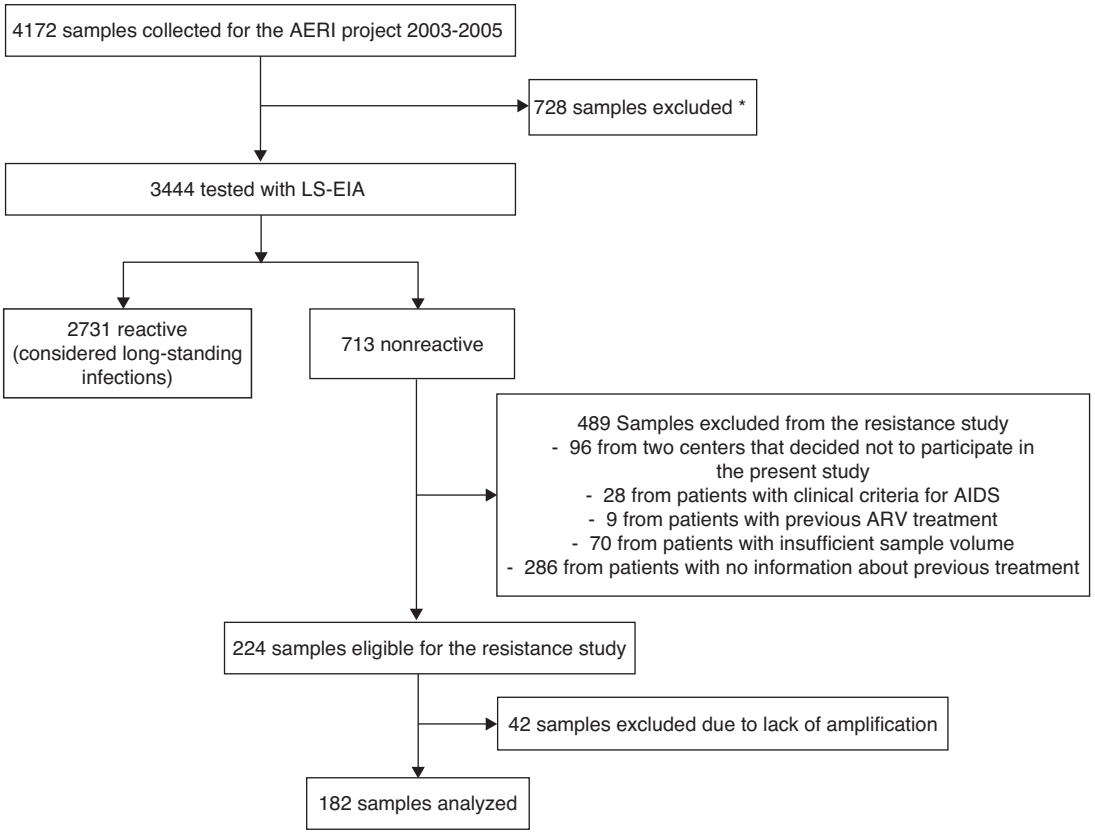

Figure 1. Flowchart of samples included in our study. *Of the 728 samples excluded, 75.0% belonged to patients whose HIV infection was diagnosed more than 6 months before sample collection, 11.1% were duplicate samples, 9.2% were HIV-negative samples, 2.7% did had insufficient volume, 1.4% were negative by WB, and 0.5% were from patients aged under 18 years.

stored at –70° C. The residual volumes of serum specimens from recently infected treatment-naïve patients were sent to the two laboratories responsible for genotyping (IrsiCaixa Foundation and Hospital Clínic).

Data collection

Laboratory staff completed a data collection form to record $CD4^+$ T-cell count, HIV-1 viral load, and previous HIV-1 test results. An additional data collection form was required for each recently infected case. This form was completed by the patient's physician or a designated person and contained demographic and clinical-epidemiological variables (sex, date of birth, country of origin, date of arrival in Spain, HIV-1 risk category, sexually transmitted infections, known HIV-1 status at the time of current testing, and use of cART).

Quality control

Extensive control procedures were implemented to ensure the quality of the data. In patients identified as recent infected, their identification study numbers were sent to each participant centre to ensure that each included patient had not received antiretroviral treatment. Patients for whom no information on previous cART was available were excluded from the analysis.

Identification of recent infections

HIV-1 positive specimens were tested at the Microbiology Service of *Hospital Universitari Germans Trias i Pujol* using a modified version of the Vironostika HIV-1 EIA (bioMérieux, Durham, North Carolina, USA), in which sample dilution times and sample and conjugate incubation times were modified to render it less sensitive. Recent infection was defined as occurring within the past 170 days (95% confidence interval [CI], 144-200).[9] Since 2000, *CEEISCAT* (Center for Epidemiological Studies on STIs/HIV/AIDS of Catalonia) and the Microbiology Service of the *Hospital Universitari Germans Trias i Pujol* have been participating in an international STARHS quality control program established by the Centers for Disease Control and Prevention (FDA BB-IND # 8193).

Genotyping

HIV-1 reverse transcriptase and protease genes were genotyped using the TruGene HIV-1 Genotyping Kit, (Siemens Healthcare Diagnostics, Barcelona, Spain) at the IrsiCaixa laboratory and the ViroSeq HIV-1 Genotyping System (Abbott Molecular, Abbott Park, Illinois, USA) at the Hospital Clínic laboratory. TruGene amplifies from codon 4 to 99 in the protease gene and from codon 37 to 247 in the reverse transcriptase gene. ViroSeq amplifies codons 1 to 99 in the protease gene and codons 1 to 335 in the reverse transcriptase gene.

In order to homogenize the sequence analyses, all FASTA sequences were analyzed using the HIVDB Program (available from http://hivdb.stanford.edu, August 2008) and summarized. However, to calculate the prevalence of mutations associated with reduced drug susceptibility, we used the World Health Organization (WHO) 2009 List of Mutations for Surveillance of Transmitted Drug Resistant HIV Strains.[10] Viral subtypes were assessed on the basis of the pol sequence using the REGA HIV-1 subtyping tool, version 2 (available from http://www.bioafrica.net/subtypetool/html/, July 2009). In cases where the REGA HIV-1 subtyping tool did not assign a valid subtype, we used phylogenetic analysis. Genetic distances and evolutionary rates were computed using a Kimura 2-parameter model. Neighbour-joining phylogenetic trees of each subject's pol sequences were constructed using MEGA4.1. The reliability of phylogram clustering was assessed by bootstrapping analyses.

Statistical analysis

We calculated the proportion of the presence or absence of transmitted HIV-1 drug resistance and B and non-B HIV-1 subtypes. We provided the 95% CI using the normal approximation or the exact method when appropriate. Patients with or without resistant profiles and with B or non-B HIV-1 subtypes were compared using the Pearson chi square test or Fisher exact test. The Mann-Whitney test was performed for quantitative variables. The variables examined were gender, age at diagnosis, route of transmission, origin, year of diagnosis, $CD4^+$ T-cell count, HIV-1 viral load, co-infection with a sexually transmitted infection, and geographic area. Univariate and multivariate logistic regression models were constructed to identify the characteristics associated with infection by a non-B HIV-1 subtype.

Results

We collected 4,172 samples between 2003 and 2005. After excluding duplicates and patients with a previously known HIV-1 infection, we used LS-EIA to analyze 3,444 samples, of which 713 were nonreactive. Of these, 489 were excluded for various reasons (lack of information about previous treatment, samples from 2 centers that did not participate in the study, AIDS, previous antiretroviral treatment, or insufficient sample volume); therefore, 224 samples were eligible, and, of these, 182 were successfully genotyped (Fig. 1). Age, gender, origin, and HIV-1 risk were similar for the study patients and those who were excluded (data not shown). Most of the patients were male (80.8%), natives of Spain (76.2%), and the median age was 33.3 years. The main route of HIV-1 acquisition was sexual relations between men who have sex with men (48.3%), followed by heterosexual relations (22.5%), and intravenous drug use (13.2%). Median (interquartile range (IQR)) viral load ($\log_{10}$) was 5.0 (4.5-5.5) and median (IQR) CD4 lymphocyte count was 541 cells/mm^3 (357-698).

According to the WHO 2009 list, 7.7% (95% CI, 4.3-12.6) of individuals with recent infections (14 cases) presented mutations associated with resistance. Seven patients (3.8%; 95% CI, 1.6%-7.8%) had evidence of resistance against non-nucleoside reverse transcriptase inhibitors (NNRTIs), 6 (3.3%; 95% CI, 1.2%-7.0%) had genotypic evidence of primary drug resistance to nucleoside reverse transcriptase inhibitors (NRTIs), 3 (1.6%; 95% CI, 0.3%-4.7%) against protease inhibitors (PIs), and only 2 (1.1%; 95% CI, 0.1%-3.9%) presented mutations associated with resistance against more than 1 class of drugs. The prevalence of resistance was 10.0% (95% CI, 3.8-20.5) for 2003, 6.5% (95% CI, 2.1-14.5) for 2004, and 6.7% (95% CI, 1.4-18.3) for 2005.

Table 1
Prevalence of Mutations in the Protease and Reverse Transcriptase Genes.

Mutations	Frequency N (%)
PR gene	
L10F/I/V	19 (10.4)
A71T/V	19 (10.4)
D30N	1 (0.5)
K43T	1 (0.5)
I47V	1 (0.5)
Q58E	1 (0.5)
V82L	1 (0.5)
L90S	1 (0.5)
RT gene	
NRTIs	
A62V	5 (2.7)
V118I	5 (2.7)
M41L	3 (1.6)
G333E	3 (1.6)
D67N/E	2 (1.1)
L210M	2 (1.1)
L210W	1 (0.5)
T215D	2 (1.1)
T215F	1 (0.5)
K219Q/E	2 (1.1)
T69N	1 (0.5)
K70R	1 (0.5)
V75A/I/T/V	1 (0.5)
Y115C	1 (0.5)
E44A	1 (0.5)
NNRTIs	
E138A	10 (5.5)
V179E/D/C	7 (3.8)
K103N	5 (2.7)
K103R	4 (2.2)
A98T/G	2 (1.1)
Y188L/H	2 (1.1)
F227L/S	2 (1.1)
V106A	1 (0.5)
V108I	1 (0.5)
V90I	2 (1.1)
V106I	2 (1.1)

Mutations in bold are considered to be associated with resistance according to the list for surveillance by WHO.

According to the WHO list, the most prevalent resistance mutations—K103N (2.7%), and M41L (1.6%)—were in the reverse transcriptase gene. In addition, considering mutations reported by the HIVDB, minor mutations were common in the protein gene (L10F/I/V and A71T/V; 10.44% each), as were NNRTI-related mutations, such as E138A (5.49%), V179E/D/C (3.85%), and K103R (2.2%). A list with all the mutations observed is presented in Table 1.

Patient characteristics according to the presence or absence of transmitted HIV-1 drug resistance (WHO 2009 List of Mutations) are shown in Table 2. Patients with transmitted HIV-1 drug resistance were men (100.0%) with a median age of 33.6 years. Men who have sex with men accounted for 57.1%, and 57.1% were from Spain. An STI was diagnosed in 75%, 71.4% were subtype B, 85.7% were from the area of Barcelona, the median viral load ($\log_{10}$) was 4.7 and the median CD4 lymphocyte count was 476 cells/mm^3. There were no statistically significant differences between resistant and nonresistant groups. However, immigrants (15.0%), men (9.5%), and patients with a diagnosis of a sexually transmitted infection (8.3%) showed a higher prevalence of transmitted HIV-1 drug resistance.

Of the 182 successfully genotyped samples, 147 (80.8%) were labeled as subtype B and 35 (19.2%) were classified as other subtypes. A total of 39 sequences were not ascribed by the REGA subtyping tool and the subtype was assigned by phylogenetic analysis. The distribution of non-B subtypes was CRF02_AG (n=11), CRF01_AE (n=9), G (n=3), A (n=3), F/B (n=2), G/B (n=2), D (n=1), H/B (n=1), J/K (n=1), K/G (n=1) and C (n=1). The characteristics of patients according to HIV-1 subtype are shown in Table 3. Most of the patients

Table 2
Characteristics of patients according to the presence or absence of transmitted HIV-1 drug resistance, according the World Health Organization 2009 List of Mutations for Surveillance of Transmitted Drug Resistant HIV Strains.

	Total	Non resistant	Resistant	*P*
	182	168 (92.3) 95% CI: (87.4-95.7	14 (7.7) 95% CI: 4.3-12.6	
Sex[*]				.075
Male	147 (80.8)	133 (79.2)	14 (100.0)	
Female	35 (19.2)	35 (20.8)	0 (0.0)	
Age (years) (N=181)[**]	33.3 (27.2–37.9)	33.3 (27.2-37.8)	33.6 (28.2-42.7)	.500
<30[*]	69 (38.1)	65 (38.9)	4 (28.5)	.677
30-40	78 (43.1)	72 (43.1)	6 (42.9)	
40-50	21 (11.6)	19 (11.4)	2 (14.3)	
>50	13 (7.2)	11 (6.6)	2 (14.3)	
Route of transmission[*]				.404
Men who have sex with men	88 (48.3)	80 (47.6)	8 (57.1)	
Heterosexual	41 (22.5)	37 (22.0)	4 (28.6)	
Intravenous drug use	24 (13.3)	22 (13.1)	2 (14.3)	
Others/unknown	29 (15.9)	29 (17.3)	0 (0.0)	
Origin[*] *(N=168)*[***]				.101
Natives	128 (70.3)	120 (77.9)	8 (57.1)	
Immigrant	40 (29.7)	34 (22.1)	6 (42.9)	
Plasma RNA-HIV-1 (log_{10}) N=162)[**]	5.0 (4.5-5.5)	5.1 (4.5-5.6)	4.7 (4.2-5.3)	.093
<5.0[*]	81 (50.0)	73 (48.3)	8 (72.7)	.210
>5.0	81 (50.0)	78 (51.7)	3 (27.3)	
CD4 cell count /mm^3 (N=154)[**]	541 (357-698)	541 (358-6999)	476 (194-714)	.582
<350[*]	36 (23.4)	33 (23.0)	3 (30.0)	.540
350-500	31 (20.1)	28 (19.4)	3 (30.0)	
>500	87 (56.5)	83 (57.6)	4 (40.0)	
STI co-infection (N=96)[*]				.147
Yes	36 (37.5)	33 (35.9)	3 (75.0)	
No	60 (62.5)	59 (64.1)	1 (25.0)	
HIV subtype[*]				.477
B	147 (80.8)	137 (81.5)	10 (71.4)	
Non B	35 (19.2)	31 (18.5)	4 (28.6)	
Geographic area[*]				.624
Barcelona	165 (90.7)	153 (91.1)	12 (85.7)	
Outside Barcelona	17 (9.3)	15 (8.9)	2 (14.3)	

[*]N (%); [**]Median and interquartile range; [***]P value calculated for 168 patients with available information.
STI co-infection: Having a diagnosis of an sexually transmitted infection within 12 months before inclusion.
Barcelona: Includes Barcelona city and its metropolitan area.
Outside Barcelona: Includes Lleida, Tortosa, Reus, Vic and Palamòs.

with a non-B subtype were male (71.4%) with a median age of 35.9 years. Spanish-born patients accounted for 64.7% and 80% were from the area of Barcelona. Median viral load was 5.1, median CD4 cell count was 471 cells/mm^3, 20% reported a diagnosis of STI, and 11.4% presented transmitted HIV-1 drug resistances. Among the patients infected by a non-B subtype, the percentage of men who have sex with men, heterosexual individuals, and intravenous drug users was the same for the 3 groups (28.6%). Twelve (34%) of these patients infected by a non-B subtype were immigrants: 5 were from South America, 4 from Sub-Saharan Africa, 2 from Eastern Europe, and 1 from North Africa. The date of arrival in Spain was known in 8 cases: 6 had arrived in Spain more than 2 years previously, 1 had arrived 7 months before the HIV diagnosis, and 1 patient had arrived just 1 month before the HIV diagnosis. When comparing individuals carrying the B subtype with those carrying the non-B subtype, individuals with non-B subtypes were older (35.9 vs 32.8 years), more frequently intravenous drug users (41.7%), and more frequently from areas outside Barcelona (41.2 vs. 17.0%). These differences were statistically significant (Table 3). They also presented a lower CD4 lymphocyte count than patients carrying the B subtype. The variables entered in the logistic regression model were age, CD4 count, transmission group, and transmitted HIV-1 drug resistance. In the multivariate analysis, being over 40 years of age was the only factor associated with infection by a non-B HIV-1 subtype.

Discussion

This is the first prospective study to evaluate the prevalence of transmitted HIV-1 drug resistances in recently infected patients in the Autonomous Region of Catalonia, Spain. Using the 2009 WHO HIV mutations list, we were able to demonstrate a rate of drug resistance (7.7%) similar to that of previous studies in other Spanish and European regions.[2–4,11–13] In a recent meta-analysis including 26 studies performed in Spain, the global transmitted HIV-1 drug resistance prevalence was estimated to be 10.6% between 1997 – 2008.[14] Although this result is quite similar to ours, the revised studies have used different lists of transmitted HIV-1 drug resistance and none of them have used the WHO 2009 list, making difficult any comparison.

We observed that prevalence was higher in 2003 than in following years (10.0 vs 6.5% in 2004 and 6.7% in 2005). Although not statistically significant, this finding is consistent with those of other authors, who report a decreasing temporal trend in the prevalence of transmitted HIV-1 drug resistances.[2]

The main difficulty in analyzing genotypic information to provide prevalence rates is to establish a comparable definition of resistance.[1,12] The IAS-USA 2009[15] list is commonly used to report resistance and has been developed to analyze mutations selected by the HIV-1 B subtype. This is particularly relevant in persons infected

Table 3
Characteristics of patients according to HIV-1 subtype.

	Total	B subtype	Non B subtype	*P*
	182	147 (80.8) 95% CI: (74.3-86.2)	35 (19.2) 95% CI: (13.8-25.7)	
Sex[*]				.151
Male	147 (80.8)	122 (83.0)	25 (71.4)	
Female	35 (19.2)	25 (17.0)	10 (28.6)	
Age (years) (N=181)[**]	33.3 (27.2–37.9)	32.8 (26.9-37.2)	35.9 (29.2-43.1)	.017
<30[*]	69 (38.1)	60 (41.1)	9 (25.7)	.004
30-40	78 (43.1)	66 (45.2)	12 (34.3)	
40-50	21 (11.6)	11 (7.5)	10 (28.6)	
>50	13 (7.2)	9 (6.1)	4 (11.4)	
Route of transmission[*]				.009
Men who have sex with men	88 (48.3)	78 (53.1)	10 (28.6)	
Heterosexual	41 (22.5)	31 (21.1)	10 (28.6)	
Intravenous drug use	24 (13.3)	14 (9.5)	10 (28.6)	
Others/unknown	29 (15.9)	24 (16.3)	5 (14.3)	
Origin[*] *(N=168)*[***]				.113
Natives	128 (70.3)	106 (79.1)	22 (64.7)	
Immigrant	40 (29.7)	28 (20.9)	12 (34.3)	
Plasma RNA-HIV-1 (log_{10}) (N=162)[**]	5.0 (4.5-5.5)	5.0 (4.5-5.5)	5.1 (4.2-5.7)	.935
<5.0[*]	81 (50.0)	67 (50.8)	14 (46.7)	.840
>5.0	81 (50.0)	65 (49.2)	16 (53.3)	
CD4 cell count /mm^3 (N=154)[**]	541 (357-698)	553 (390-712)	471 (281-581)	.063
<350[*]	36 (23.4)	25 (19.8)	11 (39.3)	.059
350-500	31 (20.1)	25 (19.8)	6 (21.4)	
>500	87 (56.5)	76 (60.3)	11 (39.3)	
STI co-infection (N=96)[*]				.127
Yes	36 (37.5)	33 (40.7)	3 (20.0)	
No	60 (62.5)	48 (59.3)	12 (80.0)	
*Transmitted HIV-Drug Resistances**				.477
Yes	14 (7.7)	10 (6.8)	4 (11.4)	
No	168 (92.3)	137 (93.2)	31 (88.6)	
Geographic area[*]				.024
Barcelona	165 (90.7)	137 (93.2)	28 (80.0)	
Outside Barcelona	17 (9.3)	10 (6.8)	7 (20.0)	

[*]N (%); [**]Median and interquartile range; [***]P value calculated for 168 patients with available information.
STI co-infection: Having a diagnosis of an sexually transmitted infection within 12 months before inclusion.
Barcelona: Includes Barcelona city and its metropolitan area.
Outside Barcelona: Includes Lleida, Tortosa, Reus, Vic and Palamòs.

with subtypes other than B, which shows many more polymorphisms than the reference subtype B virus.

As in previous reports,[4] the NNRTI mutation K103N was common in our study (5 cases). This mutation is the result of a single-nucleotide polymorphism from wild-type K103. It is associated with a high level of resistance to nevirapine and efavirenz, although it does not affect the activity of etravirine.[16,17] The polymorphism K103R is not associated with the emergence of K103N; however, in combination with V179, it significantly reduces the susceptibility of the virus to nevirapine and efavirenz.

"Minor" or "secondary" mutations to new-generation NNRTIs (mutations associated with reduced activity to etravirine) were common in our study. The virological response to etravirine is a function of the number and weight of the baseline mutations. In NNRTI-experienced patients who started etravirine, darunavir, and a background regimen in the DUET studies, weighted mutation scores of 0-2, 2.5-3.5, and $\geq$4 were associated with a response of 74%, 52%, and 38%, respectively.[16]

As in other series,[1,2] thymidine analog mutations (eg, M41L, D67N/E, L210W, T215F, K219Q/E) were less common than NNRTI mutations.

Major PI mutations were rare (<0.5%). Although minor PI mutations were relatively common in our sample (>10% for L10F/I/V and for D71T/V/A), they are not included in the WHO list because they are often reported in almost all non-B subtypes.

The proportion of immigrants in our sample was 23.3% in 2003, 18.2% in 2004, and 26.7% in 2005. In Catalonia, the number of immigrants accounting for new cases of HIV rose during the study period (31.3% in 2003, 34.0% in 2004, and 39.1% in 2005).[18] Nearly 20% of our patients had non-B subtypes, and although the highest prevalence was seen in 2005 (28.9%), there were no significant trends during the 3 years of the study. In areas outside Barcelona (Lleida, Tortosa, Reus, Vic, and Palamós), subtypes other than B were more frequent than in the metropolitan area of Barcelona (41.2 vs. 17.0%). In our population of recently HIV-infected patients, the percentage of immigrants was higher in areas outside Barcelona (47.1 vs. 19.6%). The prevalence of non-B subtypes among autochthonous patients was 17.2%. This high prevalence would suggest that non-B subtypes are already circulating in our population. Several authors have reported an increase in the frequency of non-B subtypes in different Spanish regions. In Madrid, the prevalence of non-B subtypes increased from 9% in 2000 to 32% in 2007; the number of autochthonous patients infected also increased during the same period (from 4% to 10%).[19] In Galicia, the prevalence was 22.3% between 2000 and 2002,[20] and in Gran Canaria it was 22.4% between 2002 and 2005.[13]

Little is known about the clinical and biological consequences of infections by non-B subtypes. Differences in pathogenicity, transmissibility, or susceptibility to antiretroviral drugs among HIV-1 subtypes have been proposed,[21,22] and there is evidence

that similar outcomes can be found in almost all the subtypes after conventional anti–HIV-1 treatment.[23] We observed resistance mutations to be more frequent in patients with the non-B subtype, although this difference it is not statistically significant. In the multivariate analysis, the only factor associated with infection by a non-B HIV-1 subtype was being over 40 years old. However, we are not able to offer a clear explanation for this finding, probably due to the small sample size and the constraints of our current data.

Given the limitations of our study, the results should be interpreted with caution. First, we only analyzed 182 of the 713 potentially recent HIV-1 infections. The lack of data on antiretroviral therapy was the most frequent cause of exclusion,[9] although a comparison of the baseline characteristics of patients with and without a resistance test result suggests that our data are representative of the original study sample. Second, 21% of the samples identified could not be amplified. This was probably because samples were residual aliquots from serological testing and, therefore, were not optimally stored for PCR analysis. Third, the LS-EIA used to identify recent infections was validated in the B subtype. The results of this assay vary depending on the HIV-1 subtype, with a longer window period in non-B subtypes than in B subtypes. For circulating recombinant forms (CRF01_AE in particular), the mean window period was 356 days (95% CI, 318-402).[24] These data, would suggest that non-B subtypes identified as recent infections are presumed to have seroconverted within the past 12 months. And fourth, we used conventional sequencing, which underestimates minority HIV-1 variants.

Despite its limitations, our approach does have certain strengths. First, it focused on recent infections, which demonstrated a higher rate of resistance than chronic infections, thus reflecting, at least in part, the gradual disappearance of the dominant quasispecies over time. Second, analyzing all samples for recent infection and resistance avoids the bias of recruiting persons who seek more frequent testing (usually white men who have sex with men), thus preventing extrapolation to the general HIV-1–infected population. Third, the robustness of our data and the characteristics of the Catalonian health system (unrestricted free access to HIV care and treatment) mean that there is little risk of including patients with little exposure to therapy.

In conclusion, we observed a prevalence of transmitted HIV-1 drug resistance of 7.7% (95% CI, 4.3-12.6), which is consistent with results from other parts of Europe. Furthermore, a high prevalence of transmitted HIV-1 drug resistance driven mainly by K103N and NRTI mutations was found in Catalonia. We also showed the feasibility of monitoring both transmitted HIV-1 drug resistance and HIV subtypes in our setting, and the logistical pitfalls identified will be crucial if we are to improve monitoring of these parameters. Although not significant, the prevalence of non-B subtypes among recently infected patients is increasing, and this group has the highest prevalence of resistance. Although we could not establish a spatial or temporal relationship between immigration patterns, our data suggest that the non-B subtypes are already circulating in our setting. Long-term monitoring of recent infection will allow us to better describe the relationship between transmitted HIV-1 drug resistance and subtypes with imported and locally acquired HIV infection, as well as its clinical implications.

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Conflict of interests

The authors declare no conflicts of interest related to this study.

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Appendix A.

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Site-testing nongovernmental organizations: Roser Sala (Laboratori Sabater Tobella, Barcelona); Olga Díaz (Servei d'Atenció i Prevenció Sociosanitària: SAPS - Creu Roja, Barcelona); Kati Zaragoza (Stop Sida, Barcelona); Ferran Pujol and Jorge Saz (Projecte dels Noms – Joves positius, Barcelona); Mercè Meroño (Àmbit Prevenció, Barcelona); Jasmina Becerra (Associació Ciutadana Antisida de Catalunya – ACASC, Barcelona); Rosa Ros (Centre Jove d'Anticoncepció i Sexualitat – CJAS, Barcelona); Anna Avellaneda and Montse Sité (Actua Vallès, Sabadell) and Anna Rafel (Associació Antisida de Lleida).

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ARTÍCULO IV

PLOS | ONE

RESEARCH ARTICLE

Trends in Transmission of Drug Resistance and Prevalence of Non-B Subtypes in Patients with Acute or Recent HIV-1 Infection in Barcelona in the Last 16 Years (1997-2012)

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Abstract

Objectives

To evaluate the prevalence of transmitted drug resistance (TDR) and non-B subtypes in patients with acute/recent HIV-1 infection in Barcelona during the period 1997-2012.

Methods

Patients from the "Hospital Clínic Primary HIV-1 Infection Cohort" with a genotyping test performed within 180 days of infection were included. The 2009 WHO List of Mutations for Surveillance of Transmitted HIV-1 Drug Resistance was used for estimating the prevalence of TDR and phylogenetic analysis for subtype determination.

Results

189 patients with acute/recent HIV-1 infection were analyzed in 4 time periods (1997-2000, n=28; 2001-4, n=42; 2005-8, n=55 and 2009-12, n=64). The proportion of patients with acute/recent HIV-1 infection with respect to the total of newly HIV-diagnosed patients in our center increased over the time and was 2.18%, 3.82%, 4.15% and 4.55% for the 4 periods, respectively (p=0.005). The global prevalence of TDR was 9%, or 17.9%, 9.5%, 3.6% and 9.4% by study period (p=0.2). The increase in the last period was driven by protease-inhibitor and nucleoside-reverse-transcriptase-inhibitor resistance mutations while non-nucleoside-reverse-transcriptase inhibitor TDR and TDR of more than one family decreased. The overall prevalence of non-B subtypes was 11.1%, or 0%, 4.8%, 9.1% and 20.3 by study period (p=0.01). B/F recombinants, B/G recombinants and subtype F emerged in the last

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period. We also noticed an increase in the number of immigrant patients (p=0.052). The proportion of men-who-have-sex-with-men (MSM) among patients with acute/recent HIV-1 infection increased over the time (p=0.04).

Conclusions

The overall prevalence of TDR in patients with acute/recent HIV-1 infection in Barcelona was 9%, and it has stayed relatively stable in recent years. Non-B subtypes and immigrants proportions progressively increased.

Introduction

Antiretroviral therapy (ART) has dramatically changed the natural history of HIV infection. Most naïve patients who begin ART today suppress viral replication and achieve functional restoration of the immune system. However, during treatment, almost one-quarter of patients experience virological failure and often have resistant HIV isolates [1]. The widespread use of ART and the increased survival of patients receiving it make the transmission of resistant HIV strains likely to occur. Resistant strains have been reported in infections acquired through sexual contact, vertical transmission and exposure to infected blood [2, 3]. Thus, transmitted drug resistance (TDR) has become a relevant public health problem. Active surveillance of TDR provides important information about the factors involved in the transmission of resistant HIV strains and in the selection of ART components. It plays a major role in the design of strategies to control the evolution and emergence of resistance [4].

Worldwide, the prevalence of resistance in acute or recent HIV-1 infections ranges from 5% to 24.5%[2, 5]. In Spain, the multicenter studies performed have very small samples [6, 7], and there are no previous reports from Barcelona.

HIV-1 subtype B infections have traditionally been predominant among the infected European population, particularly in men-who-have-sex-with-men (MSM). However, the prevalence of non-B subtypes is increasing in developed countries, as result of international travel and population migration [8, 9].

The aims of this study were to estimate the prevalence of antiretroviral resistance mutations and non-B subtypes in a cohort of 189 consecutive patients with acute or recent HIV-1 infection in a tertiary teaching hospital in Barcelona, Spain, and to describe the pattern of changes over a 16-year period (1997–2012).

Materials and Methods

Study population

The study population comprised patients from the "Hospital Clinic Primary HIV-1 Infection Cohort" consecutively evaluated within 180 days after HIV infection at the Hospital Clínic, Barcelona, Spain, between January 1, 1997 and December 31, 2012. The inclusion criteria were detectable viremia with a negative HIV serology result or documented seroconversion within the 6 months prior to the first evaluation. In symptomatic patients with several exposures, the date of infection was assumed to be 14 days before the beginning of symptoms. For asymptomatic seroconverters, the date of infection was assumed to be the midpoint between the last negative test result and the first positive one. At the time of genotyping, patients with an estimated time of infection of less than 30 days were defined as 'acute infection' and those with an estimated time of

infection between 30 and 180 days as 'recent infection'. Patients with resistance tests performed beyond 180 days after the suspected day of infection were excluded from the analysis.

Patients were classified into 4 periods according to the year of diagnosis: 2009–2012 (widespread availability in Barcelona of 4 new drugs: darunavir, etravirin, raltegravir and maraviroc) and three earlier periods of equal duration: 1997–2000, 2001–2004 and 2005–2008.

Virological analyses

HIV serology was determined using a microparticle enzyme immunoassay (AxSYM, Abbott Laboratories, Illinois, USA) and confirmed by line immunoassay (Inno-LIA HIV I/II Score, Innogenetics, Ghent, Belgium). Viremia was measured using the Cobas Amplicor Monitor (Roche Molecular Systems, Branchburg, New Jersey, USA) or the Versant HIV-1 RNA 1.0 Assay kPCR (Siemens Healthcare, Erlangen, Germany) with a limit of detection of 50 or 37 copies/mL, respectively. Genotypic mutations of both the reverse transcriptase gene and the protease gene from viral RNA were detected using the ViroSeq HIV Genotyping System v.2 (Abbott Laboratories, Illinois, USA) and an ABI3100 sequencer. HIV- 1 subtype characterization was first performed by using the REGA HIV-1 Subtyping Tool of the Stanford database (available from http://dbpartners.stanford.edu/RegaSubtyping/) and confirmed by Neighbor-Joining (NJ) phylogenetic analyses by using the MEGA version 6 program [10]. NJ phylogenetic trees were inferred under the Kimura 2-parameter (K2-P) nucleotide substitution model and reliability of the obtained tree topology was estimated with the bootstrap method with reference sequences obtained from the HIV Sequence Database, Los Alamos National Laboratory (LANL; www.hiv.lanl.gov). HIV-1 sequences suspected to be recombinants in the NJ phylogenetic tree were analyzed by bootscan analyses with Simplot 3.5.1 software [11]. The amino acid substitutions selected by highly active antiretroviral therapy (HAART) and associated with drug resistance were identified using the 2009 World Health Organization (WHO) list of mutations for surveillance of drug resistance [12]. Drug mutations for integrase inhibitors were not analyzed since they are not routinely performed for patients with primary infection in our institution. Mutations not included in the 2009 WHO list, but associated with resistance to rilpivirine according to Stanford HIV drug resistance database were also reported due to the clinical relevance.

Statistical analysis

The chi-square test or the Fisher exact test was used, as appropriate, to compare categorical variables, and the Mann-Whitney or Klustal-Wallis tests were used, as appropriate, to compare continuous variables. Links between resistant genotype and sex, age, risk factors, symptoms, CD4 and CD8 cell counts, and viral load were tested. All p values were considered significant at <0.05. As described above, 4 time periods were analyzed: 1997–2000, 2001–4, 2005–8 and 2009–12. The correlation between the increase in non-B HIV-1 subtypes and the increase in immigration during the 4 study periods was evaluated using Spearman's Rank Correlation Coefficient. All statistical analyses were performed using SPSS software, version 17.

The study was approved by the Institutional Review Board (Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi-Sunyer-IDIBAPS-). All patients signed the informed consent form.

Results

Patient characteristics according to analyzed periods

During the study period, 5,109 newly diagnosed patients underwent a first evaluation at our center; of these, 199 (3.89%) met the criteria for primary HIV infection (PHI) and were enrolled in the "Hospital Clínic Primary HIV-1 Infection Cohort". Ten patients were excluded due to the

Table 1. Baseline characteristics of patients and study periods.

		Period				
N (%)	**total 189 (100)**	**1997–2000 28 (14.8)**	**2001–2004 42 (22.2)**	**2005–2008 55 (29.1)**	**2009–2012 64 (33.9)**	**p**
Gender*						0.011
Male	175 (92.6)	26 (92.9)	34 (81)	53 (96.4)	62 (96.9)	
Female	14 (7.4)	2 (7.1)	8 (19)	2 (3.6)	2 (3.1)	
Age (n = 188)**	33 (28–39)	30 (26–36)	31 (27–37)	34 (28–38)	34 (27–39)	0.275
<30*	63 (33.5)	13 (46.4)	16 (38.1)	14 (25.5)	20 (31.7)	
30–40	82 (43.6)	10 (35.7)	20 (47.6)	27 (49.1)	25 (39.7)	
40–50	33 (17.6)	3 (10.7)	4 (9.5)	12 (21.8)	14 (22.2)	
>50	10 (5.3)	2 (7.1)	2 (4.8)	2 (3.6)	4 (6.3)	
Route of transmission*						0.004
MSM-bisexual	153 (81)	21 (75)	30 (71.4)	47 (85.5)	55 (85.9)	
Heterosexual	21 (11.1)	2 (7.1)	8 (19)	6 (10.9)	5 (7.8)	
IDU	10 (5.3)	5 (17.9)	4 (9.5)	1 (1.8)	0 (0)	
Unknown	5 (2.6)	0 (0)	0 (0)	1 (0)	4 (6.3)	
Origin*						0.052
Native	120 (63.5)	24 (85.7)	24 (57.1)	36 (65.5)	36 (56.3)	
Immigrant	59 (31.2)	2 (7.1)	14 (33.3)	18 (32.7)	25 (39.1)	
Unknown	10 (5.3)	2 (7.1)	4 (9.5)	1 (1.8)	3 (4.7)	
Symptomatic*						0.626
yes	162 (85.7)	23 (82.1)	34 (81)	49 (89.1)	56 (87.5)	
no	27 (14.3)	5 (17.9)	8 (19)	6 (10.9)	8 (12.5)	
Plasma HIV-1 log10RNA (n = 188)**	5.17 (4.51–5.80)	5.04 (4.39–5.80)	5 (4.43–5.40)	5.45 (4.75–5.62)	5.41 (4.50–5.89)	0.168
<5.0*	78 (41.5)	13 (46.4)	21 (50)	19 (35.2)	25 (39.1)	
>5.0	110 (58.5)	15 (53.6)	21 (50)	35 (64.8)	39 (60.9)	
CD4 cell count/ul (n = 188)**	494 (375–619)	494 (376–637)	584 (438–740)	506 (377–618)	402 (309–562)	0.009
<350	45 (23.9)	6 (21.4)	8 (19)	10 (18.5)	21 (32.8)	
350–500	52 (27.7)	8 (28.6)	8 (19)	15 (27.8)	21 (32.8)	
>500	91 (48.4)	14 (50)	26 (61.9)	29 (53.7)	22 (34.4)	
Acute or Recent infection at genotyping*						0.21
Acute (infection of <30 days)	23 (12.2)	2 (7.2)	1 (2.4)	9 (16.4)	11 (17.2)	
Recent (infection between 30 and 180 days)	166 (87.8)	26 (92.8)	41 (97.6)	46 (83.6)	53 (82.8)	
Resistant strain (any mutation)*	17 (9)	5 (17.9)	4 (9.5)	2 (3.6)	6 (9.4)	0.2
Non-B subtypes*	20 (10.6)	0 (0)	2 (4.8)	5 (9.1)	13 (20.3)	0.01

* n(%)

** median (IQR)

MSM: men-who-have-sex-with-men

IDU: injective drug user

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genotypic resistance test being performed beyond 180 days post-infection; thus 189 patients were included in the final analysis. Baseline characteristics of the whole population studied and according to periods are described in Table 1: 92.6% were male, median (interquartile range) age was 33 (28–39) years; for 81%, the main route of transmission was sexual relations between MSM, and 31.2% were immigrants, most of whom from Latin America (56%), followed by other Western European countries (15%) and Eastern European countries (10%). There were no patients from Sub-Saharan Africa. According to the predefined analyzed periods, 28 patients were

included in 1997–2000, 42 in 2001–4, 55 in 2005–8 and 64 in 2009–12. The proportion of patients with PHI out of the total of newly HIV-diagnosed patients increased over the time and was 2.18%, 3.82%, 4.15% and 4.55% for the 4 periods, respectively (p = 0.005).

Genotypic drug resistance

According to the WHO list of mutations, 9% of patients had a strain with 1 or more resistance mutations. The prevalence of TDR in patients with acute or recent HIV-1 infection decreased until the period 2005–8, and slightly increased in 2009–12. The proportion of resistance was 17.9% for the period 1997–2000, 9.5% for the period 2001–4, 3.6% for the period 2005–8 and 9.4% for the period 2009–12 (p = 0.2). The increase in the last period was found in nucleoside-reverse-transcriptase-inhibitor (NTRI) associated mutations (3.6% in 2005–8 and 4.7% in 2009–12) and in protease inhibitor (PI) associated mutations (1.8% in 2005–8 and 4.7% in 2009–12). Non-nucleoside reverse transcriptase inhibitor (NNRTI) associated resistance mutations, and resistance mutations to more than one family, however, decreased in the last period (Fig 1A). Overall, there were no differences in rates of TDR between the MSM and the heterosexual population (9.2% and 9.5% respectively, p = 0.628). There was no TDR among injection-drug-users (IDUs).

On the basis of the WHO proposed mutations, NRTI mutations were found in 11 patients (5.8%), NNRTI mutations were found in 6 patients (3.2%) and PI mutations were found in 9 patients (4.8%). Resistance to at least 2 drugs from the different families was observed in 7 patients (3.7%). Some resistance mutations, such as Q151M or K65R, were not found. Details of mutations found in each period are shown in Table 2. The E138A mutation, not listed by WHO 2009 list but associated to rilpivirine resistance was detected in 4 patients, 3 in the 2005–2008 period and 1 in the 2009–2012 period. No other rilpivirine associated mutation was detected.

HIV-1 Subtypes

Non-B subtypes were identified in 20 cases (10.6%). HIV-1 non-B subtype distribution increased over the 4 periods: 0%, 4.8%, 9.1% and 20.3%, respectively, with a clear linear trend (p = 0.01, Fig 1B). Both immigrant and non-B subtype proportions increased over the time (Spearman's Rank Correlation Coefficient = 0.8, p = 0.2). Non-B subtypes included subtypes A, n = 1; C, n = 2; F, n = 2; G, n = 1; A/G recombinant, n = 1; B/G recombinants, n = 2; B/F recombinants, n = 3; CRF01_AE, n = 2; and other recombinants (B/C recombinant, B/CRF06_cpx, CRF19_cpx (D segment), CRF02_AG/CRF09_cpx, CRF14_BG, CRF02_AG/Subtype B recombinant), n = 6 (Fig 2). All the Subtype F, B/G recombinants and B/F recombinants were reported in the last period (2009–2012).

The proportion of males was comparable between patients with subtype B and non-B (92.9% vs. 90%; p = 0.647), but regarding the route of transmission, MSMs were significantly higher among patients with B subtype (82.8% vs. 65%) and heterosexuals among those with non-B subtypes (20% vs. 10.1%; p = 0.001). There were no IDUs infected with non-B subtypes. The proportion of immigrants was not different among patients infected with B and non-B subtypes (30.8% and 35% respectively, p = 0.524). Patients infected by a non-B subtype were slightly older (36 vs. 33 years old) and age categories differently distributed (p = 0.037). Finally, TDR mutations were found in similar proportions among patients infected with subtype B and non-B strains (8.9% and 10% respectively, p = 0.697). A comparison of patients infected with B and non-B subtypes is shown in Table 3.

Discussion and Conclusions

In this study, the prevalence of TDR for antiretroviral drugs in patients with acute or recent HIV-1 infection (less than 6 months) in a single reference center in Barcelona over a period of

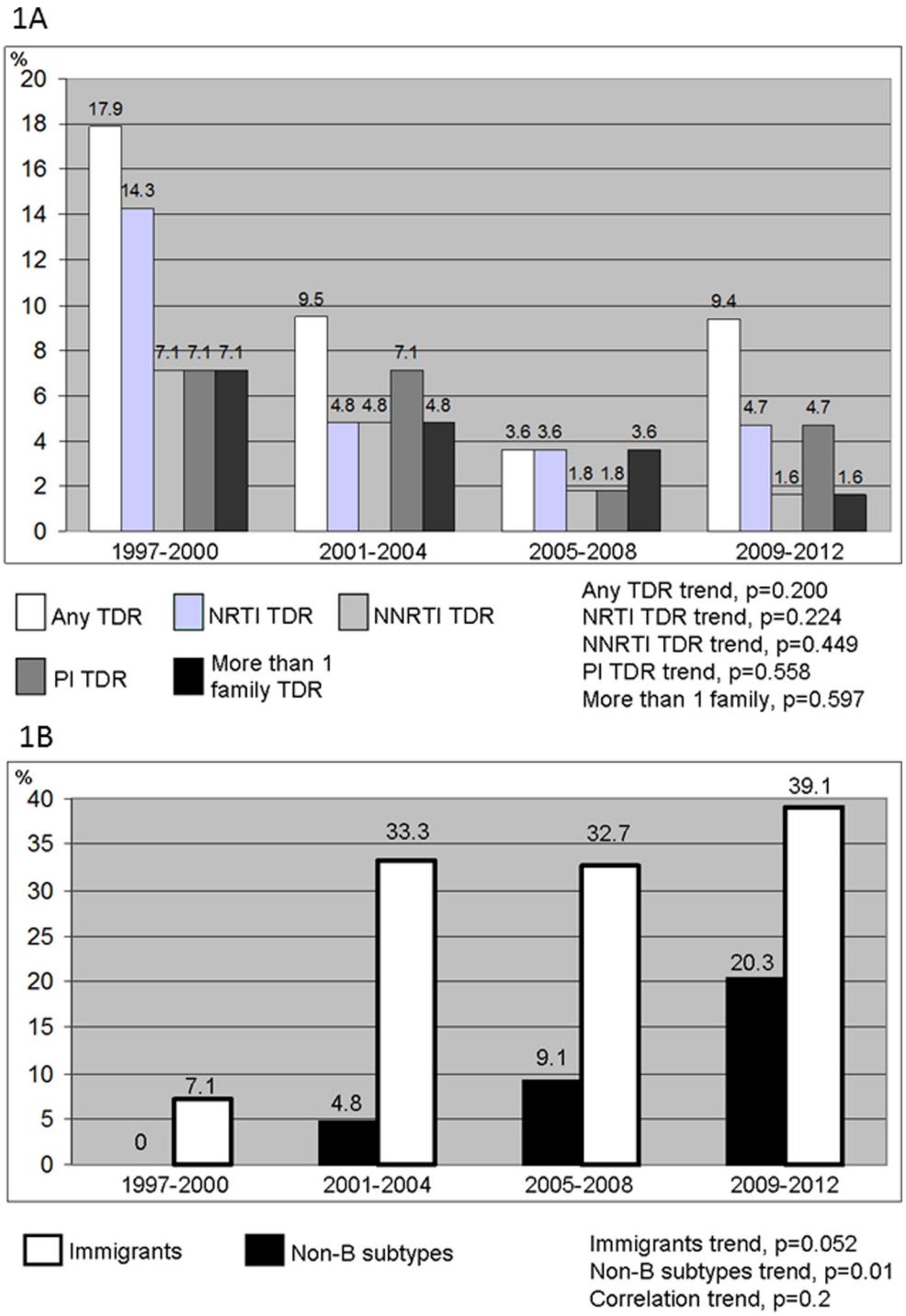

Fig 1. Fig 1A: Prevalence of transmitted drug resistance mutations by study period and drug family according to the 2009 WHO list. Fig 1B: Prevalence of non-B HIV-1 subtypes and immigrants by study period. Footnote figure: NRTI indicates nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

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Table 2. Details of mutations found according to antiretroviral family and period of study.

		Period			
Mutations N (%)*	**total**	**1997–2000**	**2001–2004**	**2005–2008**	**2009–2012**
	189	**28**	**42**	**55**	**64**
NRTI					
M41L	6 (3.2)	2 (7.1)	2 (4.8)	0 (0)	2 (3.1)
D67N	3 (1.6)	1 (3.6)	0 (0)	1 (1.8)	1 (1.6)
T69N	2 (1.1)	1 (3.6)	0 (0)	0 (0)	1 (1.6)
K70R	2 (1.1)	2 (7.1)	0 (0)	0 (0)	0 (0)
M184V	1 (0.5)	1 (3.6)	0 (0)	0 (0)	0 (0)
L210W	3 (1.6)	1 (3.6)	1 (2.4)	1 (1.8)	0 (0)
T215F/Y	3 (1.6)	2 (7.2)	1 (2.4)	0 (0)	0 (0)
T215S/D/L	2 (1.1)	0 (0)	0 (0)	2 (3.6)	0 (0)
K219E/Q	4 (2.2)	2 (7.2)	0 (0)	1 (1.8)	1 (1.6)
NNRTI[&]					
K101E	1 (0.5)	0 (0)	1 (2.4)	0 (0)	0 (0)
K103N	3 (1.6)	1 (3.6)	1 (2.4)	0 (0)	1 (1.6)
Y181C	3 (1.6)	1 (3.6)	1 (2.4)	1 (1.8)	0 (0)
G190A	1 (0.5)	0 (0)	1 (2.4)	0 (0)	0 (0)
PI					
M46I/L	5 (2.7)	2 (7.1)	2 (4.8)	0 (0)	1 (1.6)
I54L	1 (0.5)	1 (3.6)	0 (0)	0 (0)	0 (0)
V82A/F/I/T	7 (3.7)	3 (1.6)	2 (4.8)	0 (0)	2 (3.1)
L90M	2 (1.1)	0 (0)	1 (2.4)	1 (1.8)	0 (0)

* Only mutations found in at least one case according to WHO list of TDR are listed.

[&] E138A mutation, not listed by WHO 2009 list but associated to rilpivirine resistance was detected in 4 patients (2.1%), 3 (5.4%) in the 2005–2008 period and 1 (1.6%) in the 2009–2012 period.

NRTI: Nucleoside/nucleotide reverse transcriptase inhinitors

NNRTI: Non-nucleoside reverse transcriptase inhibitors

PI: Protease inhibitors

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16 years (1997–2012) was 9%. In a meta-analysis including 26 studies performed in Spain [13], those performed between 1996–2003 reported prevalence values of TDR that decreased from 26.7% to 6.7%. This wide range can be explained by differences in methodology. However, data from the studies performed between 2004–8 showed a narrower range: 11% to 2.9%[13]. De Mendoza et al. showed a decrease in the prevalence of resistance in PHI in other Spanish cities from 20% in 1999 to 3.4% in 2001 [14]. The authors claimed that these changes were due to the decrease in the number of patients with detectable viremia due to HAART and to the increase in new infections transmitted by immigrants from areas with no access to ART (many of whom were carrying HIV-1 non-B infections)[14]. However, access to ART has increased in developing countries. This is consistent with our results up to 2008, but we noticed a new increase in overall TDR in the last period (2009–12), although this increase was only seen for some antiretroviral families (PI and NRTI), while NNRTI TDR and multi-drug resistance transmission decreased. However, the increased single PI resistance mutations have very limited clinical consequences, considering the high genetic barrier of these drugs. A boosted-PI regimen is the regimen of choice when ART needs to be initiated in a patient with PHI and where the resistance test is still unavailable [15]. Moreover, we found the E138A mutation, associated with reduced susceptibility to rilpivirine in 4 patients of our cohort. This is related to the

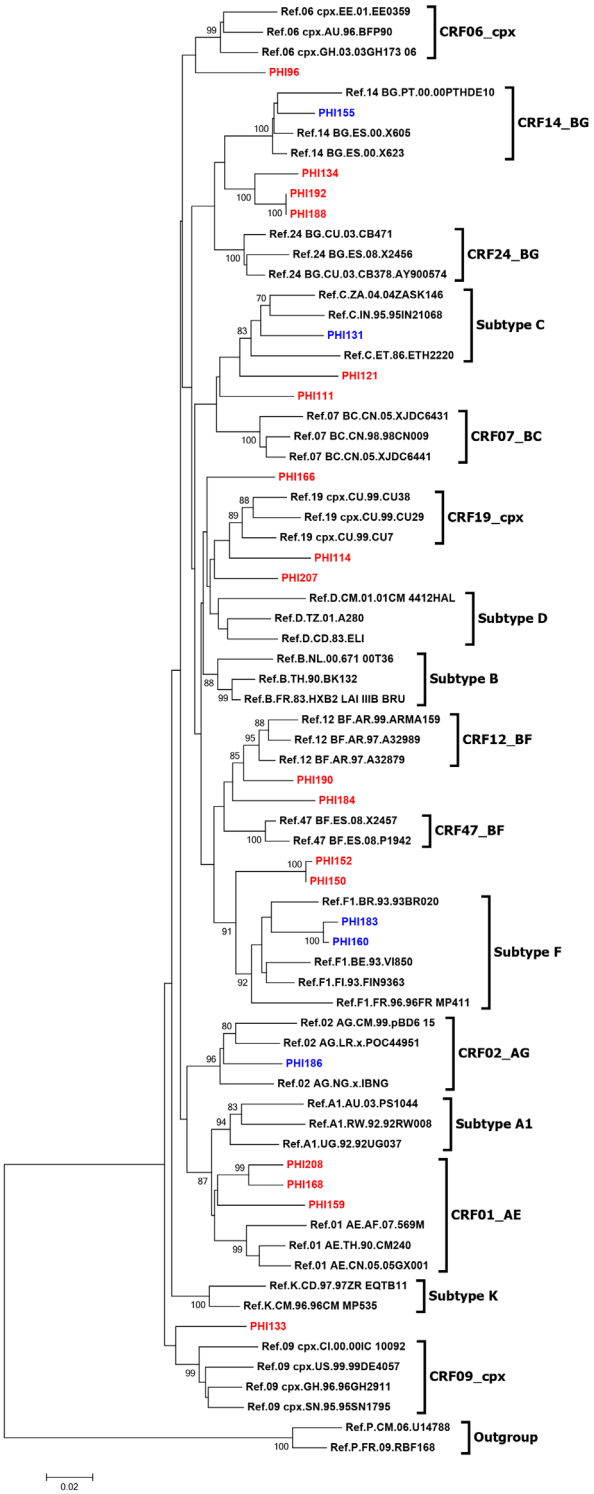

Fig 2. Phylogenetic tree analysis of the non-B HIV-1 pol sequences. Footnote figure: The phylogenetic inferences were performed by the Neighbor-Joining algorithm under the Kimura-2 parameter nucleotide substitution model with bootstrap. HIV-1 pure subtype and CRFs reference sequences were obtained from Los Alamos National Laboratory (LANL; www.hiv.lanl.gov). Bootstrap values of 70% or greater provide reasonable confidence for genotype assignment (sequences represented in blue). Sequences represented in red were suspected to be recombinants and re-analyzed by bootscanning (data not shown).

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Table 3. Patients characteristics according to subtype of infection (B or non-B).

N (%)	total 189 (100)	B subtype 169 (89.4)	Non-B subtypes 20 (10.6)	p
Gender*				0.647
Male	175 (92.6)	157 (92.9)	18 (90)	
Female	14 (7.4)	12 (7.1)	2 (10)	
Age (n = 188)**	33 (28–39)	33 (28–39)	36 (29–47)	0.037
<30*	63 (33.5)	58 (34.3)	5 (26.3)	
30–40	82 (43.6)	77 (45.6)	5 (26.3)	
40–50	33 (17.6)	27 (16)	6 (31.6)	
>50	10 (5.3)	7 (4.1)	3 (15.8)	
Route of transmission*				0.001
MSM-bisexual	153 (81)	140 (82.8)	13 (65)	
Heterosexual	21 (11.1)	17 (10.1)	4 (20)	
IDU	10 (5.3)	10 (5.9)	0 (0)	
Unknown	5 (2.6)	2 (1.2)	3 (15)	
Origin*				0.524
Native	120 (63.5)	107 (63.3)	13 (65)	
Immigrant	59 (31.2)	52 (30.8)	7 (35)	
Unknown	10 (5.3)	10 (5.9)	0 (0)	
Symptomatic*				1
yes	162 (85.7)	145 (85.8)	17 (85)	
no	27 (14.3)	24 (14,.2)	3 (15)	
Plasma HIV-1 log10RNA (n = 188)**	5.17 (4.51–5.80)	5.17 (4.60–5.80)	5.50 (4.89–5.92)	0.533
<5.0*	78 (41.5)	71 (42.3)	7 (35)	
>5.0	110 (58.5)	97 (57.7)	13 (65)	
CD4 cell count/ul (n = 188)**	494 (375–619)	490 (380–614)	512 (372–619)	0.175
<350	45 (23.9)	39 (23.2)	6 (30)	
350–500	52 (27.7)	50 (29.8)	2 (10)	
>500	91 (48.4)	79 (47)	12 (60)	
Resistant strain (any mutation)*	17 (9)	15 (8.9)	2 (10)	0.697

* n(%)

** median (IQR)

MSM: men-who-have-sex-with-men

IDU: injective drug user

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polymorphic nature of the mutation, since it was found in patients infected years before the the drug became available. Indeed, prompt initiation of ART during PHI has not only clinical but also epidemiological consequences: PHI patients are a significant source of HIV transmission [16]. Thus early therapy may decrease transmissibility [15–17].

While some studies suggest that the prevalence of TDR mutations was decreasing in Spain until 2009 [18], data published by Yebra et al.[19] for 354 HIV-1 infected patients diagnosed between 1999 and 2007 in 4 Spanish HIV/AIDS clinics (3 in Madrid and 1 on the Canary Islands) seems to contradict those results. The overall prevalence of TDR was 13.8%, and the authors did not observe a decreasing temporal trend, but an increasing one. Moreover, they found a significantly higher prevalence of NNRTI resistance mutations among patients infected by non-B subtypes [19]. This must be considered in the context of the expansion of ART access in developing countries, in which NNRTI-based regimens (particularly efavirenz) are

the most frequently prescribed. The most recent results of the CoRIS Spanish Cohort found 7.9% of TDR according to the WHO list of mutations [20], which is consistent with our results.

The results of studies from France and Italy also showed a decrease until the early/mid-2000s [21, 22]. However, in Germany and in France in more recent studies, TDR seems to be stabilizing to levels comparable to our report in the last period (2009–12)[23, 24]. In Europe, the most important data sets come from the SPREAD Programme, which prospectively investigates TDR among patients with newly diagnosed HIV-1 infection in 20 European countries and Israel. Data from 1996–2002 showed a prevalence of 13.5% among recently infected patients [25]. During the following study period, 2002–5, the prevalence seems to be stabilizing at 8.4%[26]. In a more recently published, large multi-cohort European study, TDR showed a rate of around 10%[27]. These values are comparable with the 9% of global TDR found in our study, and with the 9.4% of the last period.

Surveys in the United States report an increase in the prevalence of TDR rates during recent years. In 2005 surveys, the TDR rates were 25%[5], while previous reports showed a rate of 8.3% for 1997–2001 (26). Little et al. reported an increase from 3.4% in 1995–8 to 12.4% in 1999–2000 in 10 US cities [28]. The National HIV Surveillance system reported TDR rates of 14.6% in 2006 [29] and 16% in 2007 for 10 states and 1 county in the US [30]. In a more recent study performed in New York City in patients with acute or recent infection (median 66.5 days), TDR prevalence was 14.3%[31]. Rates can be influenced by different definitions and methodologies, but resistance could also vary according to demographic factors and access to health care and antiretroviral treatment. The US Surveillance study performed in 2006 showed that the prevalence of mutations varied with ethnicity and risk behavior: 14% in white MSM compared with less than 5% in Hispanic or African-American heterosexual men or African-American women [32], probably due to differences in access to health care in the US [33]. In Catalonia, the health care system is universal and free and provides ART to all those who need it, reducing the impact of these differences. In our cohort, we did not observe resistance in IDUs. Possible explanations are that IDUs are being infected by individuals not exposed to ART and that this transmission route is becoming less frequent. Indeed, there were no patients with PHI who had acquired the HIV infection through injection use in the last period (2009–12) of our study. However, the descriptive design and the small number of patients in this study prevent us from drawing definite conclusions for this risk group of patients.

Resistance mutations may persist for a variable time following transmission [34, 35]. Nevertheless, NRTI-associated mutations can gradually disappear, particularly those reducing viral fitness (such as M184V). A study performed in the framework of the Swiss HIV Cohort Study found that M184V minority variants were present in 8.2% of acute/recently infected patients and only in 2.5% of chronic/established infections [36]. Thus, the timing of the genotypic test may also explain differences in detected rates both in European and American studies.

Non-B subtype infections account for 20–40% of new HIV diagnoses in some European countries [37–39]. It is noteworthy that, in our study, no cases of infection by these HIV-1 non-B variants were reported in 1997, and they then progressively increased with time. A retrospective Spanish study performed outside Catalonia from 1995 to 2003 found that non-B subtypes were recognized in 43.2% of HIV-1-infected subjects with epidemiological suspicion of infection by non-B subtypes [40]. In another Spanish study including 198 seroconverters from different cities outside Catalonia, between 1997 and 2004, the frequency of non-B subtype HIV infections was only 7.6%, and no cases were found before 2002 [6]. Another region with high rates of non-B HIV infections is Galicia (a province with predominantly Portuguese and African immigration)[41]. Indeed, Pernas et al. recently reported a 37.8% of non-B variants in northwest Spain, and subtype F was the most prevalent. These patients may also have and impaired virological response [42]. In our report, we found an increasing trend over time of non-B

subtype HIV infections in Barcelona. B/F recombinants, B/G recombinants and subtype F were exclusively found in the last period. The MSM population, which represented almost 90% of the cases of PHI in our study in the last period (2009–12), seems to be particularly affected by this emergence. Indeed, although MSM was the most prevalent risk factor in all periods, the proportion among the total patients with PHI increased over time, suggesting a clear issue of transmission in early phases of HIV infection in this group. A correlation was observed between the increase in immigration and the increase in non-B subtypes. Therefore, it may be hypothesized that immigrants are infected locally by B subtypes, whereas Spanish-born patients become infected by non-B subtypes during sexual contact with immigrants infected by non-B variants.

Our study has several limitations. First, the limited number of patients infected by intravenous drug use limits the extrapolation of our conclusions. Second, the fact that some mutations are identified as resistant does not necessarily indicate clinical resistance. Last, the number of patients included per period progressively increased. A small bias in selection criteria for recent infection (within the 180-day period) cannot completely been excluded, which might have reduced the detection of some resistance mutations in a determined period. The study does, however, have several strengths. The number of patients with PHI included is very large for a single-center study. It has been performed over a long period of time, allowing us to analyze also epidemiological and risk trends in a longitudinal way.

Antiretroviral resistance is a frequent, dynamic, and complex phenomenon that should be considered from both individual and public health perspectives. The heterogeneous nature of data might be due to temporal and geographic variations, differences in the resistance tests used, timing of sampling, local prevalence of HIV subtypes, and scarce access to health care. It is necessary to establish a consensus regarding the definition of a resistance mutation and recent HIV infection in order to ensure the quality and comparative value of data.

In conclusion, the overall prevalence of resistant HIV-1 strains in acute and recent HIV-1-infected patients in Barcelona was 9%. Non-B subtypes are emerging in our population and the proportion of immigrants among patients with acute or recent HIV infection is increasing. These data should be taken into account when starting combined antiretroviral therapy in this setting and in the development of strategies for the prevention of HIV transmission, particularly in groups such as MSM.

Supporting Information

S1 File. Transmission of drug resistance and Non-B HIV-1 subtypes dataset.
(XLS)

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Author Contributions

Conceived and designed the experiments: JMM JMG LZ TP. Performed the experiments: MP MAM TP. Analyzed the data: ML OS AR FA JA. Contributed reagents/materials/analysis tools: MP MAM TP. Wrote the paper: JA OS DN CM JMG JMM. Read and agreed with the final version of the manuscript: JA OS DN MP ML AR FA MAM CM LZ JMG TP JMM. Developed the phylogenetic analyses: MGC.

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ARTÍCULO V

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RESEARCH Open Access

Acute retroviral syndrome and high baseline viral load are predictors of rapid HIV progression among untreated Argentinean seroconverters

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Abstract

Background: Diagnosis of primary HIV infection (PHI) has important clinical and public health implications. HAART initiation at this stage remains controversial.

Methods: Our objective was to identify predictors of disease progression among Argentinean seroconverters during the first year of infection, within a multicentre registry of PHI-patients diagnosed between 1997 and 2008. Cox regression was used to analyze predictors of progression (LT-CD4 < 350 cells/mm^3, B, C events or death) at 12 months among untreated patients.

Results: Among 134 subjects, 74% presented with acute retroviral syndrome (ARS). Seven opportunistic infections (one death), nine B events, and 10 non-AIDS defining serious events were observed. Among the 92 untreated patients, 24 (26%) progressed at 12 months versus three (7%) in the treated group (p = 0.01). The 12-month progression rate among untreated patients with ARS was 34% (95% CI 22.5-46.3) versus 13% (95% CI 1.1-24.7) in asymptomatic patients (p = 0.04). In univariate analysis, ARS, baseline LT-CD4 < 350 cells/mm^3, and baseline and six-month viral load (VL) > 100,000 copies/mL were associated with progression. In multivariate analysis, only ARS and baseline VL > 100,000 copies/mL remained independently associated; HR: 8.44 (95% CI 0.97-73.42) and 9.44 (95% CI 1.38-64.68), respectively.

Conclusions: In Argentina, PHI is associated with significant morbidity. HAART should be considered in PHI patients with ARS and high baseline VL to prevent disease progression.

Background

Cohort studies addressing primary HIV infection (PHI) have been used as a tool to study the natural history of HIV and to estimate the incidence of AIDS-defining events, as well as other non-associated AIDS comorbidities. It is increasingly recognized that early host-virus interactions may influence the later course of disease [1,2]. Therefore, follow up of patients immediately after seroconversion may help identify prognostic markers useful in the evaluation of therapeutic approaches.

To date, most studies of HIV seroconverters have been performed in Europe or North America [3-5]. Scarce information exists on this issue from resource-limited settings, particularly in South America, where there are different host, social and viral (i.e., subtype) characteristics that may alter the course of HIV infection [6-8].

In Argentina, it is estimated that there are approximately 130,000 persons living with HIV/AIDS, but only half of them are aware of their status. In 2008, more than 4000 new HIV infections were reported [9]. However, information regarding patients diagnosed during the early stages of infection is limited. To address this

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situation, a multicentre registry of patients with primary HIV infection in Argentina was started in 2008 [10,11].

This paper describes the epidemiological, clinical, immunological and virological characteristics of the first 134 patients enrolled in our cohort with the aim of identifying potential markers associated with HIV progression.

Methods

Study population

Grupo Argentino de Seroconversión [10,11] is an ongoing multicentre Argentine observational cohort of patients diagnosed during primary HIV infection. This cohort was started in 2008 and includes two data sets: the first one includes patients diagnosed between 1997 and 2007, and the second prospectively follows patients diagnosed after January 2008.

Inclusion criteria for enrolment in the cohort are: age > 16 years at first evaluation, confirmed diagnosis of primary HIV infection, and first medical and laboratory evaluation (i.e., CD4 cell count and plasma HIV RNA) within six months of the probable date of infection. Primary HIV infection is defined as: (1) detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western blot assay [12]; or (2) positive Western blot with a negative test within the previous six months Hence, it includes both acute and recent HIV-infection patients.

Structured questionnaires are used for baseline and follow-up visits. Clinical and laboratory information is updated every six months until death or loss to follow up.

In this paper, we report on patients who were diagnosed up to 31 December 2008. Analysis of disease progression was limited to the first year of infection.

Ethical considerations

The *Grupo Argentino de Seroconversión* study protocol was approved by the Huésped Foundation Ethics Committee. All patients followed prospectively signed written informed consent before enrolment. Patients studied retrospectively signed consent at their first follow-up visit, if still alive.

Definitions

We defined PHI as "symptomatic" if one or more symptoms associated with acute retroviral syndrome were present [13,14]. "Severe symptomatic PHI" was defined as presence of B or C events, (according to the Centers for Disease Control and Prevention 1993 classification [15]), any other serious non-AIDS-related events, or death at the time of HIV seroconversion.

In symptomatic patients, the date of infection was estimated as 14 days before the onset of symptoms. In asymptomatic patients, the date of infection was estimated as the midpoint between the last negative and the first positive test or one month before the date of the indeterminate or negative Western blot assay [16-18].

HIV progression was defined either by clinical (B or C events [15]), or immunological (CD4 cell count < 350 cells/mm^3) criteria, whichever occurred first. We chose these endpoints based on the current national and international recommendations for initiation of antiretroviral therapy [19,20]. Analysis of disease progression was limited to those patients who did not start treatment within the first 120 days of infection.

Statistical analysis

Quantitative variables were described using mean and standard deviation (SD) in cases where the underlying distribution was normal; median and interquartile ranges (IQR) were used for variables without normal distribution. Differences were analyzed using Student's t-test for independent samples or the non-parametric Wilcoxon Rank Sum test.

Categorical variables were described using proportions and percentages. Differences between proportions were analyzed with the Chi-square test, or Fisher's exact test. Differences were considered statistically significant for $p < 0.05$, two-tailed tests. Univariate analysis was performed for the variables hypothesized as risk factors for events under study. All the variables of interest for the study were included in the multivariate analysis. Cox regression analysis was performed and the hazard risk (HR), 95% confidence interval (CI) and p value were calculated for each variable.

Progression-free survival time was measured from the estimated date of infection to the date of progression. For those patients who did not experience an event, data was censored at their last visit within their first year of infection or at treatment initiation. Time until an event was studied using Kaplan-Meier survival analysis, and the log rank test was applied for significance. Overall median time estimates, as well as median time by arm and corresponding 95% CI, are given. Kaplan-Meier plots are shown. Data analysis was performed with SPSS 15.0, 2007 (Chicago, Illinois).

Results

Baseline characteristics

As of December 2008, 134 patients with primary HIV infection were enrolled in the cohort; 99 retrospectively and 35 prospectively. Baseline characteristics are summarized in Table 1. Most patients were male (n = 109) with a median age of 32 years (IQR 25-39). More than half of the patients (53%) defined themselves as men who have sex with men (MSM), while 50 (37%) reported heterosexual exposure. Only one patient reported intravenous drug use as the probable route of infection.

Table 1 Baseline characteristics of *Grupo Argentino de Seroconversión* cohort (N = 134)

Characteristic	All (N = 134)	Symptomatic PHI		p
		YES (n = 99)	NO (n = 35)	
Age at HIV diagnosis, mean years (SD)	33.4 (10.7)	33.8 (10.37)	32.2 (11.64)	0.44
Male sex, n (%)	109 (81.3)	79 (79.8)	30 (85.8)	0.61
High school education or more, n (%)	79 (75.2)	59 (72.8)	20 (83.4)	0.3
Born in Buenos Aires, n (%)	74 (67.9)	56 (67.5)	18 (69.2)	0.61
Employed, n (%)	82 (70.7)	62 (70.5)	20 (71.4)	0.89
Reason for HIV test, n (%)				
Physician's suspicion	61 (48.4)	56 (59.6)	5 (15.6)	**< 0.001**
Patient request	42 (33.3)	27 (28.7)	15 (46.9)	
Routine	23 (18.3)	11 (11.7)	12 (37.5)	
Risk factor for HIV transmission, n (%)				
MSM	71 (53)	51 (51.5)	20 (57.1)	0.788
Heterosexual	50 (37.3)	38 (38.4)	12 (34.3)	
IDU	1 (0.7)	1 (1)	0 (0)	
Missing	12 (9)	9 (9)	3 (8.6)	
HIV RNA, median log_{10} copies/mL (IQR)	4.87 (4.11-5.51)	5.12 (4.49-5.69)	4.36 (3.43-4.95)	**< 0.001**
CD4 cell count, median cells/mm^3 (IQR)	479 (341-682)	466 (327-609)	533 (425-814)	**0.019**
HAART initiation, n (%)	42 (31.3)	39 (39.4)	3 (8.6)	**0.003**

MSM-men who have sex with men; IDU-injection drug user; HAART-highly active antiretroviral therapy

Most of the patients (n = 74) were from Buenos Aires city and its surroundings suburbs, areas that concentrate 44% of people living with HIV/AIDS in Argentina [9]. Seventy-five percent of patients completed at least high school and 29% were unemployed. HIV testing was requested based on a physician's clinical suspicion in 48% of cases and because of patient's request in 33% of cases. In 18% of cases, HIV seroconversion was diagnosed in patients undergoing periodic HIV testing. Of note, three patients were diagnosed during pregnancy. The source of transmission could be identified in 52 cases. In 28 (54%) of these, a stable HIV-positive partner was identified.

At first evaluation, the Western blot test was negative in 12 patients (9%) and indeterminate in 53 (40%). In 26 of these cases, a virologic test (p24 antigen or HIV viral load) defined the diagnosis. All cases with initial negative or indeterminate Western blot had HIV infection confirmed by subsequent seroconversion. The remaining 69 (51%) patients with a reactive Western blot had a negative test within the previous six months.

The first laboratory evaluation (HIV viral load and CD4 cell count) was done at a median of 66 days (IQR 48-112) after the probable date of exposure to HIV. Median HIV-1 RNA VL was 4.87 log_{10} copies/mL (IQR 4.11-5.51) and the median absolute and percentage CD4 cell count were 479 cells/mm^3 (IQR 341-682) and 23% (IQR 17-28), respectively. Baseline CD4 cell counts were < 350 and < 200 cells/mm^3 in 27% and 6.25% of patients, respectively. A total of 42 patients (31%) started HAART during the acute phase, with a median time of 84 days (IQR 53-110), from the probable date of infection: 39 due to symptomatic infection, and in three asymptomatic cases, due to pregnancy. Since indication of HAART during PHI is considered optional in Argentina [20], the decision on whether to start treatment or not depended on the physician in charge.

Morbidity and mortality associated with acute HIV infection

Ninety-nine patients (74%) presented with acute retroviral syndrome, lasting a median of 16 days (IQR 8-29). Twenty-six of them developed severe symptoms: seven opportunistic infections (three *Pneumocystis jiroveci* pneumonia, one histoplasmosis, one cryptococcal meningitis, one esophageal candidiasis and one pulmonary TB); nine B events (thrush, herpes zoster) and 10 non-AIDS defining severe events. The latter included aseptic meningitis, rhabdomyolysis with multi-organ failure, acute hepatitis, Bell's paralysis and guttate psoriasis.

Thirty-five patients (26.2%) required hospital admission. One patient developed chronic hydrocephaly and cognitive impairment secondary to cryptococcal meningitis and another suffered fatal disseminated histoplasmosis.

Factors associated with severe symptomatic seroconversion were CD4 cell counts lower than 350 cell/mm^3 (p = 0.001) and viral loads higher than 100,000 copies/mL (p = 0.001). HIV testing was requested more frequently by physicians based on clinical suspicion rather than patients' initiative (OR 5.06; 95% CI 1.83-14.04). We found no association between age, gender, birth place, risk factor or year of diagnosis with regard to severity of symptoms (Table 2).

12-month morbidity and mortality

Untreated patients

Among the ninety-two patients who did not start HAART during acute HIV infection, 24 (26%, 95% CI: 17.5-36.3) patients presented with disease progression within the first year of infection: 12 had clinical progression (five AIDS-defining events and seven B events) and 12 exhibited immunological progression (CD4 cell count < 350 cells/mm^3). The median time between the probable date of infection and the event presentation was 182 days (IQR 67-233). One patient who developed a non-Hodgkin lymphoma within six months of HIV infection died shortly after diagnosis.

Among untreated patients, progression was observed in 20 out of 60 symptomatic patients and in 4 out of 32 asymptomatic patients. Using Kaplan-Meier curves, estimated rates of progression at 12 months of follow up were 34% (95% CI 22.5-46.3%) among symptomatic untreated patients versus 13% (95% CI 1.1- 24.7%) in the asymptomatic group. The difference between the two curves was statistically significant (p = 0.04) (Figure 1). The hazard ratio of disease progression for untreated persons with symptomatic primary HIV infection compared with asymptomatic seroconverters was 8.44 (95% CI 0.97-73.42).

Factors associated with faster progression among untreated patients during the first year of infection were symptomatic primary HIV infection (p = 0.046), higher viral load at baseline and at six months from seroconversion (p = 0.04 and 0.008, respectively), as well as lower baseline CD4 cell count (p = 0.002). No association was found with age at seroconversion, gender, mode of HIV acquisition and year of infection. In the multivariate analysis (Table 3), only symptomatic primary HIV infection (p = 0.049) and baseline viral load higher than 5 $\log_{10}$ copies/mL (p = 0.022) remained as independent predictors of faster progression; relative risks 8.44 (95% CI 0.97-73.42) and 9.44 (95% CI 1.38-64.68), respectively. Baseline CD4 and viral load at six months were no longer associated with increased risk of progression in the multivariate model.

Table 2 Factors associated with severe symptomatic PHI (univariate analysis) (n = 26)

Risk factor	OR (95%CI)	p
Age at seroconversion > 30 years	1.36 (0.63-2.92)	0.495
Male sex	2.52 (0.63-10.04)	0.246
Mode of HIV transmission (MSM)	1.14 (0.51-2.55)	0.58
Diagnosis based on physician suspicion	5.06 (1.83-14.04)	**< 0.001**
CD4 cell count < 350 cells/mm^3	3.72 (1.83-7.58)	**0.001**
HIV RNA > 100,000 copies/mL	3.72 (1.58-8.77)	**0.001**
Year of diagnosis ≥ 2005	0.79 (0.37-1.70)	0.619

MSM-men who have sex with men

Evolution among treated patients

Among those patients who started HAART within the first 120 days of HIV infection, only three (7%) presented with HIV progression (one C event, one B event and one CD4 cell count decrease to < 350 cells/mm^3 despite HAART initiation) within the first year of infection. The difference to the 26% progression rate seen in the untreated group was statistically significant (p = 0.01). Of note, the C event was pulmonary TB, which is endemic in Argentina.

Discussion

This study is the first report from the only multicentre cohort of HIV seroconverters in Argentina and one of the few descriptions of HIV-1 progression from seroconversion in Latin America.

In our cohort, the proportion of patients with symptomatic disease was similar to previous series [13,17,21,22]. Of note, one-quarter presented with serious clinical manifestations associated with seroconversion. Even though these have been previously reported [23-26], our results regarding the relatively high frequency of serious clinical manifestations during primary HIV infection are rather unusual. In our study, severe PHI was strongly associated with higher baseline viral load and low CD4 cell count, which is also consistent with other reports [27-29]. Likewise, during acute HIV infection, opportunistic infections are usually associated with low CD4 cell count. In our study, however, four out of five AIDS-defining events registered after the first 60 days of HIV infection were associated with CD4 counts greater than 200 cells/mm^3 (Table 4), thereby highlighting the need to consider opportunistic infection even in patients with moderate immune deficiency.

Most of our patients were young males, with MSM being slightly overrepresented compared with the current proportion in the local HIV epidemic, where heterosexual intercourse is the most common mode of HIV transmission [9]. Greater awareness regarding acute retroviral syndrome (ARS), the higher frequency of testing among this population, and the inclusion in the cohort of a voluntary counselling and testing centre, where most of the attendants are MSM, could have influenced our results. In addition, medical prejudice could have

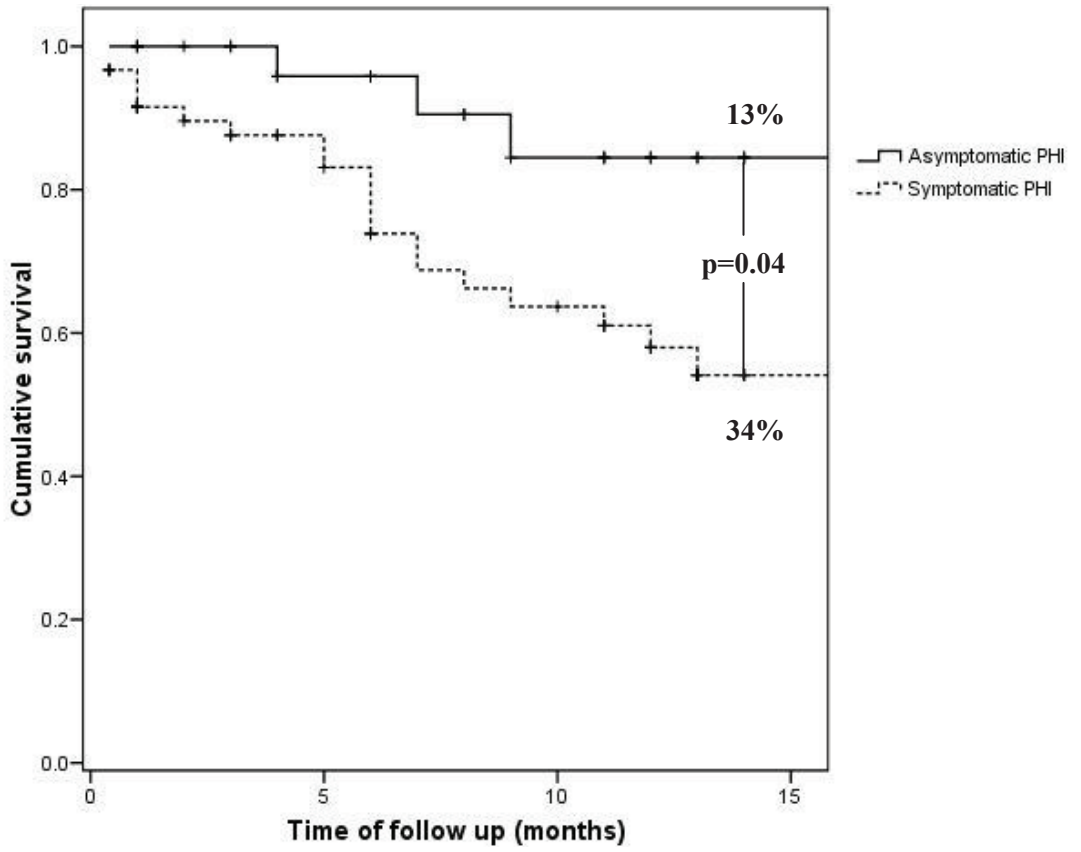

Figure 1 Time to progression of HIV disease among untreated patients from the *Grupo Argentino de Seroconversión*. Progression-free survival from onset of HIV infection among untreated patients with or without symptomatic primary HIV infection.

resulted in higher recognition of ARS in MSM patients than in the heterosexual population. This could also partly explain the lower proportion of women in our cohort compared with Argentina's overall HIV population [9] (19% vs. 39%), limiting the generalization of our findings.

One-quarter of the patients who did not start HAART during the acute phase met clinical or immunological criteria (< 350 CD4 cells/mm^3) [19,20] to initiate HAART during the first year of HIV infection. This observation is particularly relevant as one-third of the patients were already excluded in the progression analysis due to HAART initiation during the acute HIV phase, which resulted in the exclusion of a considerable proportion of symptomatic patients with risk of progression. The progression rate described here is much higher than in earlier epidemiological reports [30], which estimated a window of several years before the

Table 3 Predictors of disease progression in untreated patients (unadjusted and adjusted analysis) (n = 92)

Risk factor	Unadjusted HR (95%CI)	p	Adjusted HR (95%CI)	p
Symptomatic PHI	1.41 (1.08- 1.83)	**0.046**	8.44 (0.97-73.42)	**0.049**
Age at seroconversion > 30 years	1.40 (0.93- 2.10)	0.159	4.42 (0.91-21.47)	0.065
Mode of HIV transmission (MSM)	1.38 (1.02-1.86)	0.081	0.99 (0.11-8.64)	0.995
Baseline CD4 cell count ≤ 350 cell/mm^3	3.81 (1.64-8.86)	**0.002**	3.14 (0.47-20.78)	0.236
Baseline HIV RNA ≥ 100,000 copies/mL	1.91 (1.08-3.39)	**0.043**	9.44 (1.38-64.68)	**0.022**
HIV RNA at 6 months ≥ 100,000 copies/mL	9.88 (1.30-75.20)	**0.008**	2.24 (0.19-26.14)	0.520
Male sex	1.07 (0.89-1.29)	0.752	3.33 (0.16-67.54)	0.433
Year of diagnosis ≥ 2005	0.81 (0.61-1.09)	0.146	2.10 (0.20-21.99)	0.537

PHI-primary HIV infection; MSM-men who have sex with men

Table 4 AIDS-defining events during the first year of infection

Subject	Event	Time from HIV infection to event (days)	CD4 cell count (cells/mm^3)	Outcome
1	PCP	15	27	Resolved, HAART initiated
2	PCP	15	13	Resolved, HAART initiated
3	Cryptococcal meningitis	60	227	Cognitive impairment secondary to chronic hydrocephaly
4	Disseminated histoplasmosis	32	42	Death
5	Esophageal candidiasis	9	134	Resolved, HAART initiated
6	Pulmonary TB	28	419	Resolved with TB treatment
7	PCP	25	199	Resolved, HAART initiated
8	Cytomegalovirus disease	92	278	Resolved, HAART initiated
9	Non-Hodgkin lymphoma	210	28	Death
10	Pulmonary TB	203	553	Resolved with TB treatment
11	Cryptosporidiosis	120	570	Resolved
12	Kaposi's sarcoma	230	828	Resolved, HAART and quimiotherapy initiated

PCP-*Pneumocystis jiroveci* pneumonia; TB-tuberculosis

need for HAART initiation. However, a recent study by CASCADE cohort investigators [31] found that nearly 30% of their patients had ≤ 500 CD4 cells/mm^3 12 months after infection.

Symptomatic PHI and baseline HIV RNA > 100,000 copies/mL were identified in our study as predictors of disease progression in the multivariate model. These findings are consistent with prior studies [2,3,28,29,32]. While high viral loads during acute HIV infection are typically described [33,34], low plasma levels of HIV RNA have also been reported [7,35]. Comparisons across cohorts are difficult. However, an interesting finding of our study was that compared with European and North American cohorts of seroconvertors [3,4], baseline HIV RNA was higher and closer to levels seen in reports from African [8] and Asian [2] countries.

Although some differences in early laboratory values may be accounted for by differences in the quantitative methods used or the length of seroconversion intervals, first viral load measurement in our cohort was done at a median of 66 days from the probable date of infection, similar to most of the published studies [2-4,8]. There is growing evidence that initial viral load measurements, as well as the subsequent course of HIV infection, may be affected by viral [36-39] and host factors, including age, gender [40,41], race [42] and genetics [43,44].

In our cohort, the relative risk of disease progression in patients with baseline viral loads of > 100,000 copies/mL was almost 10-fold. Taking into account that more than 40% (59/134) of the patients enrolled in our cohort presented with initial viral load levels above this threshold, the impact of this finding as a prognostic factor on the subsequent course of infection deserves to be highlighted. Viral load at six months, however, did not correlate with progression; likewise, neither did CD4 cell count at baseline or six months, which underscores the need to identify other markers of progression at this early stage of infection.

Recent evidence suggesting an increase in HIV virulence over time [31,45-47] could not be corroborated, as patients who seroconverted before or after 2005 presented with similar median CD4 cell count (481 cells/mm^3 vs. 477 cells/mm^3; p = NS) and disease progression (p = 0.537). However, the relatively small size of our cohort prevents us from formulating definite conclusions on this topic.

Our study has several limitations. First, it is possible that current clinical practice in Argentina limited identification to only the most symptomatic patients, which could have contributed to the faster progression seen in our cohort. In our country, universal access to HIV testing is guaranteed by law, but there are structural, social and economic barriers to access. It is estimated that at least 50% of infected people still remain unidentified [9]. Except for antenatal care, testing is usually conducted in specialized centres. HIV testing in emergency rooms, for example, is usually not accessible. These practices could have resulted in HIV testing being requested only in those patients with a more severe clinical picture, or with evident epidemiological risk. Although we cannot rule out this possibility, 26% of patients in our cohort were asymptomatic.

Second, many of the symptomatic patients started HAART during PHI, and were therefore excluded from the analysis. This could have lead to a more conservative estimate of the risk of disease progression. Third, inclusion of patients with different seroconversion intervals (i.e., acute and recent HIV infection) could have influenced our results. However, we compared rates of progression between pre- and post-seroconversion patients and found no meaningful differences (32% vs. 22%; p = 0.39).

In addition, due to the retrospective-prospective design of this study and the availability of stored blood samples only for a subset of patients enrolled after 2008, we could not study biological factors affecting immune dysregulation, such as viral tropism [39,48], specific HLA haplotypes [48,49] and regulatory T cells [50,51]. Our research group is currently conducting other studies to understand the role of these biologic factors in the course of HIV infection.

Finally, information regarding viral subtype and genotypic analysis were not available for all patients and therefore it is not presented here. It is possible that HIV subtype could influence viral load set point and subsequent course of HIV infection [36-38]. We are currently studying the potential influence of the two most prevalent subtypes of HIV-1, B and BF [52-56], on disease progression in our country.

Conclusions

In conclusion, the data presented here have direct implications for providing HIV care in Argentina. First, acute retroviral syndrome was associated with faster progression, significant morbidity and, in some cases, with HIV-associated mortality. Therefore, awareness needs to be raised among physicians to include HIV in their differential diagnosis of febrile illness, especially in high-risk groups, such as serodiscordant couples, sexual workers, injection drug users and MSM. Likewise, HIV should be considered in any sexually active person who presents in the emergency room with flu-like syndrome as nearly 1% of them may have acute HIV infection [57,58].

Furthermore, this data should be taken into consideration when making decisions on treatment initiation. Patients with acute retroviral syndrome or high baseline viral load should be considered for treatment initiation, as our data suggest that approximately one-third of them will require treatment in the following year; new evidence also suggests benefits of earlier treatment initiation [59,60].

Combined with other ongoing research in this field, the data presented here could provide valuable information on the complex interplay between virus and host factors in HIV pathogenesis that could aid in the development of better algorithms, new therapeutic approaches and the design of preventive interventions.

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Authors' contributions

MES, OS, NL and PC designed the study, and analyzed and interpreted the data. MES also wrote the first draft of the manuscript. RV contributed to the design of the study. MES, OS, NL, CC, AK and PC revised the manuscript critically for important intellectual content. All authors participated in data collection, and revised and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ARTÍCULO VI

PLOS ONE

RESEARCH ARTICLE

Structured Treatment Interruptions and Low Doses of IL-2 in Patients with Primary HIV Infection. Inflammatory, Virological and Immunological Outcomes

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Abstract

Background

Interventions during primary HIV infection (PHI) can modify the clinical course during the chronic phase. The long-term effect of structured treatment interruptions (STI) followed by low doses of interleukin-2 (IL-2) in treated PHI patients is unknown.

Methods

Twelve PHI patients with viral load (VL) <20 copies/mL, CD4 cells >500 cells/mm3, and CD4/CD8 ratio >1, on antiretroviral therapy (ART) initiated within the first 90 days of infection and continued for at least 12 months were included. They underwent four STI and were then allocated (week 0 of the study) to ART alone or ART plus low doses of IL-2. ART was stopped once VL <20 copies/mL ('final stop'). Primary endpoints were VL<3000 copies/mL and CD4 cells >500 cells/mm3 at 48 weeks; secondary endpoints were immune activation, inflammatory markers until 48 weeks and the time before resuming ART (CD4 <350 cells/mm3 or AIDS) after 'final stop', compared between groups.

Results

Ten out of 12 patients were males, median age was 35 years and the main risk was men-who-have-sex-with-men. Only one out of 12 patients (in the STI group) maintained VL<3000 copies/mL and CD4 cells >500 cells/mm3 without ART at 48 weeks. All other virological and immunological parameters were comparable between groups at week 0, 'final stop' and week 48. However, the proportion of CD8-CD38+ cells, tumor necrosis factor and srIL-2 were higher in the IL-2 group at 'final stop' and week 24. All these differences

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vanished during follow-up. At 5 years after the final stop 3 out of 6 patients in the IL-2 group and 6 out of 6 patients in the STI group have resumed ART (P = 0.19).

Conclusions

STI and IL-2 failed to achieve virological control after ART interruption. STI were not deleterious in long-term follow-up, an important issue for eradication and functional cure trials.

Trial Registration

ClinicalTrials.gov NCT02300623

Introduction

Potent combination antiretroviral treatment (ART) during primary HIV infection (PHI) results in strong viral suppression rates, rapid recovery of CD4 T cells[1, 2], and a reduction of viral reservoirs[3, 4]. Moreover, ART in this clinical phase preserves HIV-specific T-cell helper (Th) and cytotoxic T lymphocyte (CTL) immune responses, improves surrogate markers of disease progression[5–7] [8] and reduces HIV transmission[9, 10]. The fact that ART during PHI preserves HIV-specific CD4 helper T-cell response[5–7] is remarkable, as this response is usually absent in chronically-infected patients, even in those receiving successful ART since the early asymptomatic phase of chronic infection[5, 11]. In fact, ART-treated PHI patients exhibit HIV-specific Th and CTL immune responses in a similar manner as long-term non-progressors (LTNP) or "elite controllers" that spontaneously control HIV replication[12, 13].

Soon after the introduction of potent ART, observational case reports identified patients receiving ART during PHI that controlled HIV replication after ART discontinuation and reported that this control was associated with strong HIV-specific cell-mediated immune responses[14, 15]. These cases lead to the hypothesis that brief exposures to the autologous virus during supervised or structured treatment interruptions (STI) in patients receiving ART since PHI might act by boosting HIV-specific immune responses, which could provide immune control of viral replication. STI were also evaluated in several clinical trials during chronic HIV infection[16–18]. The encouraging results obtained in a small clinical trial using three STIs in subjects receiving ART since PHI[7] and similar experimental findings in primates[19] further fueled interest in this approach and in other possible immunotherapeutic procedures to induce/boost HIV-specific immune responses[20–22]. Unfortunately, however, the viral control after STI in the mentioned trial with ART-treated PHI patients was found to have a limited durability[23]. Nevertheless, recently, two reports have renewed further interest in administering ART very early during PHI as a considerable proportion of patients can control HIV replication after ART cessation[24, 25]. The proportion of these post-ART controllers was considerably high compared to that of spontaneous "elite" controllers, and the former lack some genetic characteristics that are overrepresented among the latter, suggesting that the key for controlling HIV viremia was the early initiation of ART[24, 25].

In our trial, we used IL-2, one of the first cytokines discovered to promote T-cell growth [26], as an adjunctive immunotherapy aimed at favoring the clonal expansion of HIV-specific Th and CTL responses. We utilized daily s.c. ultra-low IL-2 dose, which has been demonstrated to be not only nontoxic and safe but also effective in stimulating immunoreactivity in patients with AIDS and with AIDS-related malignancies[27–29].

These considerations provided the rationale for this pilot clinical trial to evaluate the impact of STI during PHI with or without the addition of low-dose recombinant IL-2 to boost HIV-1 specific immune responses and achieve a control of HIV viremia. Although STI in chronic HIV infection has shown to increase the risk of opportunistic infection and death [30], it can also enhance host immune control of viral replication [17] and no deleterious effect was shown during PHI in a recently published study[31]. This issue is particularly important in the context of the trials for HIV-functional cure, in which ART interruptions need to be performed to test the control of viremia by the immune system. We report here the virological, immunological and inflammatory markers up to 48 weeks of follow-up and the time to require resuming ART (CD4<350/μl or AIDS events) in the long-term follow-up.

Methods

Patient population

Enrolment started in March 2000 and finished in November 2001. Long-term follow-up for the last included patient finished in April 2012. All patients with PHI on stable effective ART for at least 12 months were invited to participate. The flow diagram of the study is shown in Fig 1.

Diagnosis of PHI was defined by:

-A detectable plasma viral load (PVL) or p24 antigen detection coupled with a negative or indeterminate LIA assay (according to CDC criteria), or

-A negative HIV-1 EIA in the preceding 90 days, or

-A positive EIA and LIA assay with acute retroviral syndrome in the 90 days preceding the start of ART plus documented negative HIV-1 EIA within the previous year.

Date of infection was assumed to have occurred two weeks before the onset of acute symptoms. In asymptomatic patients this date was calculated as the midpoint between the last negative and first positive test.

All patients started stavudine, lamivudine and non-boosted-indinavir at usual doses, according to recommendations at that time, within 90 days of HIV exposure, and had to show good virological and immunological responses, defined as undetectable PVL (<20 copies/mL in the last two controls), CD4 T cells higher than 500 cells/mm3 and a CD4/CD8 ratio >1 in the 8 months prior to enrolment.

Study Design

The study design included two phases (Fig 2). The first phase consisted of four STIs of 8 weeks each (*off-ART*), separated by at least 16 weeks of treatment–or the time necessary to return to PVL <20 copies/mL—(*on-ART*). At the end of 4th *off-ART* cycle (week 0), an interim evaluation was performed and the second phase initiated. During the second phase, the first 6 patients received ART until they reached PVL<20 copies/mL, discontinuing thereafter (final stop). The last 6 patients received ART and low doses of IL-2. ART was stopped after reaching PVL<20 copies/mL (final stop) and IL-2 after 6 months of treatment. IL-2 was prescribed at a dose of 750,000 UI/m^2 daily and was self-administrated in all patients after training with a specialized nurse; administration was checked and verified during follow-up visits.

In both groups, ART was resumed in patients whose CD4 cell count dropped below 350 cells/mm3 in two consecutive determinations or in patients who developed opportunistic infections. Evaluations were performed at interim analysis (end of 4th off-ART, week 0) and 24 and 48 weeks after the end of 4th off-ART. A long-term follow-up analysis was also performed

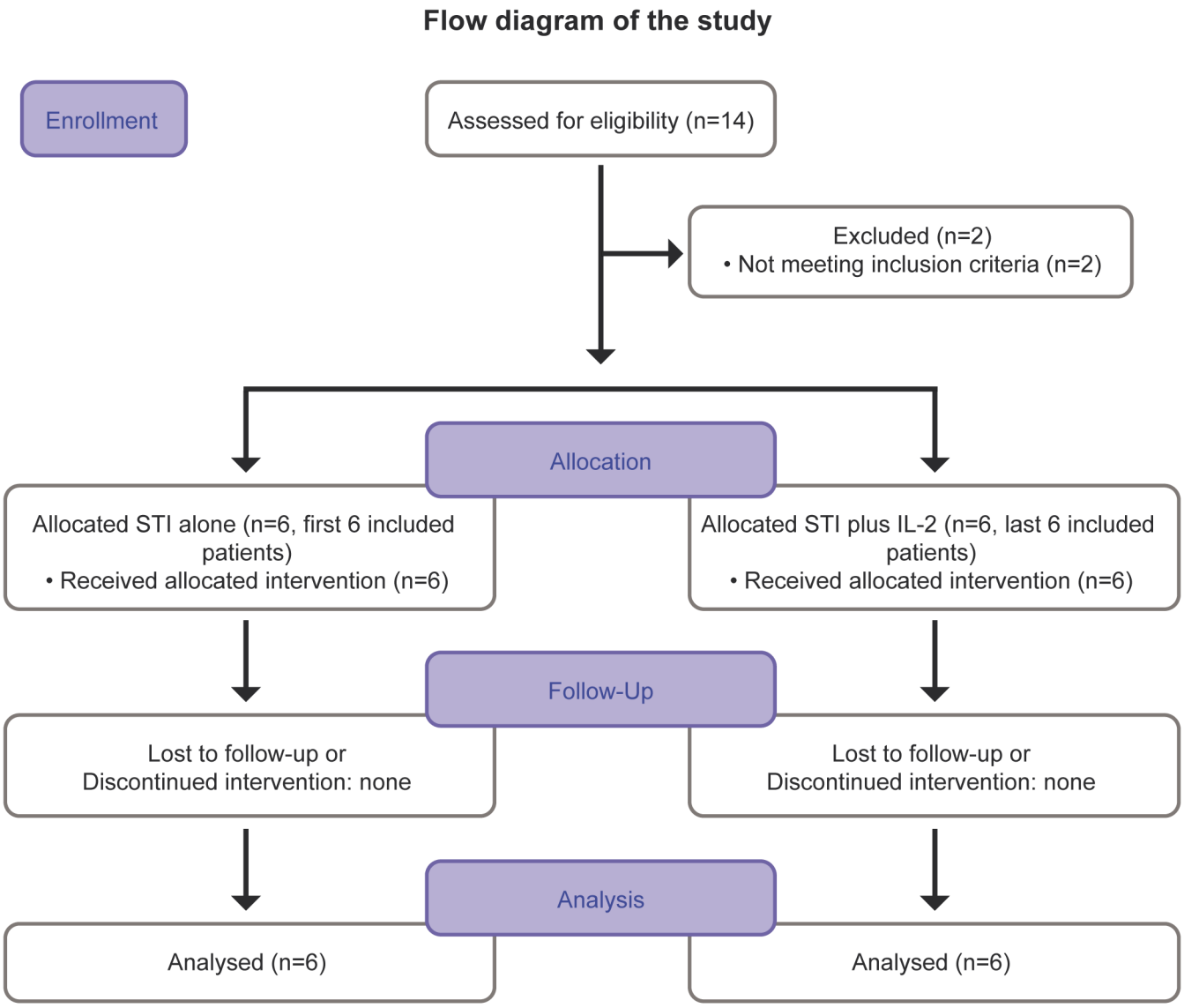

Fig 1. Flow diagram of the trial.

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up to 9 years from the final stop. It included survival rate, clinical events, time to resume ART, CD4-CD8-CD4/CD8 ratio. This was performed, as standard of care, during follow-up visits.

Measurements and evaluation

At enrolment the investigators recorded patient medical histories and performed a clinical examination. During the follow-up, clinical status and adverse events were recorded. Safety parameters, plasma HIV-1 RNA load, and CD4 and CD8 T cell counts were obtained at weekly intervals during off-ART and until PVL lowered below 20 copies/mL and monthly thereafter. Viral reservoir (HIV proviral DNA) was not performed.

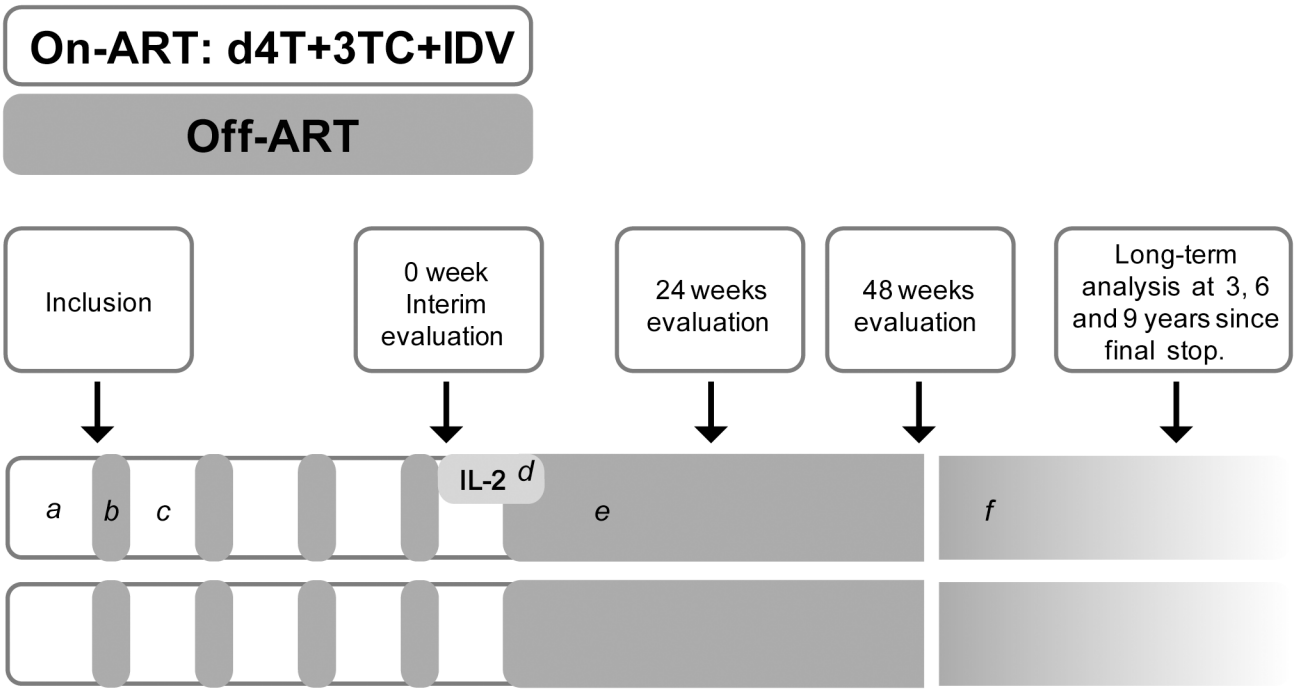

Fig 2. Design of the study. Footnote: (a) Eligible individuals were patients treated within 90 days of HIV exposure on stable ART for at least 12 months. Patients had to show good virological and immunological responses (at least 2 viral load <20 copies/mL and CD4 T cells higher than 500 cells/mm3 with a CD4/CD8 ratio >1). All individuals started four structured treatment interruption cycles (b) of 8 week each (*off-ART*), followed by four cycles (c) of treatment (*on-ART*). After the last treatment interruption (week 0) 6 patients started self-administrated IL-2 at a dose of 750,000 UI/m2 daily for 6 months (d). Treatment was interrupted in both arms when patients reached viral load <20 copies/mL (e). Analyses were performed at 24 and 48 weeks and after a long term follow-up period (9 years). During this period (f) treatment was restarted in patients whose CD4 cell count dropped below 350 cell/mm3.

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Laboratory methods

HIV serology was determined with a microparticulate enzyme immunoassay (MEIA) AxSYM system (Abbott Laboratories, North Chicago, IL) and confirmed with LIA (Inno-LIA HIV I/II Score. Innogenetics. Ghent, Belgium). PVL was determined using the Amplicor HIV-1 Monitor Ultra Sensitive Specimen Preparation Protocol Ultra Direct Assay (Roche Molecular Systems, Inc., Somerville, NJ) with a limit of detection of 20 copies/mL. Those samples below the detection limit were retested with a lower limit of detection of 5 HIV-1 RNA copies/mL as described[32]. HIV-1-RNA quantification in tonsillar tissue was performed in each patient at baseline as previously described[33]. Subpopulations of CD3+, CD4+, and CD8+ T cells, as well as proportion of CD8/CD38+ cells were determined by flow cytometry.

Genotypic mutations of both reverse transcriptase (RT) and protease (PR) genes from viral RNA were tested using the ViroSeq HIV Genotyping System v.2 (Abbott Laboratories, North Chicago, IL) and ABI3100 sequencer. Samples for genotype were drawn at baseline and when patients presented PVL higher than 1,000 copies/mL after the final ART discontinuation. Resistance mutations were re-interpreted according the HIV2007 IAS consensus. Viral subtype was inferred comparing the "fasta sequence" in REGA HIV-1 Subtyping Tool—Version 2.0 [34]. The HLA class I genotype was determined by reverse sequence-specific oligonucleotide (SSO) (RELIDynal, Madrid, Spain). Allele definition was automatically assigned by the RELI SSO Pattern-matching Program software and was manually supervised. Tumoral necrosis factor (TNF) and soluble receptor for interleukin 2 (srIL2) levels were measured by EIA based techniques (Immunotech, France).

Lymphocyte Proliferation Assays

Freshly isolated PBMCs were washed twice and resuspended at 2×10^6/mL in a serum-free medium X-VIVO 10. Cultures were plated in triplicate at 2×10^5/well in 7-day assays, in 96 round-bottomed microplates (TPP, Trasadingen, Switzerland). Cells were cultured in the absence or presence of Pokeweed mitogen 10 μg/mL (Sigma) and 5 μg/mL of HIV-1 recombinant proteins gp160 and p24 (Protein Sciences, Meriden, CT). Incorporation of tritium-labeled thymidine was assessed for the last 18 hours of culture (Betaplate LKB, Wallac, Sweden). Results were expressed as mean counts per minute (cpm). The stimulation index (SI) was calculated for each sample using the formula: SI = mean cpm for cells with stimulus/mean cpm for cells without stimulus. Positive antigen-specific responses were defined as >3,000 cpm and SI >3. For analytical purposes, results were expressed as 'positive' or 'negative' CD4 proliferative responses to HIV-1 P24 protein.

HIV-1-Specific CD8+ T-Cell Responses

An ELISPOT assay (enzyme-linked immuno spot assay) was used to measure HIV epitope-specific CD8+ T-cell interferon-release from cryopreserved PBMC samples[6]. A mean of 16 (range: 3–27) different HLA class I–restricted synthetic peptides from gag, pol, env, and nef proteins were tested in each individual according to the HLA genotype. Results were expressed as total Spot Forming Cells (SFC)/10^6 PBMC and considered as positive with more than 500 SFC/determination.

Statistical analysis

The primary endpoint was the proportion of patients who maintained a PVL <3,000 copies/mL and CD4 cells >500/μL at 48 weeks from the end of the 4th STI (allocation to ART alone or ART plus IL-2, week 0). An interim analysis was performed at week 0 and at week 24.

Virological and immunological parameters during follow-up and changes from baseline were summarized by median and interquartile range (IQR) values. For the purpose of analysis, undetectable PVLs (<5 and <20 copies/mL) were considered equivalent to 5 and 20 copies/mL, respectively. The PVL values underwent log10 transformation before analysis.

The χ^2 test and the Fischer exact test were used, as appropriate, to compare categorical variables. Continuous variables were compared between subgroups using the Mann-Whitney test. For long-term analysis and time free of ARV after final stop, Kaplan-Meier curves and the log-rank test were used. All p were considered significant at $p<0.05$. For missing values related to endpoints, the most recent available determination for this variable was considered and specifically mentioned in the results section. All statistical analyses were performed with Stata and SPSS software.

Ethics Statement

The study was approved by the Hospital Clínic Ethics Committee Board, and by the Spanish Regulatory Agency ('Agencia Española del Medicamento'). All participants gave their written informed consent before enrolment. Registration of the trial in clinicalTrails.gov (NCT02300623) was retrospectively performed (registration was not compulsory at the time of the study conception).

Results

Patient selection

Fourteen patients were enrolled (Fig 1). Two patients were prematurely discontinued. Twelve patients completed the protocol and were included in the final analysis. All patients included in this analysis completed the four STIs and the follow-up period up to 8 years from the 48 weeks

analysis. The STI periods started on 3/27/2000 for the first patient. The last patient completed the 48 weeks of follow-up on 4/20/2004.

PHI characteristics

Of the 12 patients, 10 were male, median age was 35 years (IQR 28.5–2.54) and the main risk category was homosexual/bisexual intercourse. PHI was symptomatic in 9 of 12 patients, and the median interval between the estimated date of infection and ART initiation was 11 weeks (IQR 5.5–12), median VL was 103,500 copies/mL (IQR 23,379–207,000), median CD4 cell count 536.5 cells/mm3 (IRQ 391–612.5) and CD4/CD8 ratio 0.735 (IQR 0.425–0.970), all variables being comparable between the two groups. All patients were infected with HIV-1 B subtype virus, none had evidence of transmitted antiretroviral drug resistance and all patients were in Fiebig 4 phase at the time of ART initiation. None of the patients showed genetic variation in the CCR5 co-receptor gene, HBV or HCV co-infection.

Characteristics at inclusion

The median time of treatment was 22.5 months (range 12–45), RNA-VIH-1 was below 5 copies/mL in 11/12 patients (patient #14 had 12 copies/mL), and the median CD4 count was 1,067 cells/mm^3, (IQR 848–1328) with a median increase of 487 cells/mm^3 since PHI. All but one patient had tonsillar RNA-HIV-1 below 40 copies/mg (patient #17 had 104 copies/mg). Baseline data at inclusion are shown in Table 1.

Primary endpoint

Only one out of 12 patients (in the STI group) maintained VL<3000 copies/mL and CD4 cells >500 cells/mm3 without ART at 48 weeks.

Viral dynamics during the STI cycles and follow-up period

Viral load rebounded during STI and at follow-up in all patients except for patient #2, who maintained viral load <5 copies/mL until 48 week after the ART interruption. The peak of PVL rebound was highest in the first STI, PVR rose to 150,500 copies/mL (IQR, 25,650–364,500 copies/mL) and decreased to stable levels during the following off-ART cycles: 23,250 (5,260–50,450) copies/mL in 2^{nd}, 19,200 (1,655–112,200) copies/mL in 3^{rd} and 20,000 (1,360–54,650) in 4^{th} (Table 1). There was a trend to an increased doubling time of PVL during the off-ART cycles (11.07 days in the 1^{st} off-ART cycle and 24.45 days in the 4^{th} p = 0.09). After resuming ART, all patients showed rapid declines in PVL in each treatment phase. The number of patients fulfilling *Responder* criteria was 2 out of 12, 4 out of 12, 5 out of 12 and 4 out of 12 patients during 1^{st} to 4^{th} off-ART cycle respectively. After 24 weeks of follow-up, 2/6 patients in the IL-2 arm and 2/6 patients in the non-IL-2 arm maintained PVL <3,000 c/mL and after 48 weeks only one (in the STI group, although reported at 40 weeks) out of 12 patients did. Between weeks 40 and 60, all patients presented VL around log4. VL dynamics after 4^{th} STI are shown in Fig 3 and Table 2.

Lymphocyte dynamics and cytokine levels during STI cycles and follow-up period

CD4 cells dropped in every STI but never below 500 T CD4 cells/μl. Immunological, virological and cytokine evolution at the end of 4^{th} STI (interim evaluation), the end of ART (Final Stop), week 24 and week 48 is shown in Table 2. Briefly, CD4 cell count, CD4/CD8 ratio and CD8-CD28+ cells were all comparable for both groups at the end of 4^{th} STI, the end of ART (final stop), week 24 and week 48. However, the proportion of CD8-CD38+ cells was significantly

Table 1. Baseline characteristics at inclusion and CD4 T cells and viral load during STI.

		Total (n = 12)		STI (n = 6)		IL-2 (n = 6)		p value*
PHI characteristics		**n**	**%**	**n**	**%**	**n**	**%**	
Risk Category								0.545
	MSM/ bisexual	8	67	5	83	3	50	
	Heterosexual	3	25	1	17	2	33	
	IDU	1	8			1	17	
Gender								1
	Male	10	83	5	83	5	83	
	Female	2	17	1	17	1	17	
		median	IQR	median	IQR	median	IQR	p value**
Age (years)		35	28.5; 42.5	36.5	28; 47	32.5	29; 36	0.52
CD4 T cells/mm^3		1,067	848; 1328	1,064	566; 1415	1,067.5	1012; 1097	0.973
CD4/CD8 ratio		1.59	1.31; 1.80	1.64	1.58; 1.78	1.31	1.10; 1.86	0.648
CD4 T cells and viral load during STI								
CD4 T cells (Nadir during STI) cells/ mm^3								
1st STI		641	606; 776	666	626; 804	637	550; 749	0.485
2nd STI		546	443; 899	718	383; 870	597	114; 667	0.486
3rd STI		681	448; 870	586	465; 1040	738	106; 823	0.699
4th STI		592	347; 916	592	433; 968	528	204; 730	0.485
HIV RNA (Peak during STI) copies/mL								
1st STI		150,500	25,650; 364,500	78,250	21,100; 596,000	363,500	30,200; 182,000	0.394
2nd STI		23,250	5,260; 50,450	7,780	130; 26,000	42,000	10,400; 197,000	0.1
3rd STI		19,200	1,655; 112,200	14,400	2,020; 19,200	98,350	1,290; 558,000	0.31
4th STI		20,000	1,360; 54,650	13,740	1,240; 42,400	22,100	1,480; 197,000	0.485

*Fisher's exact test

**Wilcoxon Rank Sum test

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higher at the end of ART (final stop) for the IL-2 group. TNF and srIL-2 were also higher in the IL-2 group and the end of ART (final stop) and at 24 weeks of follow-up. These differences vanished at 48 weeks of follow-up. CD4 cell dynamics after 4th STI and until week 48 are shown in Fig 3 and Table 2.

Specific CD4 and CD8 immune responses

None of the 12 patients presented specific CD4 responses before STI. This response increased to 5/6 patients at 3rd STI cycle for the STI group and to 4/6 patients at 2nd STI for the IL-2 group. However, they decreased progressively for both groups (2/6 for the STI group and 1/6 for the IL-2 group at week 0-end of 4th STI-), and, at week 48, all but one patient in the IL-2 group had lost these HIV-specific CD4 responses.

Regarding HIV-specific CD8 responses, 1/12 presented specific CD8 responses before STIs (in the IL-2 group), which also increased in both groups during STI. The number of patients presenting HIV-specific CD8 responses was comparable in both groups in 1st, 2nd and 3rd

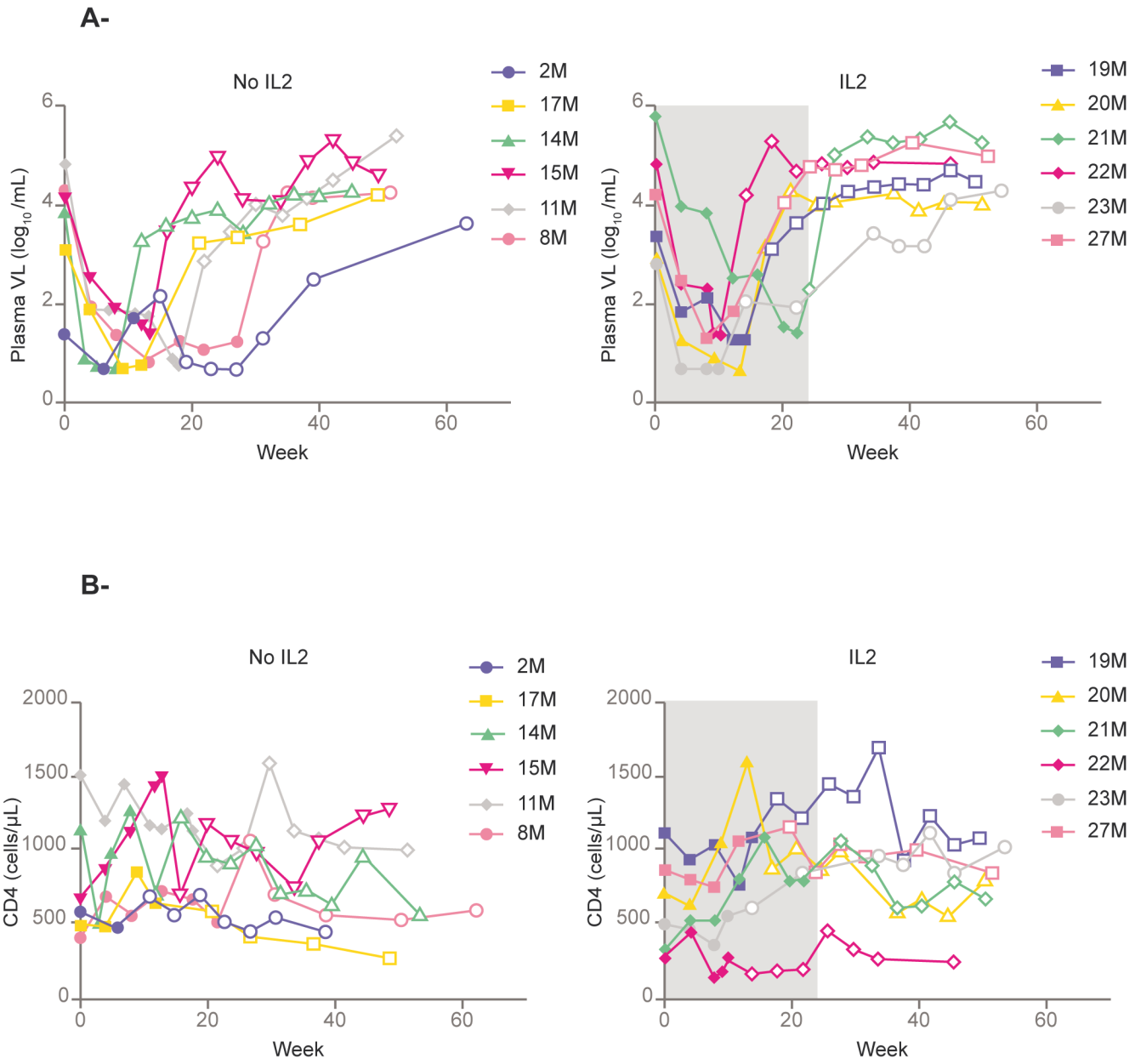

Fig 3. A) Plasma HIV Viral Load evolution after 4th STI cycle. B) CD4+ T cell evolution after 4th STI cycle. Footnote: The period of IL-2 administration is shown in grey. The filled points represent periods on ART. Empty points are determinations without ART.

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STI (p = 0.558, p = 1, p = 0.558 respectively). However, at week 0-end of 4th STI- these responses decreased to zero patients for the IL-2 group compared to 4 in the STI group (p = 0.014), and at 48 weeks of follow-up, they were absent in all 12 patients (Table 2).

Considering the 12 patients together, there were no statistically significant differences in the VL doubling time among those patients eliciting a strong CD8 response compared to those eliciting a weak CD8 response (p = 0.530, p = 0.180, p = 1, p = 0.283 for 1st, 2nd, 3rd, 4th STI respectively).

Development of ART resistance mutations

After the 4 STI cycles, only one patient developed K70R resistance mutation.

Table 2. Inflammation, immune activation, immunological and virological evolution following the end of 4th STI (week 0) until week 48 of follow-up.

	Week 0 (end 4th STI)		End of ART (final STOP)		Week 24		Week 48	
CD4 (cells/mm^3)								
STI group	622	(514–1,167)	1,116	(669–1,297)	928	(601–1,079)	668	(514–1,033)
IL-2 group	613	(278–864)	933	(559–1092)	865	(832–906)	690	(669–936)
CD4/CD8 ratio								
STI group	1.05	(0.90–1.60)	1.55	(0.80–1.60)	1.35	(1.00–1.55)	1.05	(0.75–1.30)
IL-2 group	0.83	(0.77–1.00)	1.1	(0.92–1.87)	0.93	(0.67–1.28)	0.77	(0.48–0.87)
CD8/CD28+ (%)								
STI group	57	(37–60)	56	(39–65)	59	(33–61)	52	(34–62)
IL-2 group	49	(36–64)	38	(34–58)	47	(26–50)	51	(42–53)
CD8/CD38+ (%)								
STI group	57	(52–64)	47	(52–64)*	47	(52–64)	53	(44–63)
IL-2 group	46	(36–57)	61	(53–67)*	56	(53–69)	47	(44–59)
HIV Viral Load (log10/mL)								
STI group	4.13	(3.64–4.64)	<1.30		3.36	(2.89–3.93)	4.26	(4.15–4.60)
IL-2 group	3.98	(2.96–4.83)	<1.30		4.52	(3.76–5.08)	4.58	(4.19–5.01)
HIV Viral Load<3000 copies/mL								
STI group		2 (33%)	N/A		2(33%)		1(17%)	
IL-2 group		3 (50%)	N/A		2(33%)		0	
TNF levels (pg/mL)								
STI group	30	(29–33)	24	(21–26)*	26	(16–29)*	19	(17–32)
IL-2 group	36	(21–42)	39	(37–49)*	42	(27–73)*	44	(29–64)
srIL-2 (pM)								
STI group	61	(61–82)	59	(35–60)*	60	(39–67)*	76	(39–91)
IL-2 group	76	(42–104)	124	(116–249)*	124	(101–127)*	114	(101–132)
Specific CD4 responses (positive antigen-specific anti-P24 protein responses) number of patients/total patients								
STI group		2 out of 6			1 out of 6		none	
IL-2 group		1 out of 6			2 out of 6		1 out of 6	
Specific CD8 responses (>500 SFC/10E6 PBMC) number of patients/total patients								
STI group		4 out of 6*			1 out of 6		none	
IL-2 group		none*			none		none	

Values are median and IQR

*p<0.05

ART: Antiretroviral treatment

STI: structured treatment interruptions

srIL-2: seric receptor for IL-2

SFC: Spot Forming Cells

PBMC: Peripheral blood mononuclear cells

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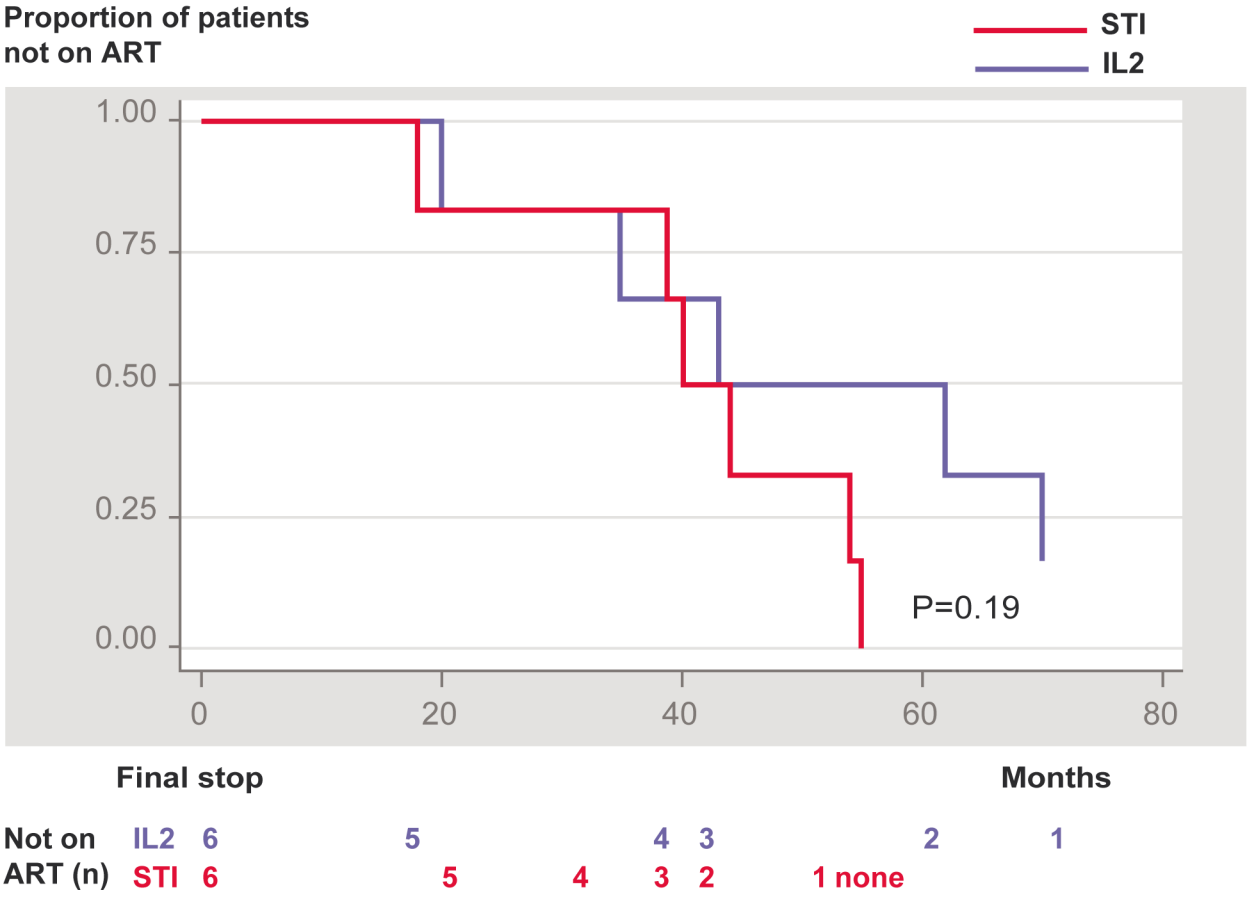
Low IL-2 dose tolerability

Overall injections were well tolerated, but all patients presented mild injection-site reactions. One patient presented cellulitis. No adverse effects on liver tests, red blood cells, white blood cells or lipid profile were seen (data not shown).

Long-term clinical and immunological follow-up

One patient in the IL-2 group died at 7 years and 10 months following the final stop due to metastatic colon cancer, aged 60. Overall 11/12 patients were alive at 9 years after the final stop. No other patient presented an AIDS-related opportunistic infection or neoplasm or other non-AIDS defining cancer. All patients in the STI group resumed ART at the end of the long-term follow-up and 5 out of 6 in the IL-2 group (one patient did not need to resume ART during the 9 years following the final stop, keeping CD4 cells consistently between 600 and 1100 cells/μl and VL to range around log4). Most of the patients in both groups resumed ART with a NNRTI (efavirenz)-based regimen. At the time of resuming ART, CD4 cells were comparable: 291/mm^3 for the STI group and 325/mm^3 for the IL-2 group (p = 0.855); CD4 percentage and CD4/CD8 ratios were also comparable for both groups. Immunological parameters remained comparable during long-term follow-up. Nine years after final stop the number of CD4 and CD8 cells and CD4/CD8 ratio were not different between groups: CD4 cells = 769.5/mm^3 for the STI group and 844.5/mm^3 for the IL-2 group (p = 1), CD4 percentage = 38.88 for the STI group and 38.7 for the IL-2 group (p = 0.394) and CD4/CD8 ratio = 1.20 for the STI group and 0.95 for the IL-2 group (p = 0.522). Time to resume ART after the final stop is shown in Fig 4.



Kaplan-Meier curves of IL-2 and STI groups after final stop. Log-rank test was used for statistical analysis.

ART: antiretroviral treatment.

Fig 4. Proportion of patients not receiving ART after the final stop.

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There was a trend to delayed resuming of ART in the IL-2 group. At 60 months after final stop, 3 out of 6 patients in the IL-2 group were not on ART compared to none in the STI group, but the difference did not reach statistical significance (p = 0.19).

Discussion

The most recently published reports suggest that a favorable immunological outcome is obtained when ART is initiated very early in the course of primary HIV infection [25, 35, 36]. Moreover, in recent years it has been shown to have had a big impact in transmission for patients with PHI/recent HIV infection, adding epidemiological aspects to the arguments for ART during this phase[35, 37, 38]. ART in primary infection may also improve patients' quality of life[39]. However, although all guidelines suggest treating symptomatic PHI patients, the indication is less clear for those who are asymptomatic. When ART is initiated, most guidelines recommend lifelong therapy due to the risk of viral rebound (and, probably, loss of any advantage) if treatment is stopped. In cases where ART is stopped, several immunological strategies have been evaluated as having impact on the clinical and immunological evolution of HIV infection and delaying the resuming of ART. Among these strategies, STI might boost HIV immunity, and several studies have analyzed this approach, including the use of STI alone or associated with other immune modulating therapies. A recently published study evaluated the impact of Peg-IFN and STI on PHI, but apart from lower peaks of HIV viremia during STI, no favorable immunological differences were seen in the long-term follow-up[31]. In our study, unfortunately, we also failed to prove any immunological benefit: at 48 weeks of follow-up all patients in both groups had lost the specific anti-HIV immune responses. According to our definition criteria of *Responder*, only one patient in the STI group had less than 3,000 copies of HIV-1 RNA, thus failing to show control of viral replication in both groups. We have seen no differences in the CD4 levels and viral load between groups. Thus, our 12 patients rebounded during the follow-up, with a relative decrease in the PVL with the following interruptions, but none of them evolved as a post-treatment controller, which has been calculated to occur in around 10% of the patients treated during PHI[25]. However, all our patients evolved clinically well when ART was resumed, thus there was not a deleterious long-term effect of STI. This is particularly important for the design of HIV functional-cure trials, in which, sooner or later, ART must be stopped to test spontaneous viral control but without harming patients not showing such viral control. However, we did not measured viral reservoirs, which could have been increased with STIs.

The IL-2 group showed higher immune activation and higher levels of inflammatory markers during the initial 24 weeks of follow-up. It is not clear whether this higher immune activation and inflammation impact the clinical outcome negatively in the long-term follow-up, but the only death, due to a non-AIDS defining cancer, was noted in the IL-2 group. Chronic inflammation and immune activation, among other factors, have been associated with non-AIDS defining cancers among HIV-infected patients[40]. However, in the studies conducted with IL-2 in chronic HIV-infection, with more than 3,000 patients, no increased incidence of cancer was seen[41]. On the other hand, there was a trend to delayed initiation of ART after the final stop in the IL-2 group but the numbers are too small to draw definite conclusions. The delayed resuming of ART did not prevent achieving equal immunological parameters (CD4, CD4 proportion and CD4/CD8 ratio) 9 years after the final stop between groups. However, in a life-long treatment scenario, this has very little clinical relevance. Indeed, nowadays most guidelines recommend an earlier initiation of ART, regardless of CD4 cell count.

It seems that the window to obtain an immunological advantage when treating PHI is very narrow, as suggested by a recent cohort study[36] in which patients reached 900 CD4 cells/μl more frequently when they started ART within 4 months of primary infection. In another

recently published study, Goujard et al. suggest that the precocity of ART is not the only important factor: baseline immune response against HIV is a key factor to control HIV replication efficiently in a long-term follow-up after stopping ART[35]. In another study, however, apart from early ART during PHI, no other factor was found to be associated with long-term control of viremia[24]. The PRIMO-SHM trial investigated the impact of 24 or 60 weeks of ART compared to no-ART during PHI, and they concluded that a short cycle of ART might delay the indication for treatment later [42]. In the SPARTAC study, the conclusions were similar, but the time gained to resume ART was approximately the same for patients who were on treatment during PHI, thus the benefit was very limited[43]. Therefore, it seems clear that ART should be administered as early as possible to obtain the maximum immunological advantage, but whether the addition of another immunomodulating strategy (such as IL-2) may help ART in this setting remains unknown.

As far as we know, this study is the first to evaluate the effect of daily low-dose IL-2 in association with STI during PHI. It is also one of the few studies reporting such a long period of follow-up, giving a reliable view of the intervention effect. It has, however, several limitations. First, the small size of patients in both arms probably prevented any statistically significant difference being found in the time to resume ART. Secondly, the indication to resume ART was outside of the originally selected period of follow-up of the study, and it was driven by the clinical decision of the attending physician; however, CD4 cells at resuming were not statistically different among both groups, allowing us to compare them. Third, at the time of the design of this study, a VL lower than 3000 copies/mL and a CD4 cell count higher than 500 cells/mm3 was not considered an indication for ART. Nowadays most clinicians would treat these patients, since indications for ART have evolved and most guidelines recommend ART as of diagnosis, regardless of CD4 cell count or VL. Finally, due to the lack of clinical benefit in large clinical trials, the interest on IL-2 administration in HIV infection has significantly decreased.

In conclusion, in the short term, STI proved able to boost specific immune responses, but these responses were lost 48 weeks after stopping ART. IL-2 failed to boost the specific anti-HIV T-cell immune responses. There was an increased immune activation and inflammation in the IL-2 group that also vanished during follow-up. In the long-term follow-up, there was a trend to delayed need to resume ART in the IL-2 arm, not reaching statistically significant differences. Although considering that the potential delay of ART in a lifetime treatment might not be very relevant, this small study suggests that immunological interventions during PHI may impact the long-term outcome. Probably these results cannot be translated into clinical practice, but they may encourage further research in this topic. Larger studies are needed to prove whether IL-2 or other immune modulating strategies may be useful in the setting of PHI.

Supporting Information

S1 CONSORT Checklist. CONSORT 2010 check list.
(DOC)

S1 Protocol. Original Protocol.
(DOC)

S2 Protocol. Protocol, English Translation.
(DOC)

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Author Contributions

Conceived and designed the experiments: MP MT TP TG JMG JMM. Performed the experiments: JA OS MP TP XC CM. Analyzed the data: JA FA CM JMM. Contributed reagents/materials/analysis tools: MP TP TG. Wrote the paper: OS JA DN CM FA XC MP MT TP TG JMG JMM.

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DISCUSION

El trabajo desarrollado durante los últimos 10 años ha permitido crear las cohortes de infección aguda por el VIH de Barcelona y Buenos Aires y el desarrollo de los trabajos de investigación de esta tesis doctoral, permitiendo responder a los objetivos planteados. Por ello se ha estructurado la discusión en base a dichos objetivos.

Objetivo 1: Descripción de las características clínicas de los pacientes con infección aguda por VIH-1

En los **artículos I y V** se describen las características clínicas de los pacientes con infección aguda de la cohorte del Hospital Clínic de Barcelona y en la cohorte del Grupo Argentino de Seroconversión. (Tabla 7)

La cohorte del Hospital Clínic de Barcelona incluyó 75 pacientes con diagnóstico de infección aguda o reciente, la mayoría (81%) hombres, y con una mediana de edad de 30 años, que iniciaron seguimiento en el Hospital entre 1997 y 2003. Este número representó el 2.9% de todos los pacientes atendidos durante el mismo periodo. En la cohorte evidenciamos sobre-representación de relaciones sexuales entre hombres como la vía de transmisión, que alcanzó un 72% en la cohorte, frente a un 30% de los casos en el total de pacientes atendidos en este periodo. Esto refleja probablemente un mayor grado de información sobre los síntomas en este grupo de pacientes y un mejor acceso o frecuencia de realización de las pruebas de VIH ya que diversas asociaciones de HSH realizaban actividades de promoción y prevención enfatizando la importancia de detectar la infección aguda. En la cohorte pudimos incluir 8 pacientes que habían adquirido la infección mediante uso de drogas endovenosas y una proporción significativa de

inmigrantes. Los pacientes fueron seguidos durante una mediana de 37 meses y la retención en el estudio fue muy alta (89%).

El 77 % de los pacientes fueron sintomáticos, y 17 (23%) requirieron hospitalización por fiebre (11), cuadros neurológicos graves (5) y otro paciente por poliartritis reactiva. Fueron frecuentes las infecciones concomitantes, por ejemplo, hubo 2 casos hepatitis B y 7 casos de sífilis activa entre 54 HSH y 4 casos de infecciones bacterianas graves entre 8 usuarios de drogas endovenosas que podrían haber contribuido a aumentar la sintomatología y la necesidad de internación. La búsqueda activa de infección aguda en el Hospital en casos con fiebre y posible exposición de riesgo facilitó el diagnóstico en estos pacientes que ya tenían una causa alternativa que justificaba el cuadro febril.

La mayoría de los pacientes presentaron síntomas, de los cuales la fiebre fue el síntoma predominante (76%), seguido de astenia, artralgia, linfoadenopatías y faringitis. Sin embargo, solamente el 20% de los pacientes tenían la típica tríada asociada a mononucleosis (fiebre, faringitis y adenopatías lateroervicales de más de 7 días). En esta cohorte no se identificaron síntomas graves o eventos C.

En la Cohorte Argentina el 74% de pacientes (de forma similar a la cohorte de Barcelona) presentaron síndrome retroviral agudo, que tuvo una duración media de dos semanas. La proporción de pacientes que requirieron ingreso hospitalario también fue parecida, una cuarta parte de los pacientes argentinos requirió admisión y el 19% tuvo eventos graves. Al contrario que en Barcelona, en Argentina se diagnosticaron 7 casos con infecciones oportunistas (3 casos de *Pneumocystis jiroveci*, un caso de histoplasmosis, un paciente con meningitis criptocóccica, un caso de esofagitis candidiásica y un caso de TB pulmonar). Además se diagnosticaron 9 eventos B (en general cándida oral y herpes zoster), y 10 eventos clínicos graves no SIDA (meningitis aséptica, rabdomiólisis con

fallo múltiple de órganos, hepatitis aguda, parálisis de Bell y psoriasis en gota). No todos los casos se asociaron a inmunosupresión grave, de hecho, cuatro de cada 5 eventos definitorios de SIDA se asociaron con recuentos de CD4 mayores de 200 células.

Tabla 7: Características demográficas de los pacientes incluídos en la cohorte de Barcelona y la cohorte de Buenos Aires con infección aguda y reciente por el VIH-1

Característica	Barcelona	Buenos Aires	
Número de casos	75	134	
Periodo de estudio	1997-2003	1997-2008	En la cohorte Argentina el periodo 1997-2007 se tomaron retrospectivamente
Edad	30 (RIQ 26-38)	33,4 (DS 10,7)	
Sexo (% varones)	71%	81%	
Riesgo HSH HTX UDI	 72% 17% 11%	 53% 37% 0,7%	En la cohorte Argentina 9% se desconocía vía de transmisión. En 3 casos se diagnosticó durante el embarazo.
Tiempo de la 1ª evaluación RIQ	64 (40-96)	66 (48-112)	Días desde la probable fecha de exposición
Inmigrantes (%)	17%	ND	
Ingreso hospitalario (%)	23%	26%	
Síntomas (%)	77%	74%	
Síntomas neurológicos (%)	35%	ND	Cefalea
Eventos C (n)	0	7	3 PCP, 1 TB, 1 CM, 1 CE, 1 H
Carga viral (Log10) RIQ	5,0 (4,2-5,6)	4,87 (4,11-5,51)	
CD4 (células/mm3) RIQ	576 (369-730)	479 (341-681)	
ETS concomitantes	12%	ND	7 sífilis, 1 HBV, 1 sífilis+HBV
TARV temprano*	65%	31%	
Mortalidad (n)	1	2	Barcelona: Mielitis transversa Buenos Aires: 1 Hs ,1linfoma no Hodking

PCP: Neumonía por *Pneumocystis jiroveci*, TB: tuberculosis, CM: criptococosis meníngea, CE: candidiasis esofágica, H: histoplasmosis, RIQ: rango intercuartilar, DS: desvío estándar, HSH: hombres que tienen sexo con hombres, HTX: heterosexuales, UDI: Usuarios de drogas intravenosas.

*En la cohorte de Barcelona se consideró tratamiento temprano hasta 180 días de la fecha probable de infección, en Buenos Aires hasta 120 días.

Los factores asociados con seroconversión grave fueron la presencia de una cifra de CD4 menor de 350 células/mm3 y una carga viral alta en plasma (>100,000 copias/mL). La frecuencia de eventos graves en Argentina es similar a lo que se describe en la literatura [420]. En la cohorte de Barcelona la frecuencia de personas con serología negativa al momento del diagnóstico y la cifra de linfocitos CD4 basal fue mayor que en la cohorte Argentina lo que podría representar un diagnóstico más precoz y una menor evolución clínica.

Los casos graves sin infecciones oportunistas están representados mayormente por cuadros neurológicos. La sintomatología neurológica se asocia a valores altos de carga viral y mayor progresión clínica [223]. En la cohorte española, una proporción significativa de pacientes presentó cefalea, aunque en pocas situaciones fue tan grave como para requerir estudios específicos o admisión hospitalaria. Es posible que la cefalea sea la expresión clínica de la inflamación a nivel de SNC. En un pequeño estudio, la mitad de los pacientes presentaba cefalea y el 28% tenían evidencia de inflamación con los parámetros habituales. Algunos pacientes tenían aumento de algunos marcadores de activación inmune en el líquido cefalorraquídeo (neopterina, MCP-1/CCL2 e IP-10) que reflejan la activación de macrófagos, microglía, y monocitos [421].

La respuesta al TARV fue muy buena en ambas cohortes. En la cohorte del Hospital Clínic de Barcelona casi todos los pacientes lograron valores indetectables de CV y la mediana de CD4 a los 12 meses superó las 800 células/mm3. Sin embargo, debido a los Antirretrovirales disponibles en la época los pacientes presentaron con frecuencia efectos adversos. En este estudio pudimos demostrar que el TARV precoz puede normalizar el recuento de CD4, como lo confirmaron después estudios con mayor número de pacientes [345].

Objetivo 2: Describir la prevalencia de trasmisión de resistencias a los antirretrovirales en pacientes con infección aguda por VIH-1 en Barcelona

Los trabajos realizados nos permitieron describir la prevalencia de resistencia y la distribución de subtipos virales en Barcelona y Cataluña en las últimas dos décadas.

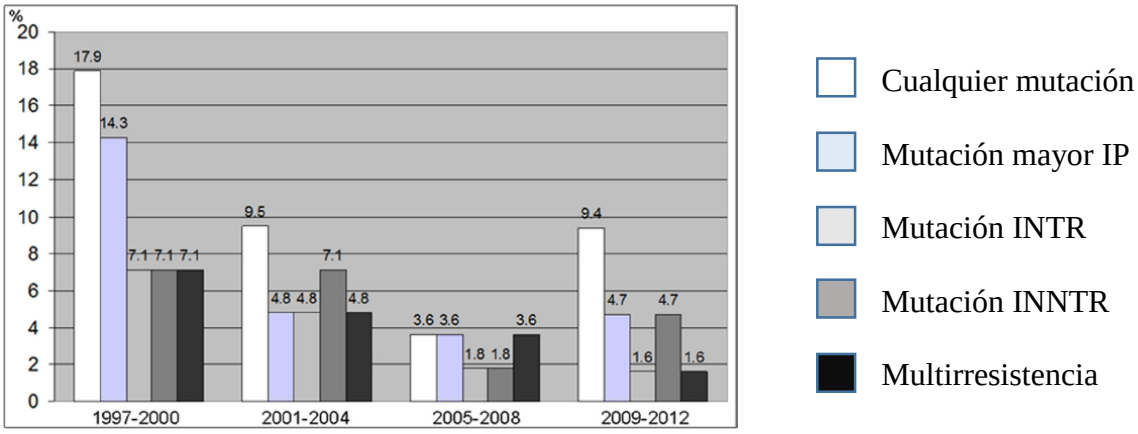
Prevalencia de trasmisión de resistencias a los Antirretrovirales

En el **Artículo III** describimos la prevalencia de trasmisión de resistencias a los Antirretrovirales en Cataluña y específicamente en Barcelona. La prevalencia en pacientes con infección reciente se sitúa entre el 6% y el 20%. Entre los 182 casos confirmados y con muestras genotipables la mayoría eran hombres (80,8%), nacidos en España (76,2%), con una mediana de edad de 33,3 años, cuyo mayor riesgo fueron las relaciones homosexuales (48,3%) seguidas de las relaciones heterosexuales (22,5%), y el uso de drogas por vía intravenosa (13,2%). La prevalencia de resistencias de acuerdo a la definición de OMS (2009) fue de 7,7% (IC95% 4,3-12,6) con tasas mayores en inmigrantes, hombres y pacientes con enfermedades de transmisión sexual. Estos valores de prevalencia son similares a otros realizados en el mismo periodo en España y otras regiones europeas [422–427] y ligeramente menor que los encontrados en un meta-análisis de 26 estudios realizados en España entre 1997 – 2008 [428].

En el **artículo IV** evaluamos la evolución de la trasmisión de resistencias a los Antirretrovirales en los pacientes con infección aguda confirmada que se evaluaron en el Hospital Clínic de Barcelona entre 1997 y 2012. Se incluyeron 189 pacientes que presentaban una prueba genotípica de resistencias dentro de los primeros 180 días de la infección. Esta gran cohorte nos permitió determinar la prevalencia y comparar la tendencia a lo largo de los años. La prevalencia global de trasmisión de resistencias fue

del 9%, disminuyendo notablemente a partir del año 2000, y permaneciendo relativamente estable desde entonces (ver Figura 7).

Figura 7: Prevalencia de resistencia primaria (Artículo IV)



Los porcentajes de resistencia encontrados se encuentran entre los valores habitualmente descritos en otros estudios nacionales [429], y confirman la reducción de la prevalencia de resistencia a partir del año 2004 [428]. Sin embargo, la comparación es difícil debido a diferencias en las metodologías utilizadas.

Los datos encontrados en ambos artículos son similares a otros países de Europa, mostrando una disminución significativa después del año 2000 y a partir de ahí prevalencias estables en alrededor del 10%, con una marcada disminución de la resistencia a INTR y cifras estables de resistencia a INNTR e IP [430]. El Programa SPREAD ha proporcionado información sobre la Resistencia en 20 países europeos en diferentes periodos. En estos estudios vemos como la resistencia ha ido disminuyendo desde un 13,5% antes del 2002 a un 8,4% en los años posteriores [422,431]. En un artículo más reciente se demostró una tasa de 9,5% [432]. En Francia la prevalencia de resistencia primaria también disminuyó en los años recientes para establecerse aproximadamente en

10%, con cifras mayores en HSH. En este estudio que incluyó 331 casos se identificaron 5 individuos con la mutación E157Q [433], una mutación polimórfica seleccionada durante el tratamiento con raltegravir y que produce una disminución de 5 veces su susceptibilidad [434], lo que enciende la alerta de la necesidad de incluir la prueba de resistencia a la integrasa a partir de ahora ya que la mayoría de guías internacionales indican que el TARV basado en los inhibidores de la integrasa son la primera opción terapéutica y por tanto su uso se generalizará en todo el mundo.

En Europa las tasas de resistencia parecen ser más bajas que en EEUU, aunque habitualmente en los estudios en este país no se utiliza la definición de la OMS para identificar las mutaciones [435]. Un estudio en la ciudad de Nueva York en 600 pacientes con infección aguda diagnosticados entre 1995 y 2010 mostró una prevalencia de resistencia del 14,3% [436] con una tendencia similar, al disminuir las mutaciones asociadas a INTR, aumento de INNTR y IP estables. En EEUU no se identificaron mutaciones asociadas a inhibidores de la integrasa durante el periodo 2007-2013 [437]. Sin embargo, en Europa y EEUU los inhibidores de la integrasa son ahora fármacos de elección para el inicio de tratamiento, por lo que debemos estar preparados para la posibilidad de que aumente la transmisión de cepas resistentes. La persistencia de las mutaciones a lo largo del tiempo dependen del *fitness* viral modificado por mutaciones específicas [438], lo que permite explicar porque por ejemplo la mutación M184V, que confiere una capacidad replicativa menor, no se observa frecuentemente en pacientes naive [439]. En general los estudios realizados durante la infección aguda demuestran tasas de prevalencia mayores que cuando se realizan durante la infección crónica [440]. También hay que tener en cuenta que los estudios realizados con métodos genotípicos identifican las principales poblaciones virales circulantes. Por lo tanto el tiempo y la metodología utilizada y la sensibilidad para detectar variantes minoritarias pueden dar lugar a diferentes valores de

prevalencia. Por ejemplo entre pacientes de la cohorte suiza que no presentaban mutaciones con las pruebas de resistencia habituales se detectaron variantes minoritarias de la mutación M184V en 8,2% de los pacientes, y esta cifra caía a 2,5% en los pacientes con infección crónica [441]. La utilización de pruebas de resistencia con alta sensibilidad para detectar variantes minoritarias puede triplicar la prevalencia. En un pequeño estudio la prevalencia de mutaciones a INNTR con el método convencional fue del 11% y aumento a 37% con piro-secuenciación [442]. En otro estudio, con 99 pacientes con infección aguda utilizando otro método de detección de variantes minoritarias (ensayo de ligación de oligonucleótidos) se detectó resistencia en 27% comparado con 5% con el método estándar, pero no pudo identificar un impacto en el tratamiento antirretroviral [443]. Hacen falta estudios prospectivos para identificar los factores adicionales que permitan predecir el riesgo de fracaso virológico (por ejemplo el tamaño de la población con mutaciones, o el valor de CV de la población con mutaciones), su persistencia después de suspender el tratamiento y el riesgo de transmisión [444].

Distribución de subtipos virales:

En ambos estudios pudimos demostrar un aumento significativo de muestras subtipo no-B a lo largo del periodo estudiado (Figura 8).

La presencia de virus subtipos no-B, no se asocia *per-se* a una mayor prevalencia de trasmisión de resistencias, pero diferencia una sub-población que sugiere una dinámica de transmisión diferente (individuos de mayor edad, UDI, que viven fuera del área metropolitana de Barcelona). Una cuarta parte de los pacientes evaluados en Cataluña con infección reciente nacieron fuera de España, lo que refleja la tendencia migratoria en esa década, pero además la vulnerabilidad de estas poblaciones ya que en muchos de los casos la transmisión ocurrió en España.

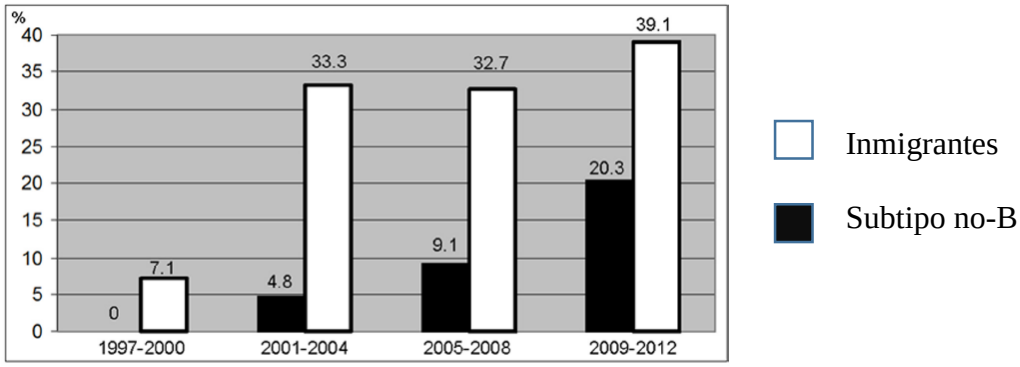


Figura 8: Prevalencia de inmigración y subtipos no-B (Artículo IV)

El aumento de inmigración buscando mejores oportunidades y mejor acceso a servicios favoreció la introducción de subtipos no-B en Cataluña, y el aumento de la frecuencia de subtipos no-B y de pacientes inmigrantes aumentaron proporcionalmente. La distribución tanto de inmigrantes como de subtipos no-B se concentra fuera del área metropolitana de Barcelona (en particular en Lleida, Tortosa, Reus, Vic, y Palamós).

El aumento de subtipo no-B a partir del 2005 es un fenómeno generalizado en toda España y en la mayoría de los países industrializados y los datos nacionales confirman la relación con la inmigración. En una encuesta de 354 casos de infección crónica el 36,2% eran subtipos no-B, con solo un 16% nativos de España. La mayoría de las muestras procedían de personas originarias de África Sub-Sahariana (50%) o de América del Sur, siendo la cepas mayoritaria las variantes CRF02_AG [445]. En trabajos similares se encuentra un aumento significativo, desde un 9% en el año 2000 al 32% en 2007 y asociado no solo a la inmigración sino también a la circulación de estas cepas virales entre población autóctona [446]. En Marsella las cepas no-B (y en particular el subtipo CRF02_AG) representa ahora 21.5% de las cepas transmitidas durante la infección aguda [447].

Estos datos son importantes para informar sobre la circulación de virus resistentes, para reforzar la importancia de analizar los patrones migratorios al planificar políticas de salud

[448] y a la necesidad de promover que a nivel mundial se realicen pruebas basales de resistencia a los antirretrovirales con el fin de seleccionar el mejor TARV inicial. Es difícil establecer si los diferentes subtipos se asocian a una mayor progresión. Aunque en Cataluña el acceso al tratamiento después del diagnóstico es universal, los migrantes presentan peor recuperación inmunovirológica y mayor riesgo de SIDA, fundamentalmente por el desproporcionado retardo [449,450].

Objetivo 3: Identificar factores asociados a la progresión clínica e inmunológica en pacientes con infección aguda por VIH-1

La experiencia acumulada durante mi estancia en Barcelona pudo ser trasladada a mi grupo de trabajo en Argentina a fin de establecer una cohorte de infección aguda en Argentina que en la fecha lleva más de 200 pacientes incluidos. Hasta el año 2010 la mayoría de los pacientes no recibieron TARV durante la fase aguda debido a los estándares de atención en Argentina en ese periodo de tiempo lo que nos permitió analizar los factores clínicos asociados a progresión.

Entre los 134 pacientes consecutivos incluidos, solo 42 comenzaron TARV durante la fase aguda. Entre los 92 pacientes sin tratamiento el 26% presentó progresión clínica o inmunológica, la mitad presento eventos B o C y la otra mitad una descenso de la cifra de linfocitos CD4 por debajo de 350 células/mm3. Hubo una muerte por linfoma no-Hodgkin. La progresión al año fue mayor entre personas con síntomas, (34%) comparada con pacientes asintomáticos (13%). En el análisis univariado, los factores asociados con progresión fueron la presencia de síntomas y la CV basal mayor de 100.000 copias/mL. Ambas variables están relacionadas ya que a mayor CV en plasma más síntomas tiene el paciente con infección aguda por el VIH-1. Estos datos reafirman descripciones similares

donde la presencia de síntomas, un período de incubación corto o una duración larga de la fase sintomática (mayor de 15 días) se correlaciona con una progresión a SIDA más rápida de los pacientes [451,452]. En la cohorte CASCADE se describieron 1.108 pacientes con fecha estimada de seroconversión de los cuales 366 progresaron a SIDA o muerte durante el seguimiento. El riesgo de progresión aumentaba en más de 2 veces si el pacientes había presentado síndrome retroviral agudo (HR 2,2) o si había tenido menos de 350 CD4 durante los primeros meses de infección (HR 2) [453].

La presencia de síntomas, tal como he comentado previamente, está fuertemente ligada al valor de la CV en plasma que durante esta fase puede ser particularmente alta: 10^6-10^7 copias de ARN viral/mL. En un estudio se pudo comprobar que por cada síntoma que estaba presente (considerando fiebre, vómitos, cefalea, artralgia, mialgias, odinofagia, astenia, exantema o astenia) había un aumento de 0,4 log10 de CV en plasma. El valor al cual se establece el *set-point*, los CD4 bajos y los casos más sintomáticos se asociaron a mayor progresión y muerte [454]. En nuestro estudio, el síndrome retroviral agudo se asoció a mayor progresión clínica (mayor morbilidad) y en algunos casos a mayor mortalidad, lo que refuerza la necesidad de aumentar el nivel de alerta en los médicos para diagnosticar la infección aguda por el VIH-1 en pacientes con síndromes febriles agudos. Es muy importante también implementar sistemas de vigilancia de infecciones de transmisión sexual, donde se pueda buscar sistemáticamente infección aguda por VIH en HSH, debido a la alta carga que presentan de estas infecciones [455] y la alta incidencia de VIH (2,5 por 100 personas/año) con una tendencia creciente [456].

En nuestro estudio pudimos identificar la CV inicial, independientemente del *set-point*, como uno de los factores predictivos de progresión clínica y/o de la caída de las células CD4 lo que es consistente con otros estudios [457–460]. La muestra para la CV se tomó mucho antes del establecimiento del *set-point* (mediana de 66 días de la fecha probable de

infección) y el valor de carga viral alta se asociaba con un riesgo de progresión 10 veces mayor. Por el contrario, la CV a los 6 meses no se correlacionó con progresión, como tampoco lo hicieron ni los CD4 basales ni a los 6 meses. En otro estudio en pacientes no tratados se demostró que la reducción inicial de la viremia y el *set-point* resultante fueron factores independientes de progresión a sida [246]. Así, la progresión a SIDA fue significativamente más rápida en los pacientes que tuvieron un aclaramiento inicial lento (< 0,63 log10/mL o < 4.260 copias/mL por mes) o en los que el nivel de viremia fue más elevado (> 4,4 log10/mL o > 25.000 copias/mL).

El impacto de la CV basal en la evolución es significativo incluso en aquellos que inician TARV, como lo demuestran Mugavero et al. en 2000 pacientes seguidos aproximadamente por 3 años, en los cuales se observa que por cada logaritmo de aumento de la CV basal se observaba un aumento del 44% del riesgo de mortalidad, incluso ajustando por otras covariables [461].

Existen otros factores, además de la CV basal, que modulan la respuesta a la infección, el valor del *set-point* y el riesgo de progresión. Entre estos se incluyen la pérdida o grado de recuperación de respuestas inmuno-específicas, las respuestas humorales, el grado de inmunoactivación y algunas características virales específicas. La creación de la Cohorte y el Grupo Argentino de Seroconversión permitieron la exploración de alguna de estas variables que comentamos brevemente a continuación:

a) La inmunidad celular de cada individuo está determinada por la posibilidad de responder a cada antígeno a través del CMH que codifica las moléculas HLA-1 y algunos alelos específicos HLA. Se ha podido demostrar que un alelo determinado (HLA-B57) se correlaciona con una menor CV, menor frecuencia de síntomas y un mejor pronóstico a largo plazo [135]. En el Grupo Argentino de Seroconversión,

la progresión acelerada se asoció a una mayor frecuencia de CCR5-CF2 (16,7% vs. 0%,) y HLA-A*11 (16,7% vs. 1.2%) y a una menor frecuencia de HLA-C*3 (2,8% vs. 17,5%). La CV alta se asoció a la presencia de HLA-A*11, HLA-A*24, y a la ausencia de HLA-A*31 y HLA-B*57 [462]. Estudios de otros grupos tienen resultados similares o complementarios. Entre 421 seroconvertidores de África se demostró las personas con HLA-B*57 presentaban valores de *set-point* más bajos, mientras que los portadores de HLA-B*18 y HLA- B*45 tenían valores más altos de CV [463]. En China también el los alelos B*57 tenían un efecto beneficioso, demostrado en un estudio entre 126 HSH, donde se pudo además demostrar que los alelos B*44, y en particular el alelo homocigota para Bw4 se asociaban a valores bajos de *set-point* [464]. Otras variantes protectoras que modifican el control viral de la infección, ya sea afectando vías innatas o adaptativas incluyen HLAB*1302, B*27, B*5801 y B*8101 [465]. En estos estudios es importante evaluar las diferencias entre los géneros. En un análisis incluyendo 521 seroconvertidores se identificaron alelos que se asocian a un valor bajo de *set-point* solamente en mujeres (HLA-A*03:01 y HLA- C*18:01) [466].

b) La respuesta celular es crítica para el control de muchas infecciones virales. En la infección por VIH la activación de los linfocitos T y la destrucción preferencial de las células CD4 son puntos claves de la patogenia y la compleja interacción de estas respuestas continúa siendo objeto de intensas investigaciones [263,467,468]. La importancia de la respuesta citotóxica de las células T CD8+ VIH-inmuno-específicas ha sido ampliamente reconocida. La reducción inicial espontánea de la CV se asocia con la emergencia de las respuestas CD8 VIH específicas, que permiten el establecimiento del *set-point*. Las primeras respuestas son dirigidas a un número limitado de epítopes y con el tiempo el control inicial se pierde debido

a la generación de mutaciones y escape viral. Un estudio que siguió longitudinalmente 622 personas para evaluar estas respuestas demostró que ni la amplitud ni la magnitud se asociaban al *set-point*, pero que una respuesta dirigida a epítopes Gag se asociaba a una progresión más lenta [164,469]. Nuestro grupo también pudo establecer esta relación entre las respuestas tempranas anti-Gag y el control de la progresión de la infección mediante preservación del compartimiento celular CD4 [470]. Una subpoblación de células CD4, las células Th17, un tipo celular diferente de las clásicas Th1 y Th2, se caracterizan por producir IL-17 y son claves para el mantenimiento de la homeostasis intestinal y la protección mucosa [471]. De hecho, los pacientes que progresan presentan mayor inmunoactivación a nivel de GALT y mayor pérdida de células Th17 con respecto a los pacientes EC sugiriendo que una mejor actividad regulatoria de las células T en la mucosa en estos individuos contribuyo a regular el exceso de inmuno-activación, favoreciendo la integridad de la mucosa intestinal [472]. A nivel periférico los pacientes con primoinfección presentan en forma progresiva en el tiempo una rápida disminución de la relación Th17/Treg por pérdida de células Th17 y por expansión de Treg no funcionales lo que se asoció temporalmente con un aumento de la inmunoactivación medida mediante niveles de sCD40L. Pero además, la pérdida de células Th17 se correlaciona con mayor activación de linfocitos CD8, mayor proporción de células CD8 con marcadores de agotamiento (PD-1), mayor CV y caída de células CD4 [473]. También en el Grupo Argentino de Seroconversión se evaluaron cuáles son las subpoblaciones de linfocitos CD8 que se asocian a control de la infección comparando pacientes con infección primaria, individuos EC y personas en estadio crónico. Pudimos evidenciar que la distribución de las subpoblaciones de linfocitos T-CD8 totales y VIH específicos

esta marcadamente alterada en los pacientes VIH, mucho más en pacientes crónicos que en pacientes con infección aguda o EC. En pacientes con infección aguda la mayor proporción de T CD8 naive se asociaban a una progresión más lenta mientras los factores asociados a progresión más rápida fueron una proporción alta de linfocitos T CD8 de memoria efectora (T_{EM}), una mayor relación T_{EM} /$T_{EM+}T_{TE}$ y una proporción alta de linfocitos CD8 que expresan PD-1[474]. La expresión de PD-1 se correlaciona también con inmunoactivación, disminución de la capacidad proliferativa y alta sensibilidad a la muerte celular [475]. La proporción de CD8 expresando este marcador aumenta durante el curso natural de la infección, lo que resalta la importancia de iniciar tratamiento en etapas tempranas [474].

c) Por otro lado, el rol de las respuestas CD4 específicas está menos estudiado. Las células CD4, además de su función colaboradora, tienen la capacidad de reconocer en forma directa y eliminar células infectadas mediante citolisis y expresión temprana de perforinas y granzimas [163]. Además, la expansión de las respuestas CD4 específicas se asocia con el control de la replicación viral independientemente de la presencia de respuestas CD8 [476]. En un estudio similar la amplitud, la magnitud y la dominancia de las respuestas CD4 específicas permanecieron estables durante el tiempo y la inducción de respuestas CD4 Gag específicas se correlacionaba inversamente con el *set-point* viral y progresión [477].

d) Con respecto a las respuestas humorales, no se había podido demostrar correlatos de protección hasta que recientemente el estudio con RV144 ALVAC-HIV (vCP1521) prime/AIDSVAX B/E boost proporcionó la primera evidencia de protección frente a la infección por VIH inducida por vacunas aunque la eficacia estaba limitada al 31% [152]. Los análisis de correlación de riesgo demostraron que

los anticuerpos frente a la proteína env gp120 V1/V2 y niveles bajos de anticuerpos IgA Env en asociación con altos niveles de citotoxicidad celular dependiente de anticuerpos (ADCC) protegían de la infección mientras que el nivel de anticuerpos IgA de unión a Env se correlacionaban con riesgo. Es probable que la producción de IgA modifique la magnitud de esta respuesta. De hecho, nuestro grupo pudo demostrar in vitro que la IgA específica de gp120 mitiga los efectos beneficiosos de ADCC en infección aguda [478]. Inmediatamente después de la infección la actividad ADCC aumenta, se mantiene estable y disminuye con la instauración del tratamiento. Aunque en trabajos iniciales no se pudo correlacionar la actividad de ADCC con progresión, un estudio realizado en el contexto del Grupo Argentino de Seroconversión demuestra que la pérdida de IgA del plasma aumenta la actividad de ADCC y permite correlacionar este aumento con progresión [478].

e) La activación de linfocitos CD4 y CD8 se asocia con mayores valores de CV [479], y se asocia con mayor reservorio medido por mayor nivel de ADN del VIH, lo que a su vez se asocia a activación residual en la fase crónica [480]. El tratamiento temprano es capaz de reducir los niveles de activación celular pero no logra retornarlos a niveles normales. En 138 pacientes que iniciaron el tratamiento antes de los 45 días de la primoinfección el nivel basal de activación de linfocitos CD8 (CD38+HLA-DR+ CD8+) era del 73% y a las 96 semanas se redujo a 16% pero seguía siendo alta en comparación con el 9% observado en los controles negativos [76]. La inmunoactivación además promueve la inflamación que puede identificarse mediante biomarcadores. En 138 pacientes los niveles elevados de sCD14 soluble se asociaron a mayor pérdida de células CD4 y mayor mortalidad [481]. Entre 90 pacientes seguidos sin tratamiento desde las fases iniciales de la infección en los

que se evaluaron diferentes citocinas para verificar si tenían un impacto en la CV o en la caída de células CD4 (TNF-α, IFN-γ, IL-1β, IL-6, PCR y dímero D) se puedo identificar al TNF-α (a valores mayores o iguales a 8,5 pg/mL) como asociado a un *set-point* más elevado y a un descenso más rápido de las células CD4 [482]. El bloqueo de TNF-α con adalimumab no redujo la CV ni la activación de CD4 pero redujo la expresión de genes proinflamatorios, disminuyó la infiltración de células polimorfonucleares a los ganglios y los niveles de macrófagos activados y células productoras de IL-10 y el factor transformante beta sCD 163, evitando la infiltración y fibrosis ganglionar [483].

f) Finalmente, la progresión más rápida también depende de características virales como una mayor capacidad replicativa o la capacidad de generar o evitar el escape viral a la respuesta adaptativa del huésped [484]. El establecimiento de una infección primaria con un tropismo no –R5 es un fenómeno poco frecuente, pero su presencia se asoció a una caída más rápida de los linfocitos CD4 [485] y a una progresión más rápida dentro del primer año de infección [486]. La menor replicación de estas cepas en GALT y en macrófagos [487], junto a la caída más rápida de células CD4 pueden explicar la mayor recuperación de células CD4 a las 24 semanas de iniciar el tratamiento [488].

En conclusión, la evolución de la infección por VIH en ausencia de TARV depende de múltiples factores, que continúan siendo estudiados como potenciales dianas de estudios inmunológicos o tratamientos inmunomediados.

Objetivo 4: Impacto a corto y largo plazo del TARV combinado con paradas estructuradas ("autovacunación") y tratamientos inmunomediados en la infección aguda por el VIH-1

Los beneficios del TARV y los casos anecdóticos de control viral en ausencia del mismo motivaron la búsqueda de estrategias que posibiliten la suspensión de los antirretrovirales y conviertan a los pacientes en no progresores a largo plazo, a aún mejor, en EC. Inicialmente, se especuló que una exposición controlada al antígeno (VIH) en personas que habían logrado reconstituir su sistema inmune con TARV podría potenciar las respuestas inmuno-específicas, las que serían suficientes para controlar la replicación en ausencia de tratamiento, dando lugar a los concepto de "paradas estructuradas del TARV" también llamado "autovacunación", en los que se suspendía en forma intermitente el tratamiento para permitir la exposición controlada al virus autólogo [489]. Varios estudios demostraron que la suspensión se asociaba a una estimulación del sistema inmune que mejoraba las respuestas específicas [490], y en algunos pacientes que habían comenzado tratamiento durante la fase aguda se observaba un control transitorio de la viremia [341,347]. Sin embargo, las suspensiones también se asociaron con un aumento de la inmunoactivación [491], aumento de riesgo de emergencia de resistencia [492] y progresión de enfermedad, por lo que se postuló que se necesitaban intervenciones adyuvantes que potenciaran y mantuvieran las respuestas VIH específicas (proliferativas y citotóxicas) [493]. Incluso la viremia intermitente a bajo grado mejora las respuestas inmunes aunque no se observó efectos beneficiosos [494]. Esto constituyó la base racional para el diseño del estudio presentado en el **Artículo VI** en el cual se combinaron 4 ciclos de interrupciones estructuradas de tratamiento y un periodo de tratamiento de 6 meses de IL-2 a dosis bajas (750.000 UI/m2/día) al final del último periodo de interrupción estructurada. Se propuso que la IL-2 podría promover la expansión clonal de las respuestas colaboradoras y

citotóxicas específicas contra el VIH y finalmente controlar la viremia [495–500], por lo que después de 6 meses de IL2 se suspendió el TARV. En el estudio se incluyeron pacientes tratados tempranamente desde la infección aguda (mediana 11 semanas desde la fecha de infección), durante un tiempo prolongado (mediana 22 meses) y que presentaron muy buena respuesta clínica, inmunológica (CD4/CD8 >1) y virológica (CV <5 copias/mL). La administración de IL-2 se asoció con una mayor activación inmune (CD8+CD38+) y mayores niveles de marcadores de inflamación (TNF and srIL-2) pero no mejoró las respuestas inmunes específicas de los linfocitos CD4 (proliferativa) ni de los CD8 (citotóxica). De esta forma el estudio no pudo demostrar un beneficio clínico o inmunológico inmediato. Esta cohorte de pacientes se siguió varios años, observándose durante el seguimiento una ligera tendencia a la disminución de las CV y a demorar el re-inicio de TARV en los pacientes que recibieron IL-2, aunque esta diferencia no fue significativa por el pequeño tamaño muestral.

En otros estudios previos en pacientes con infección crónica la IL-2 se utilizó para intentar restaurar las células T o mejorar la respuesta antiviral. En dos estudios con casi 3000 pacientes en las ramas de tratamiento inmunomediado, la IL-2 produjo un aumento de los linfocitos CD4 pero no se pudo demostrar ningún beneficio clínico [496]. Un factor a tener en cuenta es que la mayoría de las células CD4 expandidas por IL-2 presentaron un fenotipo central, memoria o naïve, que expresan la cadena α del receptor de IL-2 (CD25), FoxP3 y tienen una moderada actividad supresora, lo que sugiere un patrón Treg [501], que eventualmente pudo haber sido la causa de la falta de mejora de las respuestas inmuno-específicas. Estos estudios sugieren que las citocinas pueden tener un impacto en algunas variables de resultado y nos estimula a seguir explorando estrategias inmunomediadas en combinación con tratamiento antirretroviral y vacunas terapéuticas en etapas más

precoces de la infección por el VIH-1 (estadios Fiebig 1 o 2) que permitan que los pacientes controlen la infección sin TARV.

Durante la infección aguda el estudio de las citocinas ha generado mucho interés [134,502]. El IFN-α tiene efectos antivirales e inmunoestimulantes que lo hacen atractivo para ser utilizado como adyuvante. Un estudio piloto en 12 pacientes demostró la capacidad del IFN más tratamiento antirretroviral para suprimir la viremia y reducir el reservorio [503]. Con estos resultados se diseñó el estudio INTERPRIM que aleatorizó a 91 pacientes con infección aguda a una estrategia de tratamiento continuo durante 72 semanas, o a tratamiento continuo por 36 semanas seguido de 3 ciclos de interrupciones estructuradas hasta la semana 72, o el mismo esquema más IFN [504]. Después de las 72 semanas las tres ramas suspendían el tratamiento y se evaluaban los resultados a los 6 meses de la suspensión. Los resultados no fueron muy alentadores, el hallazgo más importante fue que el rebote viral durante las suspensiones fue menor en el grupo con IFN pero a los 6 meses los niveles de CV, respuesta inmune y tamaño del reservorio fueron similares en los 3 grupos [504]. Otro estudio reciente, con 23 voluntarios que se asignaron a recibir 90 o 180 μg/semana de IFN junto con 5 semanas de TARV que después se suspendía durante 12-24 semanas demostró que el IFN logro mantener la CV por debajo de 400 copias/mL en el 45% de los pacientes. Los respondedores además presentaban una disminución del ADN proviral. Es posible que el IFN sea una molécula que debe seguir siendo considerada a la hora de plantear tratamientos para estimular la cura funcional.

La IL-7 es una citocina que participa en el desarrollo y la homeostasis de linfocitos T a través de la vía de señalización CD127, induciendo supervivencia y proliferación. Los pacientes con VIH tienen una reducción de los niveles de IL-7 y bajos porcentajes de CD4 y CD8 expresando CD127 [505]. La administración en forma intermitente a pacientes crónicos suprimidos que no presentaron adecuada recuperaron inmunológica mejoró las

cifras de células CD4 [506]. Una sola inyección causó una expansión importante de los linfocitos T CD4 y CD8 con un pico a los 14 días de la inyección, en general en supoblaciones de memoria central y en menor medida en naive, que no se asoció con un aumento de CD25 ni de FoxP3, sino que expresan CD31 y muestran un repertorio más amplio. La administración fue segura, y la actividad fue muy potente incluso a dosis bajas sin producir elevaciones de la CV. Al igual que con IL-2, la IL-7 promueve la inmunoactivación, con un aumento de la expresión de HLA-DR y CD38 y aumento de la expresión de Ki67 [507]. En otro estudio en pacientes crónicos a los que se los intensificó con raltegravir y maraviroc y se les administró IL-7 no se pudo demostrar una disminución del reservorio viral, ya que logró la reactivación de la producción viral desde células latentes pero no pudo inducir la muerte de las células infectadas. Este fenómeno, más la proliferación de células de memoria CD4+ resulta en un aumento absoluto del ADN viral por lo que se ha abandonado esta estrategia en los estudios de erradicación [508].

La IL-15 es una citocina que actúa uniéndose al receptor específico IL-15R α, presente en las células dendríticas presentadoras de antígeno, monocitos y macrófagos que induce la diferenciación y proliferación de células T, B y NK [509]. La IL-15 estimula la diferenciación y síntesis de inmunoglobulinas por las células B y la maduración de las células dendríticas sin estimular las células Tregs, mejorando de esta forma la inmunidad innata. Se propuso que su uso podría mejorar la capacidad de las células NK de suprimir el VIH estimulando la producción de perforinas y granzimas para eliminar células T infectadas. Este enfoque se evaluó en ratones humanizados donde se pudo inhibir la infección hasta 3 días después de la exposición [510]. Un estudio en macacos demostró que la IL-15 retardaba la supresión viral pero no pudo lograr la reconstitución de los tejidos linfoides. Además, cuando se suspendía el tratamiento, el grupo que había recibido IL-15 más TARV perdía células CD4 en forma mucho más acelerada que el grupo que solo

había recibido TARV [511]. De todas maneras, todavía se considera a la IL-15 como un potencial adyuvante para las estrategias de vacunación [512].

Se han ensayado otros enfoques terapéuticos para reducir la inflamación, prevenir la immunoactivación o mejorar las respuestas inmunes (Tabla 8).

Existe mucho interés en identificar los primeros eventos de la infección y los efectos que producen las diferentes citocinas, para modular de alguna forma estos eventos [513]. Sin embargo, ninguna de estas intervenciones ha logrado evitar la necesidad del TARV para evitar la progresión de la enfermedad.

Tabla 8: Enfoques de inmunoterapia ensayados (modificado de Smith [514])

Tipo	Tratamiento	Respuesta	Ref.
Para mejorar la respuesta inmune			
Citocinas	IL-2 más suspensión TARV	Aumento de CD4 pero no logra controlar la replicación viral	496,499,515
	IL-7 en pacientes tratados	Aumento de CD4 pero aumenta también el reservorio viral	506,507,516
	IL-21 en macacos	Aumento de NK y cél CD8. Mejoría de células Th17 y disminución de la traslocación	517,518
	IL-15 en macacos	Aumento de NK, caída de CD4 al suspender	510,511
Fármacos	Lenalidomida y pomalidomida	Aumento de las respuestas de células T	519
Para corregir la inmunoactivación			
Fármacos	Aspirina	Reduce CD38, HLA-DR y sCD14	520
	Celecoxib	Reduce CD38 y marcadores de inflamación	521
Anticuerpos	Anti-PD-1	Aumenta la expresión de citocinas	522
	Anti-CTLA4 en macacos	Disminuye TGF beta, IDO, y ARN viral	144,523
Probióticos	Pre y probióticos	Reduce ADN bacteriano e IL-6 Aumenta CD4+CD25+ pero no disminuye CD8+CD38+	524 525

CONCLUSIONES

Objetivo 1

1.- Es posible diagnosticar y crear cohortes de pacientes con infección aguda y reciente por el VIH-1 en Barcelona (España) y Buenos Aires (Argentina) que permitan realizar estudios epidemiológicos, clínicos y terapéuticos con el fin de mejorar el manejo clínico y el tratamiento de estos pacientes.

2.- La infección aguda por el VIH-1 fue sintomática en más del 70% de pacientes en las cohortes de Barcelona y Buenos Aires y se observó fundamentalmente en hombres que tuvieron sexo con hombres, siendo infrecuente en los usuarios de drogas por vía intravenosa. La presencia concomitante de otras enfermedades de trasmisión sexual obliga a hacer el cribaje de las mismas en los pacientes con infección aguda por el VIH-1 y a descartar la infección por VIH-1 en aquellos pacientes con enfermedades de transmisión sexual.

3.- Las manifestaciones clínicas fueron similares en ambas cohortes y estuvieron asociadas al nivel de CV plasmática y la inflamación. Hasta una cuarta parte de los pacientes requirieron ingreso hospitalario en ambas cohortes. Sin embargo, el desarrollo de eventos C solo se observó en la cohorte Argentina (5% de casos). El retraso diagnóstico, el tipo de virus (predominio subtipo B en Barcelona y no B en Argentina) u otras características virales (tropismo R5 vs. no R5) o de la población podrían explicar este fenómeno.

Objetivo 2

4.- La prevalencia de trasmisión de resistencias a los fármacos antirretrovirales en Barcelona ha sido del 9%, habiendo disminuido del 18% en el periodo 1997-2000 a porcentajes que oscilan entre el 4% y el 9% entre los años 2001 y 2012. La prevalencia de trasmisión de virus multirresistentes también ha disminuido notablemente, pasando del 7% al 1,5%. Esta disminución es probablemente debida a que a nivel poblacional, la mayoría de pacientes tratados está suprimidos.

5.- La resistencia a nuevas drogas (como al raltegravir) es excepcional, pero debe continuarse monitorizándose debido a que el uso de los inhibidores de la integrasa se ha generalizado en la actualidad.

6.- La prevalencia de subtipos no-B ha aumentado en Barcelona en las últimas 2 décadas y se ha asociado al aumento de la inmigración, aunque también se ha identificado un aumento de subtipos no-B en la población autóctona, lo que sugiere la trasmisión local de dichos virus.

7.- Es importante mejorar el acceso de pruebas de resistencia en personas con infección aguda por el VIH-1 que inician TARV en Argentina, donde hasta ahora no se utiliza en forma rutinaria.

Objetivo 3

8.- La cantidad de virus que se detecta en la primoinfección por el VIH se asocia a que ésta sea sintomática y la gravedad de los síntomas y ambos factores (CV y síntomas graves) se han asociado de forma independiente a una mayor progresión clínica y a un mayor deterioro inmunológico de estos pacientes. Estos hallazgos, conjuntamente con

otros estudios, justifican que todos los pacientes con primoinfección sintomática por el VIH-1 deban recibir TARV.

Objetivo 4

9.- El TARV en la infección aguda por el VIH-1 permite normalizar la cifra de linfocitos CD4 y revertir el cociente CD4/CD8 >1 como se ha observado en la cohorte de Barcelona y en otros estudios. Estos datos, justifican con los anteriores, que en estos pacientes se recomiende el inicio del TARV. Sin embargo, el TARV precoz no erradica la infección por el VIH ya que la CV reapareció en todos los pacientes incluidos en el ensayo clínico que pararon de forma estructurada del TARV.

10.- En los pacientes tratados desde la infección aguda por el VIH-1 con buena respuesta inmunológica, ni las paradas estructuradas del TARV en forma individual ("autovacunación") ni combinadas con la adición de IL-2 lograron aumentar la respuesta proliferativa y/o citotóxica específica frente al VIH, ni fueron capaces de controlar la replicación viral en ausencia de TARV. Se necesitan nuevas estrategias terapéuticas combinadas (activadores del reservorio, vacunación terapéutica y otras) para lograr la cura funcional o la erradicación del VIH-1.

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ANEXOS

1.- REVISIÓN DE LA LITERATURA

A fines de asegurar que se evaluó la última información disponible a nivel mundial se realizó una revisión sistemática de la literatura buscando todas las citas publicadas en PubMed en los últimos 6 años. Para ello se realizó la búsqueda con la siguiente sintaxis:

> "acute HIV infection"[All Fields] OR "primary HIV infection"[All Fields] OR "acute HIV-1 infection"[All Fields] OR "primary HIV-1 infection"[All Fields] OR "HIV seroconversion"[All Fields] OR "HIV-1 seroconversion"[All Fields] AND ("2010/07/01"[PDat] : "2015/06/29"[PDat])

Esta búsqueda generó 707 artículos que fueron evaluados individualmente. Se eliminaron los relacionados con casos o pequeñas series de casos, temas no relacionados con la infección aguda (por ejemplo PrEP o infección crónica), duplicados y cartas al editor. En total quedaron 301 artículos que fueron agrupados en las siguientes categorías y que fueron incluidos en esta tesis:

- 35 artículos de ciencias básicas y progresión
- 35 artículos de resistencias o transmisión de virus resistentes
- 104 artículos de diagnóstico
- 38 artículos de manifestaciones clínicas
- 27 artículos de epidemiología
- 62 artículos de TARV, paradas del TARV e intervenciones inmunomediadas

Además se revisaron las referencias de los artículos publicados.

2.- ARTÍCULOS RELACIONADOS

Esta línea de trabajo en Barcelona y Buenos Aires permitió al doctorando Omar Sued realizar otras 10 publicaciones en colaboración con diferentes grupos de investigación:

Hospital Clínic de Barcelona:

1) Ambrosioni J, Nicolas D, **Sued O**, Agüero F, Manzardo C, Miro JM. Update on antiretroviral treatment during primary HIV infection. Expert Rev Anti Infect Ther. 2014 Jul;12(7):793-807.

2) Miró JM, **Sued O**, Plana M, Pumarola T, Gallart T. Avances en el diagnóstico y tratamiento de la infección por el VIH. Carta de lectores. Enferm Infecc Microbiol Clin. 2004 Dec;22(10):643-59.

CEEISCAT (Badalona):

3) Romero A, Martró E, González V, Matas L; AERI Study group. Comparison of two serological tests for the identification of recent HIV infection: Vironostika HIV-1 Microelisa and BED capture enzyme immunoassay. Enferm Infecc Microbiol Clin. 2011 Aug-Sep;29(7):553-5.

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5) Ruiz MJ, Ghiglione Y, Falivene J, Laufer N, Holgado MP, Socias ME, Cahn P, **Sued O**, Giavedoni L, Salomon H, Gherardi MM, Rodriguez AM, Turk G. Env-specific IgA from Viremic HIV-infected Subjects Compromises Antibody-Dependent Cellular Cytotoxicity. J Virol. 2015 Oct 21. pii: JVI.02363-15.

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Hospital Universitario Vall d´ Hebrón de Barcelona

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EXPERT REVIEWS

Update on antiretroviral treatment during primary HIV infection

Expert Rev. Anti Infect. Ther. 12(7), 793–807 (2014)

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Primary HIV-1 infection covers a period of around 12 weeks in which the virus disseminates from the initial site of infection into different tissues and organs. In this phase, viremia is very high and transmission of HIV is an important issue. Most guidelines recommend antiretroviral treatment in patients who are symptomatic, although the indication for treatment remains inconclusive in asymptomatic patients. In this article the authors review the main virological and immunological events during this early phase of infection, and discuss the arguments for and against antiretroviral treatment. Recommendations of different guidelines, the issue of the HIV transmission and transmission of resistance to antiretroviral drugs, as well as recently available information opening perspectives for functional cure in patients treated in very early steps of HIV infection are also discussed.

Keywords: acute retroviral syndrome • antiretroviral treatment • eradication • Fiebig phases • functional cure • HIV guidelines • HIV infection • primary HIV infection • therapeutic vaccines • transmitted drug resistance

Primary HIV-1 infection (PHI) covers a period of around 12 weeks post-infection, in which the virus disseminates from the initial site of infection (vaginal, rectal or oral mucosa) into different tissues and organs. It is a phase of high viral load (VL) and high risk of transmission and is frequently associated with signs and symptoms resembling mononucleosis syndrome (fever, fatigue, odynophagia, lymphadenopathy, etc.) or aseptic meningitis, which are the result of uncontrolled viral replication.

When diagnosis is made in this clinical phase, most guidelines recommend treatment with antiretrovirals (ARV) in patients who are symptomatic, although the indication for treatment remains inconclusive for asymptomatic patients.

It is during these first weeks of infection when the viral reservoir, defined as the chronically infected cells that carry the genome of the virus around the organism, is established. It is also the period when the greatest depletion of the lymphoid tissue occurs. Viremia increases quickly and precedes clinical symptoms by at least 7–10 days [1].

Thus, several key immunological and virological issues feature in this critical period and therapeutic interventions in this phase may have an impact that is no longer possible to achieve later on during the phase of chronic/established HIV infection.

The ARV treatment in PHI may have advantages and disadvantages [2,3]. ARVs would shorten the duration and severity of symptoms, suppress viral replication, reduce the risk of transmission of HIV-1 [4–10], reduce viral diversity and reservoir [11,12] and preserve the immune system and specific immunity to HIV-1 [13–18], which could allow an immune control of viral replication, and finally improve prognosis. To achieve these goals, ARV should probably be initiated within the first month of infection, before virological escape occurs [19]. Additionally, treatment of acute HIV-infected patients might contribute to the reduction of the risk of transmission. By contrast, the main disadvantages include the need for indefinite treatment (since the ARV regimens that have been used so far cannot eradicate infection or restore mucosa-associated lymphoid tissue lost [20,21]), the cumulative risk of side effects, the potential risk of resistance development if compliance is not adequate, the possible reduction in quality of life, and perhaps, the unnecessary treatment of non-progressors.

There were approximately 2 million adults and children newly infected with HIV in

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2012, 30,000 of them in Europe [22]. In some European countries, it is estimated that around 2.3% of these newly diagnosed infections are less than 1 month post-infection and around 20% are less than 6 months post-infection [23–26].

In this article, we will review the main virological and immunological events during PHI, and the arguments in favor and against ARV and other therapeutic interventions during this phase, the issue of the HIV transmission and transmission of resistance to ARV (and how this affects the initial ARV choice) as well as the recently published perspectives for functional cure in patients treated during PHI.

Acute HIV infection: definition & very early events

There is some overlap and confusion with some terms and definitions such as 'Acute HIV infection', 'Primary HIV infection' and 'Recent infection'. Moreover, for some of these terms, the definition considered in different trials has also differed. Although there is no universally recognized definition, acute infection is considered that of less of 30 days (pre-serologic period). The presence or absence of the p31 band in the western blot (WB, the serological confirmatory test) allows for classification of infections of more or less than 90 days [8]. Recent infection is generally considered that of less than 6 months post-infection, but in some studies the cut-off has been established at 1 year [6]. According to the time since infection, diagnostic tools to identify a PHI differ. By definition, in the pre-serologic period (around 30 days post-infection), molecular assays (viremia) or p24 antigen in absence of HIV antibodies can diagnose acute infection. With fourth-generation enzimoimmunoanalysis (EIA) (simultaneous detection of both p24 HIV-1 protein and antibodies against HIV-1), infection can be diagnosed 1 week earlier than with those of third-generation EIA (only antibodies) [27]. After 30 days, all serologic tests become detectable and WB and immunoblot can have different patterns (negative, indeterminate or positive) according to the time post-infection. As previously mentioned, the absence of p31 band can identify infections of less than 3 months [8].

Apart from the cases in which HIV infection is directly initiated parenterally (e.g., after a blood transfusion or after sharing needles among intravenous drug users) in the vast majority of cases, the infection starts in a mucosal surface (vaginal, rectal or oral). HIV infection normally follows the principle of 'bottle neck'. A single/limited number of strains initiate the infection, and population is relatively homogeneous during the first weeks of infection. This involves mostly the CCR5 coreceptor usage. It's only under the pressure of the cellular immunity several weeks/months later that heterogeneity of strains and quasispecies begins to develop and emerge [19].

Dendritic cells are one of the first cells to encounter the virus and are a key actor in this phase, since they can become infected not only by HIV but also carry the viral particles to the lymph nodes without experiencing active replication cycles [28]. In the lymph nodes, viral replication continues in CD4 T cells and monocytes and shedding of viral particles to the general circulation become detectable 7–10 days post-infection, approximately 1–3 weeks before onset of symptoms, as previously discussed.

HIV transmission during acute infection

Several studies performed in developed countries have suggested that recently infected patients represent a significant source of new HIV infections [6,29–31]. This is the consequence of several factors. Most patients with primary HIV infection are not aware of the infection. Before the infection becomes symptomatic, patients are highly viremic for several days. Risk of transmission with a single sexual intercourse in this phase is higher that in other periods. Phylogenetic studies have clearly demonstrated that patients with acute infection are overrepresented in chains of transmission. This is particularly true for some populations such as highly sexually active men-who-have-sex-with-men (MSMs), in which the association of these factors (unawareness of infection, unprotected sex and high VL) explains this clustering of infections. There is a need to expand and improve early diagnosis for patients with acute infection, in order to cut these chains of transmission. In most of the studies, this clustering among patients with recent infection was more frequent among MSMs [6,30,31].

Detecting infection very early in these patients is, however, very difficult. Identifying asymptomatic patients requires the determination of RNA levels (pre-serological phase), and this is not available in most centers. It can be hypothesized that earlier the ARVs are prescribed, the higher the impact will be on interruption of these chains of transmission associated with highly viremic patients during PHI, and multidisciplinary efforts should be applied in this context.

Transmission of drug resistance mutations in primary/acute HIV infection

Drug resistance mutant strains are a common problem in clinical practice, as they are one of the most common causes of treatment failure. Drug resistance mutations may be the result of drug pressure, but they also may be acquired in the moment of infection. Transmitted drug resistance (TDR) has been described through sexual contact, vertical transmission and infected blood contact [32,33]. Transmission of drug resistant strains has also been described in primary/acute HIV infection. TDR has a crucial role for the epidemiology and natural history of the disease, and must be taken into account at the time of a first evaluation of a recently infected patient and for the election of an appropriate ARV regimen. It must be emphasized that TDR prevalence may vary considerably depending on whether PHI patients or patients with chronic infection are studied, since some mutations may decrease viral fitness (e.g., M184V) and be rapidly replaced by the wild-type strain, and thus not be detected in naïve patients beyond the PHI period.

TDR has become a major public health issue and this has been reflected in the epidemiological studies performed to the date. Prevalence of TDR ranges from 5 to 24%, depending on the characteristics of the study, the population studied (naïve vs

PHI, MSMs, intravenous drug users, etc.) and the country of origin. In Europe, there are several studies describing TDR epidemiology in acute/recent HIV infection: a meta-analysis from Yebra and Holguin, including 26 studies performed in Spain between 1996 and 2008, reported a decreasing prevalence, ranging from 26.7% in 1996 to 2.9% in 2008 [34]. Other studies in Spain are consistent with these results, such as the one performed by de Mendoza *et al.*, in which prevalence of resistance in primary HIV ranged from 20% in 1999 to 7.7% in 2004 [35]. Other countries such as the UK, France, Italy and Germany have published similar data in prevalence studies, observing in all of them a consistent decreasing trend: data from earlier periods (1996–2002) show higher prevalence values (16.1–13%) [36,37], whereas those from 2006 to 2008 period reflected lower prevalence, ranging from 6.3 to 9% [38–40]. Karlsson *et al.* reported a mean prevalence of 5.6% during the 2003–2010 period in Sweden, not observing any temporal trend [41]. The most important data sets in Europe come from the SPREAD Program, which prospectively investigated TDR among patients with newly diagnosed HIV-1 infection in 20 European countries and Israel. Data from 1996–2002 showed a prevalence of 13.5% among recently infected patients [42]. During the study period from 2002 to 2005, the prevalence seemed to stabilize at 8.4% [43]. A recently published large multi-cohort European study showed a TDR rate of around 10% [44]. This decreasing trend in Europe in recent years has been attributed to an increase in treated patients with undetectable viremia, as well as to a higher immigrant population coming from countries where no antiretroviral pressure is present.

In contrast to Europe, data available from the USA report an increasing trend in transmitted resistance prevalence in recent years. In a 2005 survey, the TDR rate was 25% [45], while previous reports showed an 8.3% rate during 1997–2001 [46]. Little *et al.* reported an increase from 3.4% in 1995–1998 to 12.4% in 1999–2000 in 10 US cities [47]. The National HIV Surveillance system reported TDR rates of 14.6% in 2006 [48] and 16% in 2007 for 10 states and 1 county in the USA [49]. As previously mentioned, differences in epidemiology may vary from one study to another due to the methodology used, and different definitions applied; there are also other factors such as demography, healthcare access policies in each country and antiretroviral drug pressure that might play a role.

A trend in the genotypic TDR profile has also been described in both Europe and the USA, with a decrease in the prevalence of nucleos(t)ide reverse transcriptase inhibitors (NRTIs), while the prevalence of non-nucleoside reverse-transcriptase inhibitors (NNRTIs) increased, mainly driven by an increase in the rate of K103N [42,46]. This may be explained by the increasing use of efavirenz, increasing possibilities of selecting this mutation. It also depends on the population studied since, as previously mentioned, mutations decreasing viral fitness will not be detected beyond the period of acute/recent infection, whereas others such as K103N (not decreasing fitness) are more prone to persist and be detected for longer.

Data about TDR in other continents have also been described, and prevalence ranges between 1 and 20%, depending on the ARV pressure and the characteristics of each healthcare service. Several studies in Africa (mostly sub-Saharan) showed prevalence ranging from 0.85% in Zimbabwe [50] to 16% in Angola [51]. Hamers *et al.* [52] conducted the largest multicentric study in six sub-Saharan countries (Nigeria, Zimbabwe, Kenya, Zambia, South Africa and Uganda), involving 2436 naïve patients, with a reported TDR prevalence of 5.6%. The WHO recently reported 44 surveys in 18 African countries showing an increasing trend in TDR [53]. This may be due to antiretroviral therapy (ART) implementation programs, and to specific factors contributing to resistance selection, such as lack of plasma VL monitoring, suboptimal ARV regimens and drug stock-outs [54]. Latin-American countries have reported similar data, with a prevalence of around 7%, although these studies are limited by small samples [55,56]. In Asia, prevalence ranges from 3 to 4% in China and Thailand [57,58] and 8% in Japan and Taiwan [59,60], probably reflecting the spread of ARV in these countries. In conclusion, although TDR seems to be globally decreasing, it is preferable to start with a regimen based on a ritonavir-boosted protease inhibitor until the resistance pattern is known, since TDR has been reported to range from 5 to 24% in different populations. Table 1 shows the most relevant studies of each region.

Virological & immunological issues of acute HIV infection: how reservoir is established?

After the very early events occur in the mucosa, at around 7–10 days post-transmission, viremia begins to rise (Fiebig stages I and II). Feibig stages (Figure 1) illustrate the appearance of different virological markers that allow for dating of the time post-transmission and classification of patients with PHI [19,61]. Recently, a staging system adding the result of fourth-generation EIA has allowed identification of two subgroups of patients in Feibig stage I: those with negative fourth-generation EIA had lower DNA and total viral reservoir than those with positive fourth-generation EIA [62]. Viremia peak occurs around 2–4 weeks post-infection (Feibig stages III and IV) and it's at this time that patients start to develop symptoms. At this point, however, the virus has already largely disseminated in the body, and the proviral reservoir has been established in a large number of cells and in different tissues. Although therapeutic interventions in this pre-symptomatic stage might have a great impact in clinical and immunological evolution, exposed individuals rarely seek medical advice at this point. The virus widely disseminates in tissues and organs such as the central nervous system or testicules. Memory CD4 T cells, and other cell types such as monocytes carry the pro-virus (the DNA integrated into the genome) representing the viral reservoir and this prevents elimination of the infection despite years of continuous therapy with ARV.

One of the most serious immunological consequences of this period of uncontrolled replication is the depletion of the gastrointestinal-associated lymphoid tissue (GALT). The virus

Table 1. Transmitted drug resistance prevalence according to geographical region of origin of the study.

Study (year)		Country	Patients (n)	Period studied	Type of patient	HIV risk factors
Tshabalala *et al.* (2011)	AFRICA	Zimbabwe	236	2006–2007	Naïve/Recent	ND
Hamers *et al.* (2011)		Sub-Saharan Africa[†]	2436	2007–2009	Naïve	ND
Afonso *et al.* (2012)		Angola	101	2008–2010	Naïve	ND
Hattori *et al.* (2010)	ASIA	Japan	2573	2003–2008	Naïve	MSM (68.9%), IDU (0.4%), heterosexual (20.2%)
Sungkanuparph *et al.* (2012)		Thailand	466	2007–2010	Naïve	MSM (16.7%), IDU (5.6%)
Lai *et al.* (2012)		Taiwan	1349	2000–2010	Naïve	ND
Yang *et al.* (2013)		China	610	2010	Naïve	MSM (100%)
Violin *et al.* (2004)	EUROPE	Italy	112	1996–2001	Recent/Naïve	Heterosexual (40.2%), MSM (33%)
Wensing *et al.* (2005)		Europe	2208	1996–2002	Acute/Naïve	MSM (43%), heterosexual (41%), IDU (15%)
Descamps *et al.* (2005)		France	303	2001–2002	Acute/Recent	MSM (57.7%)
			363		Naïve	MSM (31.2%), IDU (7.8%)
de Mendoza *et al.* (SPREAD 2005)		Spain	198	1997–2004	Recent/Naïve	MSM (70%), heterosexual (19.5%), IDU (10%)
Vercaruten *et al.* (2009)		Europe	2793	2002–2005	Naïve	MSM (46%), heterosexual (37%), IDU (8%)
Wittkop *et al.* (Euro-Coord CHAIN 2011)		Europe	10056	1998–2009	Naïve	MSM (50%), IDU (7.5%)
Karlsson *et al.* (2012)		Sweden	1463	2003–2010	Naïve/Recent	MSM (37%), IDU (9%)
Monge *et al.*(CoRIS 2012)		Spain	1864	2007–2010	Naïve	MSM (68.8%), IDU (6.3%)
De Gascun *et al.* (2012)		Ireland	1579	2004–2008	Naïve	ND
Descamps *et al.* (2013)		France	661	2010–2011	Naïve	MSM (47%), heterosexual (39.7%)
Ferreira *et al.* (2013)	LATIN AMERICA	São Paulo (Brazil)	225	2008–2009	Naïve	ND
Ávila-Ríos *et al.* (2011)		Mexico	1655	2005–2010	Naïve	ND
Little *et al.* (2002)	USA	USA	377	1995–2010	Acute	Sexual (77%), IDU (23%)
Weinstock *et al.* (2004)		USA	1082	1997–2001	Naïve	MSM (44.5%), IDU (10%)
Smith *et al.* (2007)		San Diego (USA)	103	2005	Naïve	ND
Wheeler *et al.* (2010)		USA	2030	2006	Recent/Naïve	MSM (46.7%), IDU (2.2%), heterosexual (6%)

[†]Nigeria, Zimbabwe, Kenya, Zambia, South Africa, Uganda.
[‡]To two or three classes.
ART: Antiretroviral therapy; IDU: Intravenous drug users; MSM: Men-who-have-sex-with-men; ND: Not defined.

Global TDR prevalence (%)	Resistance to 2 ART classes (%)	Resistance to 3 ART classes (%)	PI-R (%)	NRTI-R (%)	NNRTI-R (%)	HIV subtypes	Ref.
0.85	0	0	1 patient	1 patient	0	ND	[50]
5.6	1.2	0	1.3	2.5	3.3	C 54%, A 25%, D 11.3%, G 2.6%	[52]
16.3	8	0	0	10.5	14	C 16%, F1 14%, G 6%, D 5%, H 5%	[51]
7.7	ND	ND	2.5	4.58	0.8	B 87.9%, AE 8.4%, C 1.2%	[59]
4.9	1.1	<1	1.7	1.9	2.8	CRF01_AE 91.4%, B 4.3%	[58]
8	ND	ND	ND	ND	ND	ND	[60]
4.9	1 patient	1 patient	3.9	1	0.6	C 43%, URF 18%, CRF01_AE 18%, B 11%	[57]
16.1	1.8	0	2.7	11.6	0.89	B 93%, F1 3%, CRF02_AG 3%	[37]
10.4	19	3.5	2.5	7.6	2.9	B 70%, C 10%, G 4%, CRF02_AG 4%	[43]
14	1.98‡		4.3	10.3	3.3	B 76%, CRF02 11%	[36]
6	0.82‡		1	4.3	0.8	B 66%, CRF02 19%	
12.1	ND	ND	2	9.6	4	B 92%, CRF14_BG 3.5%	[35]
8.4	0.63	0.45	2.9	4.7	2.3	B 67%, A 9.9%, C 6.7%	[42]
9.5	ND	ND	ND	ND	ND	B 69%, Non-B 31%	[44]
5.6	0.34	0.34	0.35	2.4	1	B 41%, CRF01_AE 19%, C 15%, A 9%	[41]
8.6	1.18	0.16	2.31	3.92	3.86	B 84%, CRF02_AG 4.24%, FI 2%, D 1%	[39]
6.3	ND	ND	ND	ND	ND	ND	[38]
9	0.9	0.2	1.8	6.2	2.4	B 44.5%, CRF02_AG 20%	[40]
7.6	ND	ND	ND	ND	ND	B 76%, C 7%, F 6%	[55]
7.4	0.1	0.8	1.7	4.2	2.5	B 100%	[56]
12	3.8–10.2	0.9–9.1	8.5–15.9	1.7–7.3	99	ND	[47]
8.3	1.2	0	1.9	6.3	1.7	ND	[46]
25	6	1	ND	ND	ND	ND	[45]
14.6	1.9	0.7	4.5%	5.6%	7.8%	B 96%, C 1.3%, CRF02_AG 1%	[48]

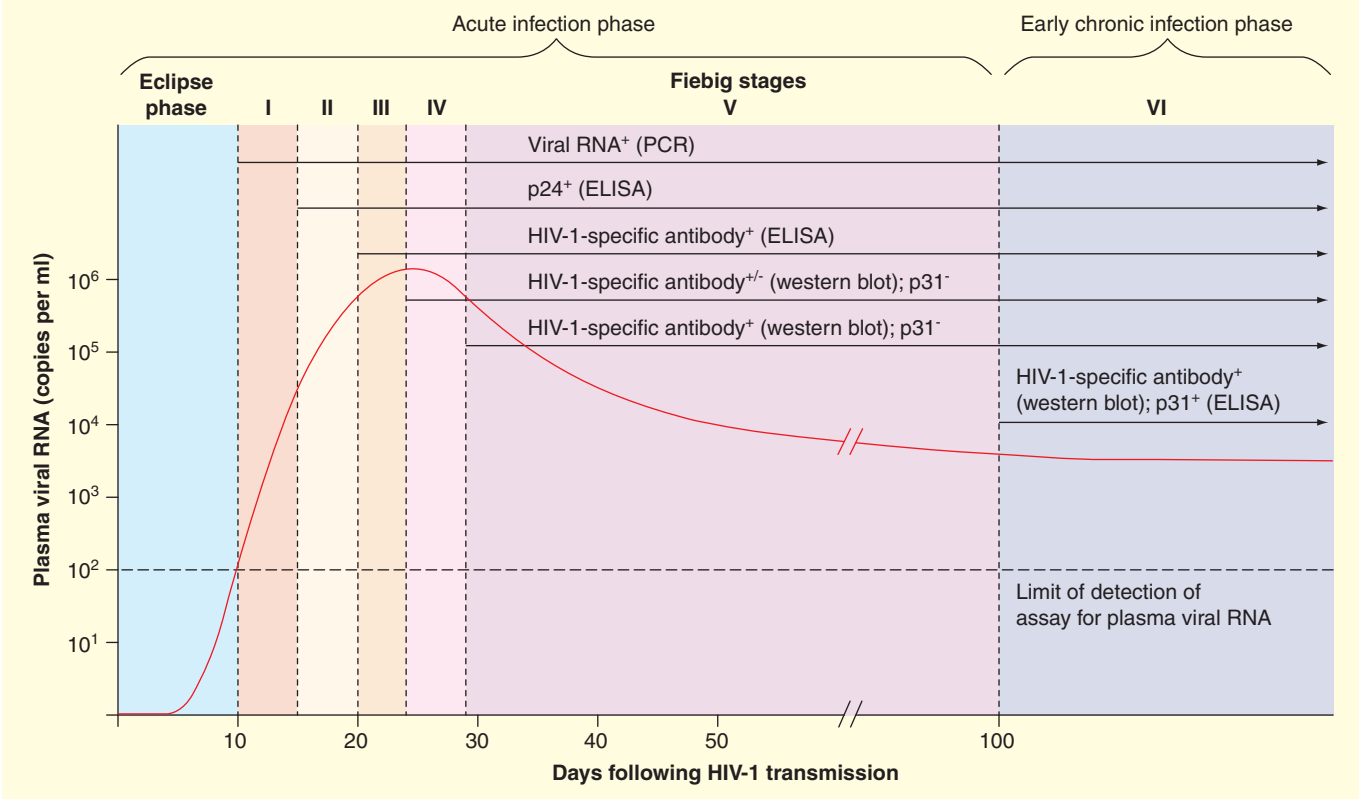

Figure 1. Plasma viral load in early phases after infection. Fiebig stages apply to the virological and immunological markers for HIV-1 detection.
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is cytopathic for activated lymphocytes, leading to death of infected cells, and rapid depletion of lymphoid tissue followed by the specific cytotoxic effect of cellular immunity, which also contributes to lymphoid depletion [63]. This loss of GALT tissue is only partially reversible, even after several years of ARV [64,65]. As a consequence, some immunological functions of the bowel, such as the limitation of bacteria and bacterial products translocation are perturbed. Some of these bacterial products may, in part, stimulate the immune system and contribute to the increased inflammatory status of chronic HIV infection [66]. Proinflammatory markers such as IL-6 at the time of seroconversion independently predict HIV disease progression in patients with PHI [67]. Several soluble biomarkers such as IL-7 or IL-15, among others, may also help predict VL set-point and the subsequent disease progression [68].

After this period of uncontrolled replication, the immune system (particularly the cellular immunity) partially controls the infection. The VL during the period in which the patients move out of the PHI is know as the 'set-point' and it is a prognostic factor for progression: those patients with >100,000 copies of HIV RNA/ml (and those who have a more symptomatic PHI, as an expression of more uncontrolled replication) will progress and deteriorate the immunological status more rapidly [69–71]; this is also true of patients with CD4 cells counts <500/µl [71]. A viral tropism other than R5 is also associated with faster progression [72] and thus, a tropism test should be performed in every patient with PHI. Although treatment is generally proposed, tropism tests may be additional important elements that help in deciding whether to initiate immediate treatment and might have to be discussed with the patient in case of reluctance. In a French cohort of PHI, 15.9% of HIV-1 strains were identified as dual-tropic and 4.9% of strains as CXCR4-tropic [73,74]. B and non-B HIV-1 subtypes may have the same response to ARV during PHI [75]. Around 1% of HIV-infected individuals very efficiently control HIV replication and do not show immune deterioration despite long periods (years or decades) without ARV and they have very low or undetectable VL during chronic infection. These patients are known as 'elite controllers', they have lower viremia during PHI and also a lower set-point [76,77].

Trials on treatment of acute HIV infection; ARV & other strategies: what have we learnt so far?

Timing of initiation of ARVs, duration of ARV therapy and optimal ARV combination during PHI have not been definitively established. Information on the impact of ARVs and other strategies in PHI is available from cohorts and randomized clinical trials.

To avoid the scenario of indefinite treatment with ARVs, several strategies have been evaluated:

- Manage the ARV for a limited period of time;
- Administer ARV intermittently, to enhance HIV-specific immune response and control viral replication;
- Combining ARV with immunosuppressants (hydroxyurea, cyclosporin A, mycophenolic acid) or cytokines (IL-2, interferon);
- Associate ARV and therapeutic vaccines.

However, none of these strategies has proven to boost the immune system enough to control viral replication in the absence of ARV (behave as post-treatment controllers in a similar way to elite controllers), so if ARVs are initiated during PHI, they probably should not be stopped.

ARV efficiency for a variable period of time has been evaluated in cohort studies and in clinical trials in which patients with acute or recent infection, treated or untreated, were compared. The results of most of these studies have failed to demonstrate a clinical, virological or immunological benefit at 48–144 weeks post-treatment cessation, while in others, only a small proportion of patients receiving ARV showed a lower set-point, better values of CD4 cells, retarded resuming ARV or maintained the specific immune response against HIV-1 [11,78–88].

The proportion of post-treatment controllers (VL <50 copies/ml) after stopping ARVs initiated during acute/recent infection has been studied in two cohorts. In the CASCADE cohort, only 11 (4%) of 259 seroconverters had no virologic rebound at 24 months of stopping ARVs. The time mediated to VL rebound (two consecutive VL >50 copies/ml) in the remaining 248 patients was 1.7 months [89]. In the French cohort PRIMO, only 14 (9%) of the 164 patients who stopped after they started ARVs during acute HIV infection kept the VL undetectable for a median of 4.5 years [90].

Recently, three clinical trials that studied the clinical benefit of different immunological or virological ARV management strategies in acute HIV infection have been published [78,91,92]. The first is the SPARTAC clinical trial, a multicenter, multinational trial including 367 patients with acute or recent infection randomized to not receiving ARV (n = 124), to receive it for 12 weeks (n = 120) or for 48 weeks (n = 123). The primary analysis variable was immunological evolution (time to have a CD4 count <350 cells/μl) or need to initiate ART indefinitely. The median follow-up of patients was about 3 years. The study failed to show significant benefits. Compared with the control arm without ARV, the 12-week group was not associated with any benefit and the 48-week ARV group delayed immunological deterioration or restarting ARVs by just over a year, benefiting more patients who started earlier (in the first 90 days post-infection). The interruption of ARV was not associated with side effects, development of resistance or compromised immune restoration when patients resumed ARV indefinitely. This study suggests that the potential benefit of ARVs during PHI vanishes rapidly, if treatment is not initiated very early. In the SPARTAC study, patients were included with an infection of up to 6 months, suggesting that this might be too late to induce post-treatment control [78]. The second study is the Primo-SHM clinical trial, a multicenter trial conducted in the Netherlands with a design similar to the SPARTAC, in which 168 patients with acute or recent HIV were randomized to ARV for 24 or 60 weeks. Patients with severe primary infection (e.g., meningitis) were only randomized to no ARV, ARV for 24 or ARV for 60 weeks. The main analysis variables were virologic (plasmatic VL at 36 weeks after completion of the ARV treatment or after infection in the control group) or time without ARV in the control group or after treatment cessation in the two treatment arms. The criteria for starting ARVs were immunological (CD4 <350 cells/μl) or clinical (AIDS). The number of patients randomized to no ARV, 24 or 60 weeks of ARV were 36, 62 and 66, respectively. Four patients were lost to follow-up. The mean (SD) of plasmatic VL at 36 weeks without ARV in the three arms of the study was 4.8(0.6) 4.0 (1.0) and 4.3(0.9) log10/ml ($p < 0.001$), although this difference was lost at 144 weeks with similar plasmatic VL in all three arms. Immune recovery was higher in patients with ARV, but the slope of decline in CD4 lymphocytes after stopping ARV was similar to patients not receiving ARV. The median (95% CI) of time without ARV in the control arm (no ARV) was 0.7 (0.0–1.8) years and in arms 24 and 60 weeks was 3.0 (1.9–4.2) and 1.8 (0.5–3.0) years, respectively (log-rank test, $p < 0.001$). The findings of this trial were that ART during acute/recent HIV transiently reduced set-point and deferred resuming ARV during chronic HIV infection [91].The third is the Setpoint Study (ACTG A5217), a randomized clinical trial, in which patients with recent infection (<180 days) were assigned to receive immediate ARV for 36 weeks with tenofovir/emtricitabine and lopinavir/ritonavir (immediate arm) or delayed ARV. The primary analysis variable was the decrease in the level of viremia at week 72 without ART (therefore comparing ARV during 36 weeks followed by 36 weeks without ARV to 72 weeks without ARV). The secondary end point was the need to initiate ARV indefinitely. The study was stopped by the data safe monitoring board because many of the patients in the delayed ARV arm had started ARV for clinical, immunological (CD4 350 cells/μl) or virological (VL >200,000 copies/ml) reasons. A total of 130 of the 150 expected participants were included. When comparing the rate of initiation of ARV indefinitely in both arms, immediate ARV only slightly delayed indefinitely starting of ARV ($p = 0.035$) [92].

Therefore, the findings of these three clinical trials are that in clinical practice, if ARVs are initiated in acute or recent HIV-1 infection, they must be indefinite. Last but not least, quality of life seems to be increased in patients with PHI treated with ARV [93]. This is not surprising considering the better tolerability of the currently available ARV regimens and the possibility to choose among several dose-fixed combinations, simplifying the long-term adherence to therapy.

In a recently published study, complete immune reconstitution (defined as 900 CD4 cells/μl) was achieved only in patients starting ARV during the first 4 months post-infection. In those starting after 4 months, recovery of CD4 cells was incomplete for those patients starting ARV with <500 CD4 cells/μl (Figure 2) [94]. Although 900 CD4 cells/μl seems to be a very high value

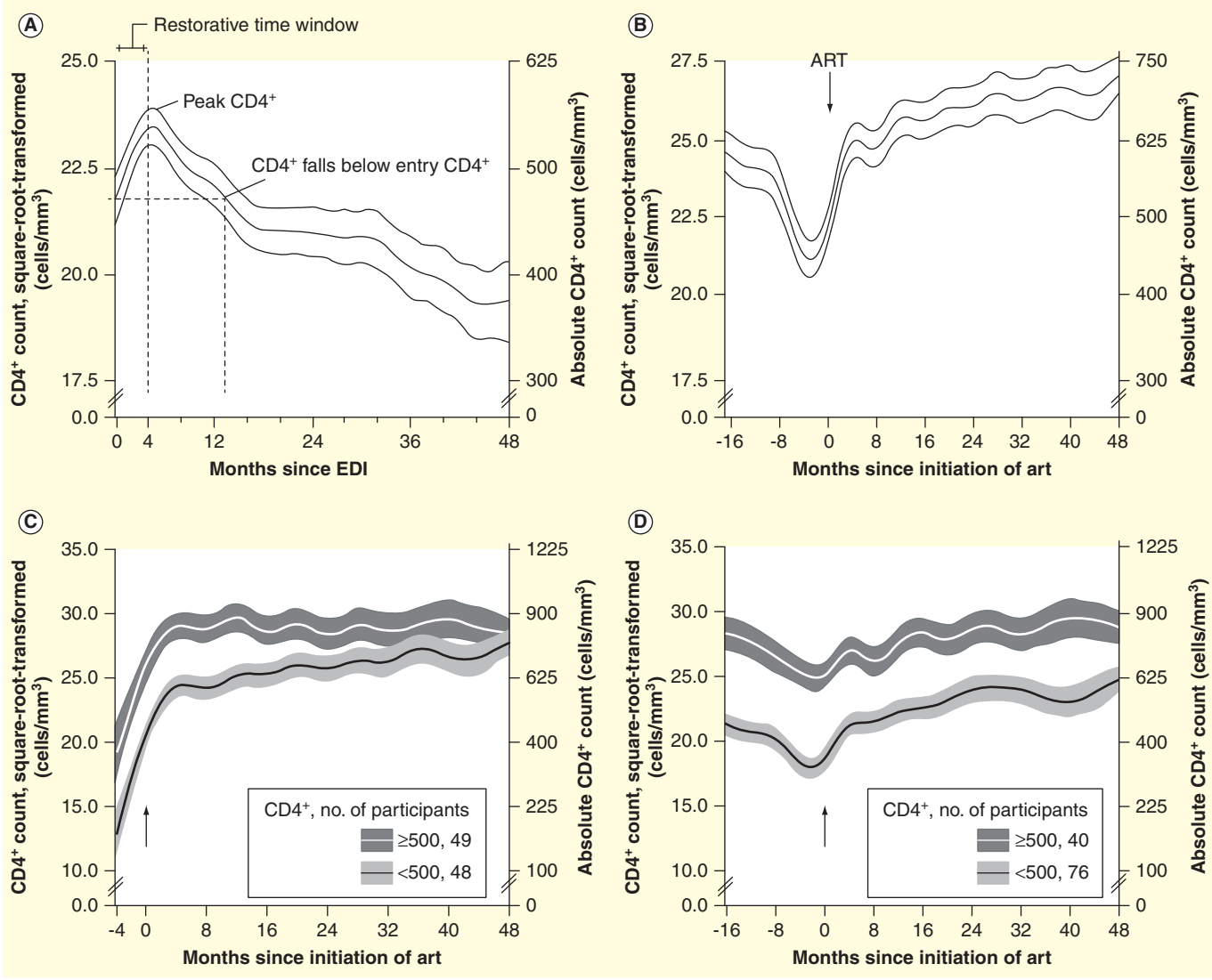

Figure 2. Likelihood of CD4 recovery to 900 CD4 cells/μl according to time of initiation of antiretrovirals after HIV infection (less or more than 4 months post-infection) and according to level of CD4 cells. (A) Study set 1. **(B)** Study set 2. **(C)** Initiation of ART ≤4 Mo after EDI (earlier ART). **(D)** Initiation of ART ≤4 Mo after EDI (later ART).
ART: Antiretrovirals; EDI: Estimated date of infection.
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(a normal level for an uninfected person), recent information from COHERE study suggest that for <750 CD4 cells, there is still an increased risk of complications related to immunosuppression [95]. Indeed, the incidence of AIDS defining illnesses was higher in individuals with a current CD4 count of 500–749 cells/μl compared with those with a CD4 count of 750–999 cells/μl, but did not decrease further at higher CD4 counts. Results were similar in patients virologically suppressed on combination ART, suggesting that immune reconstitution is not complete until the CD4 increases to >750 cells/μl [95]. Thus, taking together these two recent studies, 4 months post-infection might be considered as the 'window of opportunity' to start ARV and achieve a non-risk level of immunity (similar to a non-infected person).

The 2nd, 3rd and 4th strategies have also failed to show a clear benefit and will not be described further in this review. Structured treatment interruptions of ARV [96] or ARV associated with cytokines such as pegylated interferon or IL-2, to decrease the viral reservoir and improve immune responses have not achieved its objectives [97–99]. In some cases of intermittent ARV therapy, viremia stayed relatively low after treatment interruption in some patients, but eventually ARV needed to be resumed [100]. Studies conducted with immunosuppressive drugs (hydroxyurea, cyclosporine, mycophenolic acid) have also failed to show clear efficacy and, sometimes, were shown to be more toxic [101–105]. Therapeutic vaccines are beyond the scope of this review.

HIV functional cure. Recent insights: the VISCONTI cohort & the Mississippi baby

It has been hypothesized that if ARVs are administered very early during PHI, it might help the immune system to control viral

Table 2. Guidelines recommendations on antiretrovirals during acute/recent HIV infection.

Guideline	Year	Country/region	Acute/early infection	Transmission prevention	ARV regimen	Ref.
WHO	2013	International	NR	Recommended	Two NRTIs + NNRTI/PI/r/ITI	[118]
EACS	2013	Europe	Consider	Consider	Two NRTIs + NNRTI/PI/r/ITI	[115]
IAS	2012	USA	Recommended	Recommended	Two NRTIs + NNRTI/PI/r/ITI	[114]
DHHS	2013	USA	Recommended	Recommended	Two NRTIs + NNRTI/PI/r/ITI	[113]
GESIDA	2013	Spain	Consider	Consider	Two NRTIs + NNRTI/PI/r/ITI[†]	[116]
BHIVA	2012	UK	Not recommended	Consider	Two NRTIs + NNRTI/PI/r/ITI	[117]

[†]A boosted PI may be chosen if a resistant test is not available. Raltegravir may be first choice.
ARV: Antiretroviral; BHIVA: The British HIV Association; DHHS: Department of Health and Human Services; EACS: The European AIDS Clinical Society; IAS: International Antiviral Society; ITI: Integrase transfer inhibitor; NR: No recommendation is made; NRTI: Nucleos(t)ide reverse transcriptase inhibitor; NNRTI: Non-nucleoside reverse-transcriptase inhibitor; PI/r: Ritonavir-boosted protease inhibitor.

replication more efficiently promoting a status similar to that of elite controllers. Some patients who received early ARVs during PHI and interrupted them thereafter showed good control of infection and have been called 'post-treatment controllers'. If infection is treated very early, it is difficult for the reservoir to become established, for genetic variability of the virus to develop and for depletion of CD4 lymphocytes to take place. These observations might explain the control of replication when ARVs are stopped [90]. Unfortunately, only around 10% of patients treated during PHI behave as 'post-treatment controllers'. In the remainder, viral replication resumes in much the same way as in PHI, thus eliminating any potential benefit gained with ARVs [90,106].

It is not completely understood why only a small proportion of patients achieve this immunological goal after interrupting ARVs or how to identify them; however, when symptoms appear in PHI (and the patient first consults a physician), replication is already uncontrolled and the reservoir and depletion of lymphoid tissue, in particular in GALT, have been established [90].

Control of viral replication after interruption of ARVs for around a year was recently reported in a newborn exposed to HIV-1 during delivery and treated very early after exposure (around 30 h post-infection) [107]. ARVs were initiated when VL was still not very high, suggesting that this patient was in a very early phase of PHI, when viremia was still increasing. No similar cases have been reported in adults, although in a recently published article, a French PHI cohort presented the clinical and immunological characteristics of 14 post-treatment controllers [106]. In all 14 cases, ARVs were initiated relatively late in PHI (most of them Fiebig stages III–V; Figure 1) compared with the case of the newborn reported by Persaud *et al.* In addition, patients were treated with compounds that target late phases of the HIV-1 replication cycle, and in some cases, drugs that have limited access to some organs and tissues where HIV-1 is known to actively replicate (e.g., the central nervous system) [106]. 'Post-treatment controllers' have a different immunological profile (no protective HLA alleles, poor specific CD8 T-cell responses, more severe PHI) to that of 'elite controllers', suggesting that the key role for control of viral replication is early treatment with ARV. In the VISCONTI cohort, it has been suggested that some of the post-treatment controllers can continue decreasing the viral reservoir [106]. It seems that the addition of more active compounds (e.g., CCR5 inhibitors or integrase inhibitors) to a three-drug standard regimen is not associated with a better outcome in terms of virological control or reservoir size when patients are treated in Fiebig Phase III or a more advanced stage [108,109], but it might have an impact if started earlier. Lower reservoirs can be achieved by treating earlier PHI [110] and in an ongoing study in Thailand, patients in whom treatment was started very early (Feibig Phase I) had extremely low reservoirs [111].

Whether the experience of functional cure of the Mississippi baby can be reproduced in adults is unknown. The immune system of newborns differs considerably to that of adults. The proportion of the different subsets of CD4 cells is substantially different. For example, central memory T cells are much lower in infants compared with adults and effector memory T cells are almost absent. Moreover, newborns have three-times the levels of Treg. These differences and the relatively hyporesponsive adaptive immune system of newborns may have a big impact on the way HIV reservoirs are established [112]. Thus, establishment of reservoir might be much more limited by ARVs in newborns than in adults.

What do national & international guidelines recommend?

Table 2 summarizes the recommendations on ARV initiation in PHI from six HIV expert organizations and societies around the world, all of them published in the last 2 years. A strong recommendation of early initiation of ARV in acute/early infection is made in only two sets of guidelines, in the Department of Health and Human Services guidelines [113] and in the International Antiviral Society USA panel [114], both from the USA. These two sets of guidelines recommend offering treatment to persons with acute infection regardless of symptoms or CD4 count. They also point out the importance that an early treatment may have on reducing HIV transmission during acute phase, as it has been discussed in previous sections. The European AIDS Clinical Society guidelines [115] in the 2013 edition recommended for the first time offering treatment to acutely infected persons regardless of CD4 count and symptoms.

The other three guidelines reviewed here are more heterogeneous, and have weaker recommendations. The Spanish guidelines from the Gesida/Plan Nacional sobre el SIDA (GESIDA) give a strong recommendation to early start in those patients with acute infection and severe symptoms (neurological involvement, prolonged symptoms, CD4 <350 cells/μl, not-R5 tropism or high VL), and recommend considering treatment if high risk of transmission is suspected, but no recommendation is made for those patients with early asymptomatic infection, except including them on clinical trials when possible [116]. The British HIV Association guidelines reach the same conclusions about symptomatic or immunologically advanced patients. For asymptomatic patients, they express doubts about the benefits of early start of treatment on acute infection, and underline the need of more quality evidence [117]. Likewise, the British HIV Association guidelines are more conservative on transmission prevention, adducing that there is no specific evidence supporting this recommendation [117]. Finally, WHO guidelines do not offer a specific recommendation for acute infected patients, although they recognize the importance of an early diagnosis and early evaluation. They do recommend treatment to all serodiscordant couples regardless of symptoms and CD4 count in order to reduce the risk of HIV transmission to the negative partner [118].

Regarding which ARV regimen to initiate in acute/recent infection, guidelines recommend the same regimens as in chronic infection, that is, a combination of 2 NRTIs and a third agent (a NNRTI, a ritonavir-boosted protease inhibitor or an integrase inhibitor) [113–118]. Despite the lack of evidence for one combination against the others, GESIDA guideline notes that a boosted protease inhibitor may be the option of choice in cases where a resistance test is not available, due to the possibility of NNRTI-resistant strains. Raltegravir may be considered as the third agent because of the high concentrations achieved in genital secretions and the greatest capacity to reduce VL in the first 4–8 weeks of treatment compared with protease inhibitors [116]. This factor can help in reducing transmission risk during acute infection and in controlling symptoms in very ill patients. With the exception of WHO guidelines, all guidelines strongly recommend performing a genotypic resistance test and subtyping before initiating treatment [113–118].

Finally, regarding duration of treatment, evidence of when treatment should be stopped is not available. All guidelines recommend to prolong treatment lifelong once initiated, due to the high risk of VL rebound when stopped [113–118].

Perspectives on antiretroviral treatment of acute HIV infection

The improved tolerability of current ARV, the impact on transmission and the recent insights of cases with functional cure open a scenario in which advantages start to outweigh disadvantages of ARV during PHI, even if treatment has to be continued for an unknown period of time (probably lifetime). As previously discussed, when GALT tissue is depleted and viral reservoir is established, no strategy to date has proved to be effective to reverse this depletion or to effectively purge that reservoir. If ARVs are administrated early enough, it might be feasible to avoid this GALT depletion and the establishment of the reservoir, in a similar way as in the recently published case of the baby with a functional cure [107]. However, this strategy of very early ARV cannot be applied in a large scale. Patients rarely seek medical consultation in the pre-symptomatic period and only molecular tests allow detection of HIV infection in these very early phases. A patient fulfilling these characteristics has been recently reported, after being identified as HIV-infected immediately after starting therapy for pre-exposure prophylaxis. This patient has negative reservoir on therapy, but still takes ARVs [119]. By treating these patients, it might be possible to determine the 'window-of-opportunity' in which a functional cure can be achieved, avoiding the very early events of infection (GALT depletion and the establishment of the reservoir). Indeed, patients treated with ARVs in Fiebig stages I–II seem to preserve the integrity of the mucosal barrier much better than those treated in Fiebig stage III, thus decreasing immune activation, a marker of disease progression [120]. Although the simian animal model can also offer insights in this direction, SIV do not exactly reproduce the Fiebig phases in humans, and so results cannot be completely extrapolated. It seems, however, to confirm the limitation of reservoir establishment when ARVs are given very early, but showing an extremely rapid kinetics even with only some days of delay in the introduction of ARVs [121].

Conclusion & recommendations

Treatment of PHI offers several benefits in terms of controlling signs and symptoms, decreasing the VL and transmission and decreasing variability and the size of the viral reservoir if it is started early enough. However, to achieve these goals, treating at the symptomatic phase seems to be too late. No ARV is preferred; however, ritonavir-boosted protease inhibitor regimens may avoid the selection of minority resistant variants that may drive a virological failure. Integrase inhibitors may be preferred in very symptomatic patients or when transmission is an issue, due to its capacity to reduce the VL faster than other families of ARVs. Treatment should probably be continued lifelong, this is the main argument against early initiation of treatment, in particular in asymptomatic patients. ARVs introduced before 4 months post-infection may allow a complete recovery of immunity (>750 CD4 cells/μl) in most of the treated individuals. Very early treatment (during the initial increasing phase of viremia) may result in spontaneous virologic control in absence of treatment, as suggested by recent studies and clinical cases. With the availability of simpler and easier regimens, ARV is prescribed more frequently to patients with PHI.

In summary, we can make the following recommendations:
When to start ARV:

- ARV should be strongly considered in EVERY patient with PHI;
- A resistance test and viral tropism should always be performed at diagnosis of PHI independently of starting ARV;
- ARV should be started immediately in patients with symptomatic PHI (meningoencephalitis, Guillain–Barré syndrome, hepatitis, myocarditis, thrombocytopenia, etc.) or is accompanied by B or C clinical events related to immunosuppression;

- ARV should be started in asymptomatic patients not recovering CD4 cells at 4 months, with a VL >100,000 copies/ml, or with a non-R5 tropic virus;
- ARV should be strongly considered independently of clinical parameters in cases where there is a high risk of transmission (e.g., in sexually active MSMs or sero-discordant couples);
- Initiation of ARV is also recommended in indications that are independent of CD4 T-cell count and that apply to chronic HIV infection and pregnant HIV-infected women.

What to start with:

- It is recommended to start ARV with the same preferred regimens used to treat chronic HIV infection. A regimen with two NRTIs (preferably tenofovir/emtricitabine) and an integrase inhibitor has the advantage of a higher concentration of ARV in genital secretions and may reduce the VL more quickly during the first 4–8 weeks when compared NNRTI with induced protein, also reducing faster the risk of transmission;
- If the resistance test is not available when prescribing the ARV regimen, it is preferable to start with a regimen based on a ritonavir-boosted protease inhibitor until the resistance pattern is known, since studies on TDR have shown prevalence ranging from 5 to 24%;
- If ARVs are started, duration should be indefinite.

Expert commentary

Treatment of HIV infection in early stages offers several clinical, virological and epidemiological advantages compared with treatment of chronic infection. However, these advantages must be considered in the context of a probable lifelong antiretroviral treatment.

Five-year view

Considering recently available information on reduced viral reservoir, better immunological outcomes and decrease of transmission risk, along with easier and better tolerated combinations of ARV, it is likely that, in the future, every patient with acute HIV infection will be treated.

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Key issues

- Primary HIV infection (PHI) covers the period of around 12 weeks post-HIV-1 infection. Acute infection is that of less than 1 month (pre-serologic period) and recent infection is that of less than 6 or 12 months.
- Depletion of lymphoid tissue and establishment of viral reservoir occurs early on in this period.
- Patients with PHI are a significant source of HIV transmission, due particularly to the high viral load, highlighting the need for earlier diagnosis. In around 10% of cases, transmission of resistance to antiretrovirals (ARV) can occur.
- Guidelines uniformly recommend treatment for symptomatic patients; recommendation remains inconclusive for asymptomatic patients.
- No ARV regimen for treatment of PHI (non-nucleoside reverse-transcriptase inhibitor-, ritonavir-boosted protease inhibitor- and integrase inhibitor-based regimen) is preferred, but an integrase inhibitor regimen can decrease the viral load and transmissibility more quickly. If resistance tests are unavailable, a ritonavir-boosted protease inhibitor may be preferred until results are available.
- Recent cases of patients treated very early with subsequent control of the viral replication after withdrawal of ARV open the possibility of a functional cure.

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Avances en el diagnóstico y tratamiento de la infección aguda por el VIH-1

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Según la Organización Mundial de la Salud (OMS) cada día se infectan en el mundo unas 14.000 personas. Sin embargo, en pocos casos el diagnóstico se realizará durante la fase aguda de la infección por el virus de la inmunodeficiencia humana (VIH). La infección aguda por el VIH es el período comprendido entre la entrada del VIH en el organismo y la seroconversión completa, definida por una prueba de *Western blot* positiva. Este período dura aproximadamente 30 días y la mayoría de veces (40-90%) se acompaña de manifestaciones clínicas banales (fiebre, exantema, faringitis, úlceras en mucosas entre otras), de 2 semanas de duración, que se pueden confundir con otros procesos infecciosos comunitarios, entre ellos la mononucleosis infecciosa. El diagnóstico microbiológico se realiza por la ausencia de anticuerpos en plasma (prueba de análisis de inmunoabsorción ligado a enzimas [ELISA] negativa) y la presencia de una carga viral (CV) del VIH en plasma positiva (> 10.000 copias/ml). El diagnóstico de la infección aguda por el VIH es muy importante por varias razones: *a)* epidemiológicas, es el período con las mayores tasas de transmisión de la infección por el VIH y permite conocer el patrón de crecimiento de la epidemia y la tasa de transmisión de cepas resistentes a los antirretrovirales, que en España es del 10%; *b)* inmunopatológico, ya que es una oportunidad única para estudiar los mecanismos virológicos, inmunológicos, y genéticos implicados en la transmisión y patogenia de esta enfermedad; y *c)* terapéutico, ya que el inicio del tratamiento antirretroviral en esta fase podría modificar la historia natural de esta infección. Sin embargo, este es un tema controvertido y en la actualidad la mayoría de comités de expertos sólo recomiendan el tratamiento si se pueden incluir los pacientes en ensayos clínicos o si las manifestaciones clínicas son graves o duraderas.

Palabras clave: **Infección aguda. VIH. Tratamiento antirretroviral.**

Advances in the diagnosis and treatment of acute human immunodeficiency virus type 1 (HIV-1) infection

According the WHO there are about 14,000 new HIV infections a day. However, in a few cases the diagnosis will be made in the acute phase of the disease. Acute HIV infection is the period between infection with the virus and complete seroconversion, defined by a positive Western blot test. This period lasts approximately 30 days and most patients (40-90%) have mild clinical manifestations (fever, rash, pharyngitis, mucosal ulcers, among others) for 2 weeks which, because they are nonspecific, can be confused with other community-acquired infections. Microbiological diagnosis is based on the absence of serum antibodies (negative ELISA test) together with a positive HIV viral load in plasma (> 10,000 copies/ml). Diagnosis of acute HIV infection is important for several reasons: firstly, from the epidemiological point of view, this is the period with the highest rates of HIV transmission and identification of new HIV infections reveals the growth of the epidemic and the transmission rates of resistant HIV strains, which in Spain is about 10%; secondly, from the immunopathological point of view, this period provides a unique opportunity to study the virological, immunological and genetic mechanisms that play a role in the transmission and pathogenesis of this disease; and thirdly, therapeutically, starting antiretroviral therapy during this phase could alter the natural history of the disease. However, this is a controversial issue and currently most guidelines recommend treatment only if these patients can be included in clinical trials or if they show lasting or severe clinical manifestations.

Key words: **Acute infection. HIV. Antiretroviral treatment.**

Introducción

La Organización Mundial de la Salud (OMS) estima que el número total de pacientes afectados por la epidemia de virus de la inmunodeficiencia humana (VIH)/sida en el mundo a finales del año 2003 ascienden a 45 millones de casos. En el último año se diagnosticaron 5 millones de nuevas infecciones y 3 millones de personas fallecieron por sida[1]. Esto significa que cada día se infectan aproximada-

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mente unas 14.000 personas. Si se tienen en cuenta que la mayoría de estas nuevas infecciones ocurren en países con pocos recursos económicos y que la OMS estima que sólo unas 35.000 nuevas infecciones ocurren al año en los países de la Unión Europea, se puede calcular que diariamente hay unos 10 nuevos casos de infección por VIH en España. Sin embargo, en pocos casos el diagnóstico se realizará durante la fase aguda de la infección por el VIH.

Se define como infección aguda por el VIH al período transcurrido desde el ingreso del virus en el organismo hasta la seroconversión completa, definida por una prueba de *Western blot* positiva. Este período tiene una duración aproximada de 30 días, en la mayoría de las veces se acompaña de manifestaciones clínicas y cursa con niveles muy elevados de viremia, instaurándose la respuesta inmunitaria celular específica de VIH transitoria con la consiguiente reducción de la viremia. Después de 4-6 meses y en función de una combinación de factores del huésped y del virus se alcanzará una fase de equilibrio (*set point* virológico) para cada individuo que originará el inicio de la fase crónica de la infección por el VIH. El período comprendido entre la infección aguda por el VIH y la infección crónica se denomina infección reciente por el VIH (entre los 30 y 180 días).

Por diversas razones que se comentarán más adelante, pocos pacientes son diagnosticados durante la infección aguda por el VIH. Sin embargo, el diagnóstico de la infección por VIH en esta fase es muy importante por motivos epidemiológicos, inmunopatológicos y terapéuticos.

1. Desde el punto de vista epidemiológico es importante por varias razones: *a)* representa el período de la infección por VIH con mayores tasas de transmisión de la infección[2]. Entre el 30 y el 50% de las nuevas infecciones están directamente relacionadas a una fuente con una infección aguda por el VIH[3,4]. Los valores muy altos de carga viral (CV), junto a la falta de uso de métodos de barrera adecuados por el desconocimiento del diagnóstico permite explicar un riesgo de transmisión que se ha calculado en 500 veces superior al de la fase crónica[5]; *b)* es útil para calcular el patrón de crecimiento de la infección por el VIH en cada área geográfica, lo que permite tomar medidas específicas para optimizar la distribución de los recursos destinados a prevención en los sectores más expuestos[6], y *c)* finalmente, permite conocer la tasa de transmisión de cepas resistentes a los antorretrovirales, lo que puede ser muy útil a nivel colectivo (formular recomendaciones de tratamiento antirretroviral [TAR]) e individual (administrar el TAR más adecuado al paciente)[7].

2. Desde el punto de vista inmunopatológico es una oportunidad única para estudiar los mecanismos inmunológicos, virológicos y genéticos implicados en la transmisión y patogenia de la infección por el VIH y en las interacciones iniciales entre el sistema inmunitario y el virus que determinarán la evolución posterior del individuo[8]. Esta información es muy importante para desarrollar nuevos enfoques preventivos o terapéuticos más efectivos.

3. Desde el punto de vista terapéutico, ya que el diagnóstico precoz de la infección aguda por el VIH puede permitir instaurar un TAR adecuado que, por un lado, puede acortar la duración de la enfermedad sintomática en los casos graves, generalmente asociada a una viremia muy elevada[9] y, por otro, puede evitar el daño del sistema inmunitario o lograr la reconstitución de éste y cambiar la historia natural de esta infección[10].

Inmunopatología

En este apartado se resumen los cambios que acontecen en el sistema inmunitario y el virus en las primeras semanas de la infección por el VIH y que se revisan con detalle en varias publicaciones recientes[11-13].

Transmisión del VIH y fases iniciales de la infección (fig. 1)

En todo el mundo, la vía más común de transmisión del VIH es la sexual. La probabilidad de transmisión en un único encuentro sexual es muy baja (0,001%) y está relacionada proporcionalmente con el valor de CV en plasma, y resulta infrecuente con valores de CV < 1.500 copias/ml[11]. Otros factores implicados en el riesgo de transmisión son la ausencia de circuncisión, la presencia de inflamación del epitelio vaginal (enfermedades de transmisión sexual [ETS], uso de nonoxinol-9, vaginosis bacteriana) y la ectopia cervical[14-16].

El virus que se transmite en la infección aguda por el VIH por lo general se corresponde a las poblaciones macrofagotrópicas. Estas cepas requieren para la infección de los linfocitos CD4 la presencia del correceptor CCR5, por lo que se denominan cepas R5 o también cepas no productoras de sincitio. En contraposición, las cepas que aparecen en estadios avanzados de la infección requieren la expresión del cofactor CXCR4, por lo que se denominan cepas X4, linfotrópicas o productoras de sincitio[17]. El virus atraviesa el epitelio dañado e infecta a las células de Langerhans y a las células dendríticas que se encuentran en el epitelio estratificado de la mucosa y submucosa vaginal, respectivamente. Estas células son presentadoras de antígeno y expresan en su superficie DC-SIGN, una lectina a la que se adhiere el virus y que es responsable de que el VIH sea transportado al tejido linfático por la migración de las células presentadoras de antígeno. Por otra parte, estas células y los linfocitos CD4 expresan CCR5, pero no CXCR4, ya que la elevada presencia de la quimiocina SDF-1, ligando natural del CXCR4, en las mucosas y el tejido linfático hace que este correceptor esté regulado de forma negativa y no se exprese, lo que explica la elevada susceptibilidad a la infección por cepas R5 en la infección aguda por el VIH. Experimentos realizados en animales demuestran que la infección de estas células ya es evidente a las 24-72 h de la infección[18]. En las 24-48 h siguientes las células dendríticas infectadas migran a los ganglios linfáticos regionales donde, en los centros germinales, activan e infectan gran cantidad de linfocitos CD4, amplificando en forma explosiva la infección y producción viral. La CV se expande de forma exponencial, se duplica cada 0,3 días durante las primeras 2-3 semanas de la infección, y se alcanza el pico más elevado en plasma, secreciones genitales y otros compartimentos a las 4 semanas de la infección[13]. Por lo tanto, en menos de una semana el virus se disemina por vía hematógena a todo el organismo (ganglios linfáticos, sistema nervioso central, sistema digestivo, gónadas) de forma que cuando el paciente presenta síntomas, el virus ya está en todos los órganos y el reservorio viral ya se ha establecido[19]. En esos momentos es

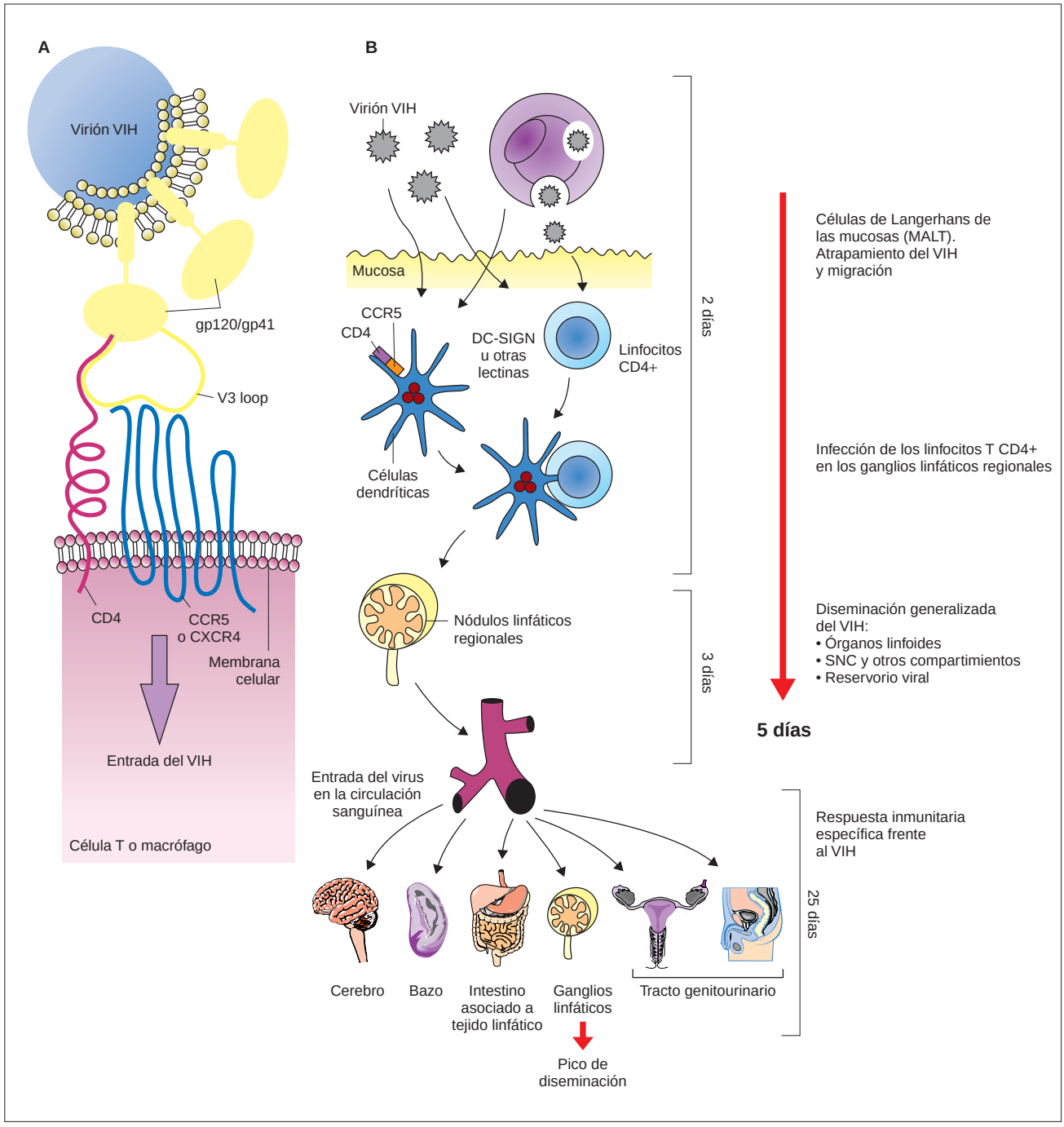

Figura 1. Transmisión de la infección por VIH y establecimiento de los reservorios. A) Interacciones entre las glucoproteínas de envoltura del VIH y la molécula CD4 y los correceptores CCR5 o CXCR4, fusión y entrada del VIH. B) Secuencia inicial de la infección por el VIH adquirida por vía sexual hasta la diseminación viral. (Tomada con autorización de Pilcher et al[13].)

cuando se está iniciando la respuesta inmunitaria adaptativa o celular que tratará de contener la infección. El reservorio viral es el responsable de que este virus no se pueda erradicar.

Dinámica viral y celular

Los linfocitos CD4 activados son la principal diana del VIH, a la vez que constituyen su principal fuente de producción. Cada día se generan entre 10^9 y 10^{10} partículas virales con una vida media de 6 h, que son las responsables de continuar el ciclo de infección de nuevos linfocitos. El balance entre la producción y muerte de estas células tiene como resultado el recuento de CD4 en estos pacientes[20]. Cada célula infectada produce 20 células hijas durante su ciclo vital, que es inferior a 24 h, por lo que cada día mueren entre 10 y 100 millones de linfocitos CD4. Este estado de inmunoactivación y recambio celular es muy importante en la infección aguda por el VIH[21].

La disminución de los linfocitos CD4 se produce por diversos mecanismos, entre los que se incluyen un efecto citopático directo, la apoptosis inducida por proteínas virales (gp120, gp41), la lisis mediada por mecanismos inmunitarios por respuesta celular específica (respuesta CTL) o inespecífica (células asesinas naturales [NK]), por anticuerpos, por el bloqueo de su producción tímica o por su redistribución con un "atrapamiento" linfático. La pérdida de la capacidad del organismo de mantener la cifra de linfocitos CD4 dentro de los valores normales es la responsable de la inmunosupresión celular que permite el desarrollo de infecciones oportunistas y tumores y la manifestación del sida[22].

Respuesta inmunitaria

Respuesta humoral: anticuerpos

Los anticuerpos se detectan en la sangre a las 4 semanas de la infección. Sin embargo, la mayoría de estos anticuerpos tienen escasa capacidad neutralizante y sólo son útiles para el diagnóstico serológico. Los anticuerpos neutralizantes suelen detectarse a partir de las 8 semanas de la infección, una vez que ya ha pasado el pico de viremia[13], y aunque inicialmente ejercen una importante presión inmunológica frente al VIH, ésta no es duradera dada la aparición de escape virológico y la diversidad viral. Estudios en monos infectados por el virus de la inmunodeficiencia del simio (SIV) a los que se les depleccionó de linfocitos B (productores de células plasmáticas y anticuerpos) demostraron que el descenso del pico inicial de CV es previo a la aparición de anticuerpos neutralizantes[23] y la infusión de gammaglobulina hiperinmune anti-VIH en pacientes infectados no ha demostrado tener un efecto beneficioso sostenido en la progresión de la enfermedad[24]. Sin embargo, en los últimos años se ha demostrado que la administración de anticuerpos monoclonales neutralizantes contra el SIV, administrados a macacos, es capaz de evitar la infección y de disminuir la progresión de aquellos animales que se infectan tras la inoculación[25,26], lo que ha abierto nuevamente el debate y la investigación en esta área.

Respuesta celular

La respuesta celular frente al VIH está formada por la respuesta específica colaboradora por linfocitos T CD4 (necesaria tanto para una adecuada respuesta humoral como citotóxica específica), linfocitos T CD8 citotóxicos (células CTL) y NK. Este tipo de respuesta es más precoz e importante que la respuesta humoral en el control de la replicación viral, y su aparición se ha correlacionado con la disminución del pico inicial de CV durante la infección aguda por el VIH[27]. La respuesta citotóxica (CTL) ha sido la más extensamente estudiada y se ha relacionado desde hace tiempo en el control de la replicación viral. Esta afirmación surgió inicialmente de trabajos *in vitro* donde se demostró que los linfocitos CD8 específicos anti-VIH producen factores solubles que inhiben la replicación viral (MIP-1α, MIP-1β, RANTES, CAF) y lisan (granzimas, perforinas) las células infectadas[28-30]. Estudios con monos infectados con SIV demostraron que la depleción de CD8 impide a los animales controlar el pico inicial de viremia, acelerando la progresión a sida[31] y que la estimulación de estas respuestas por vacunas logra disminuir la CV y retrasar la enfermedad[32]. Niveles altos de este tipo de respuesta se han observado en los pacientes que no progresan a largo plazo (NPLP)[33]. El complejo principal de histocompatibilidad (MHC) codifica las moléculas de los antígenos de histocompatibilidad (HLA) que se expresan en las células presentadoras de antígeno y que se unen a fragmentos de proteínas virales para presentarlas a las células efectoras a través de los cañones HLA-1 (CTL entre otras células) y HLA-2 (sólo los linfocitos CD4) con el fin de iniciar la respuesta inmunitaria. Se han identificado determinados alelos específicos que codifican moléculas HLA tipo 1 y que son predictores de un mejor control del virus (HLA-A2, HLA-B27, HLA-B57)[34], mientras que otros se han asociado con un peor pronóstico (HLA-B35Px)[35]. Hasta el 85% de los pacientes no progresores a largo plazo (pacientes que mantienen viremias bajas y estabilidad clínica durante varios años) o NPLP presentan el HLA-B57[36]. Sin embargo, durante la evolución de la infección la mayoría de los pacientes pierden el control de la replicación viral debido al escape inmune que la presión selectiva del sistema inmunitario provoca en el virus en fase de replicación, lo que le permite seleccionar mutaciones que le garanticen una mejor supervivencia. Se ha documentado escape viral, tanto con la respuesta humoral (evasión viral a los anticuerpos neutralizantes) como con la respuesta celular (escape al daño citotóxico de células infectadas)[37,38].

La respuesta proliferativa específica mediada por los linfocitos CD4 es básica para organizar toda la respuesta específica de patógeno en el organismo. La respuesta proliferativa es crucial para mantener la respuesta citotóxica (CTL) adecuada[13]. Sin embargo, estas células son la diana de la infección por el VIH y durante la infección aguda por el VIH se origina una activación generalizada de éstas, incluyendo los linfocitos CD4 específicos frente al VIH, con su posterior destrucción.

Reservorio

La latencia celular (provirus integrado sin una replicación activa) es una característica de todos los retrovirus. En el VIH, esta latencia se puede establecer en varios estadios, tanto antes como después de la integración en la célula infectada. Inicialmente se consideró a las células CD4 en reposo infectadas como el principal reservorio. Estas células, en pacientes en TAR y con la replicación suprimida, se estiman en 1-100 millones de células (1% de los linfocitos CD4) que pueden ser activadas dando lugar a la producción de virus que conservan su capacidad infectiva como se ha demostrado en estudios *in vitro*[39].

Los linfocitos CD4 junto con los macrófagos y células de otros tejidos forman el reservorio viral, que es el principal obstáculo para la erradicación del virus con TAR[40].

Manifestaciones clínicas

En 1985, Cooper et al[41] publicaron la primera descripción de los síntomas atribuibles a la infección aguda por el VIH en 12 pacientes homosexuales que presentaron un cuadro compatible con una "mononucleosis infecciosa" (fiebre, faringitis y exantema) con serología negativa para el virus Epstein-Barr (VEB) y en los que se confirmó la infección por VIH. Desde entonces este síndrome se conoce como "síndrome retroviral agudo", "síndrome de serocon-

versión aguda", "primoinfección por el VIH" o "infección aguda por el VIH".

El período de incubación de la infección por el VIH varía entre una y 3 semanas (típicamente 14 días) y la duración del período sintomático es de 7-10 días, raramente más de 2 semanas. En diferentes estudios, la prevalencia de síntomas durante la infección aguda por el VIH varía entre el 40 y el 90%. En estudios prospectivos de pacientes expuestos con grupo control (p. ej., pacientes atendidos en centros de atención de enfermedades de transmisión sexual), la presencia de síntomas analizados en forma individual en el grupo de pacientes infectados fluctúa entre el 53 y el 88%, pero hasta el 50% de los pacientes sin infección refieren síntomas similares, lo que demuestra su inespecificidad. Algunos estudios han observado que los síntomas de la infección aguda por el VIH son menos frecuentes en los adictos a drogas por vía parenteral (ADVP).

El espectro clínico de la infección aguda por el VIH varía desde cuadros banales, que se atribuyen a una virosis inespecífica, hasta cuadros graves con afectación neurológica. Los síntomas más frecuentes incluyen fiebre, exantema, úlceras orales y/o genitales, linfoadenopatías, astenia marcada, artromialgias y meningitis aséptica (tabla 1)[12]. Cuando se valora la gravedad de los síntomas, menos de la mitad de los pacientes consideran el cuadro suficientemente importante como para consultar a un médico. Algunos pacientes requieren ingreso hospitalario para su valoración y, en general, consultan por síntomas neurológicos o un síndrome febril prolongado.

La fiebre es el síntoma más frecuente (80-87% de los casos) y el primero en aparecer en todas las series. La duración es variable y oscila entre 3 días y 3 semanas. La temperatura suele ser muy elevada al inicio, desciende lentamente, y la febrícula puede persistir varios días. Por lo general se asocia a sudoración nocturna y astenia importante, que obliga a un reposo prolongado[42].

El exantema suele aparecer en las 24-48 h posteriores al inicio de la fiebre, con una frecuencia que oscila entre el 21 y el 57% de los casos. Puede ser generalizado o afectar sólo a la cara y el tronco (fig. 2), tiene características de máculas papulosas o eritematosas y en ocasiones se presenta como urticaria, descamación de palmas y plantas o alopecia. Se describe con mucha menor frecuencia en razas no caucasianas[43].

La afectación faríngea también se presenta con mayor frecuencia en las series de pacientes de raza blanca, en los que aparece hasta en el 60% de casos. Las lesiones más frecuentes son el dolor inespecífico, el edema, el enantema o las ulceraciones. Es poco frecuente el hallazgo de exudado[44].

Con menos frecuencia se han comunicado linfoadenopatías cervicales, axilares, occipitales o generalizadas, que en algunos casos pueden persistir durante varios meses, incluso años después de la seroconversión.

Las manifestaciones gastrointestinales son en su mayoría diarrea, náuseas o vómitos. También se ha comunicado dolor abdominal y casos de pancreatitis[45].

Las manifestaciones neurológicas de la infección aguda por el VIH incluyen la meningitis viral, la meningoencefalitis, la mielitis, la neuropatía periférica y el síndrome de Guillain-Barré. Globalmente estos cuadros representan entre el 8 y el 12% de los pacientes y son la causa más frecuente de ingreso hospitalario. Se han documentado casos de depresión, parálisis facial, neuritis óptica, trastornos cognitivos o psicosis aguda.

TABLA 1. **Síntomas de la infección aguda por el VIH**

Síntoma	Frecuencia (%)
Fiebre	53 a 87,5
Exantema	9 a 57,5
Úlceras orales	7,5 a 37
Artromialgias	24 a 54
Faringitis	15 a 44
Pérdida de peso	32
Astenia	68 a 72,5
Cefalea	54 a 55
Sudoración nocturna	50 a 51
Adenopatías	7 a 37,5

VIH: virus de la inmunodeficiencia humana.

Además, algunos pacientes pueden presentar de forma simultánea infecciones relacionadas con su conducta de riesgo e incluso infecciones de las categorías B o C del Center for Disease Control and Prevention (CDC)[12]. En este sentido, hay que descartar en los pacientes que adquirieron la infección por vía sexual otras enfermedades de transmisión sexual (p. ej., sífilis) o en los ADVP las infec-

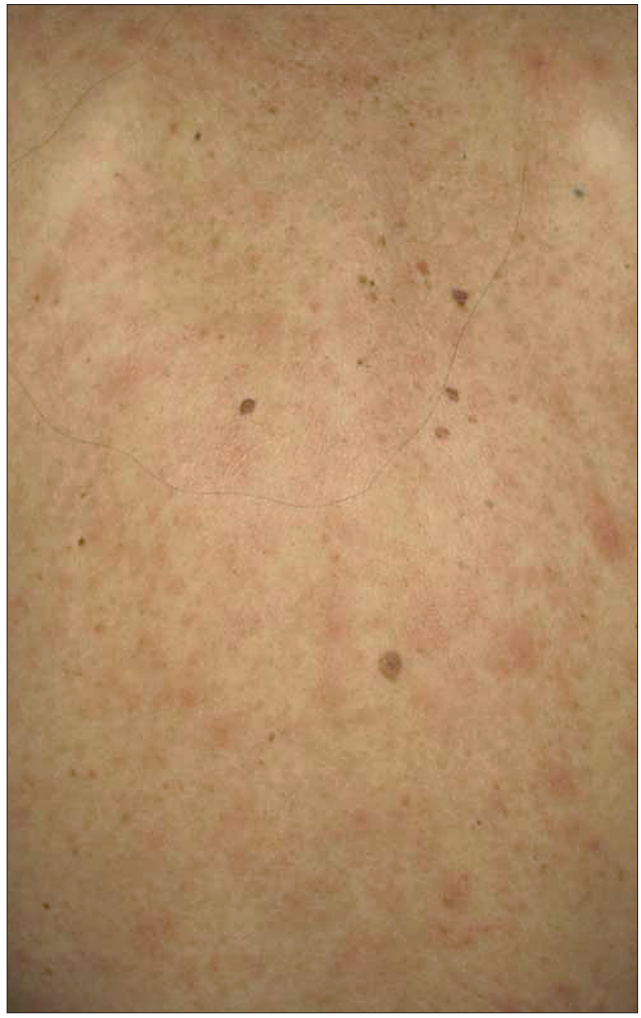

Figura 2. Exantema en la región dorsal del tronco de un paciente con infección aguda por el VIH.

ciones relacionadas con esta práctica (p. ej., endocarditis infecciosa). Con respecto a las infecciones relacionadas con el VIH, en general se trata de episodios de herpes simple o zóster, candidiasis oral o esofágica[46,47] pero también se han descrito casos de tuberculosis, de toxoplasmosis cerebral[48], de neumonía por *Pneumocystis jiroveci*[49] (antes *P. carinii*) o criptosporidiosis[50]. La mayoría de estos casos se observaron en pacientes con infección aguda por el VIH que tuvieron una disminución importante de los linfocitos CD4[51].

Especificidad de los síntomas

Es muy importante conocer que síntomas son los mejores predictores de una infección aguda por el VIH en los pacientes expuestos con el fin de sospechar rápidamente esta infección. En un estudio prospectivo se evaluaron los síntomas que presentaron 258 estudiantes universitarios que consultaron tras una exposición sexual de riesgo, 40 de ellos presentaron una infección aguda por el VIH confirmada. Analizados en forma individual, los síntomas más sensibles fueron la fiebre (80%) y la astenia (68%) mientras que los más específicos fueron la pérdida de peso de 2,5 kg (86%) y la presencia de úlceras orales (85%), pero la sensibilidad de estos dos últimos fue menor al 40%. La asociación de síntomas que fue más específica fue la combinación de "fiebre con exantema" (especificidad, 91%). Sin embargo, fue poco sensible (sensibilidad, 46%)[52]. En otro estudio realizado en mujeres de Kenia que consultaban por infecciones de transmisión sexual, la presencia de dos o más síntomas o signos (el estudio consideraba fiebre, vómitos, diarrea, astenia, linfadenopatías inguinales y *Candida* vaginal) se correlacionó con la infección aguda por el VIH con valores similares de sensibilidad (51%) y especificidad (83%)[53].

La combinación de síntomas muy frecuentes pero poco específicos y la escasa sensibilidad de los que se relacionan con la infección aguda por el VIH hace que no pueda formularse una definición de caso con un valor predictivo positivo alto y obliga a mantener un alto nivel de sospecha, especialmente en pacientes seronegativos con una potencial exposición reciente al VIH.

Relación entre síntomas y pronóstico de la enfermedad

Se ha sugerido que la presencia de una infección aguda por el VIH sintomática, un período de incubación corto antes del inicio de los síntomas o una duración larga de la fase sintomática (mayor de 15 días) se correlaciona con una progresión a sida más rápida de los pacientes[54,55]. La presencia de síntomas está fuertemente ligada al valor de la CV en plasma. En un trabajo se pudo comprobar que por cada síntoma que estaba presente (considerando fiebre, vómitos, cefalea, artralgia, mialgias, odinofagia, astenia, exantema o astenia) había un aumento de 0,4 $\log_{10}$ de CV en plasma.

Por otra parte, un estudio en pacientes no tratados demostró que la reducción inicial de la viremia y el *set point* resultante fueron factores independientes de progresión a sida[56]. Así, la progresión a sida fue significativamente más rápida en los pacientes que tuvieron un aclaramiento inicial lento (< 0,63 $\log_{10}$/ml o < 4.260 copias/ml por mes) o en los que el nivel de viremia fue más elevado (> 4,4 $\log_{10}$/ml o > 25.000 copias/ml). Este estudio demuestra que la respuesta inmunitaria inicial tiene un papel importante en la historia natural de la infección por VIH y puede permitir identificar potenciales pacientes para recibir TAR.

El nivel de viremia plasmática depende tanto de factores virológicos (capacidad replicativa del virus) como del individuo (capacidad del sistema inmunitario del individuo de controlar la viremia sin tratamiento). La inmunidad celular de cada individuo está determinada por la posibilidad de responder a cada antígeno a través del MHC que codifica las moléculas HLA-1 y algunos alelos específicos HLA se han relacionado con mejor o peor pronóstico tal como se ha explicado previamente. También durante un episodio de infección aguda por el VIH se ha podido demostrar que un alelo determinado (HLA-B57) se correlaciona con una menor CV, menor frecuencia de síntomas y un mejor pronóstico a largo plazo[57].

Alteraciones en las pruebas de laboratorio

Las anomalías más comunes de laboratorio incluyen alteraciones hematológicas y de las pruebas de función hepática[12]. Hasta el 90% de los pacientes presenta alguna alteración de las pruebas hematológicas[58]. La trombocitopenia es característica y se observa hasta en el 45% de los pacientes y se ha asociado a la presencia de anticuerpos antiplaquetas[59]. La linfopenia es común y en ocasiones se detecta la presencia de linfocitos activados o de monocitosis. Raramente se observa linfocitosis o neutrofilia[60]. El 20% de los pacientes presentan alteración de las pruebas de función hepática, en particular un aumento de las transaminasas y de la fosfatasa alcalina. En estos casos debe descartarse una coinfección simultánea de virus hepatotropos[61]. Algunos pacientes con síntomas musculares pueden presentar aumento de las enzimas creatincinasa (CK) y lacticodeshidrogenasa (LDH)[62].

Desde el inicio de los síntomas de la infección aguda por el VIH se observa un descenso de los linfocitos CD4, que primero se debe a una redistribución hacia el compartimento linfático y posteriormente es consecuencia de su destrucción por apoptosis. De forma paralela se observa un aumento de los linfocitos CD8, a expensas del fenotipo memoria, con una inversión del cociente CD4/CD8[63]. Este proceso se acompaña de una activación del sistema inmunitario con un aumento de la fracción CD8/CD38[64]. Otros indicadores de activación del sistema inmunitario son el aumento de la β_2-microblobulina, la neopterina y los receptores solubles de interleucina 2 (IL-2), que se acompañan de un aumento del interferón gamma y de la IL-1B y de un descenso de IL-2[65].

Diagnóstico diferencial

El diagnóstico diferencial de la infección aguda por el VIH es amplio y en la mayoría de casos deben descartarse otros procesos infecciosos[12]. El cuadro clínico de fiebre asociado a faringitis y adenopatías (clásico de la mononucleosis infecciosa) se observa sólo en el 15% de los pacientes con infección aguda por el VIH, por lo que la sospecha no debe limitarse a esta forma de presentación[66]. En estos pacientes, sin embargo, la frecuencia de pruebas falsamente positivas para VEB es excepcional, lo que hace relativamente fácil el diagnóstico diferencial entre ambas[67]. En el estudio efectuado en Estados Unidos en pacientes en los que se sospechó una mononucleosis infecciosa por el VEB y las pruebas son negativas, la prevalencia de infección aguda por el VIH fue de alrededor del 1%[13].

Otras infecciones que deben incluirse en el diagnóstico diferencial son las ETS como la sífilis (en etapa de secun-

darismo) o la gonococia, las infecciones por citomegalovirus o los virus de la gripe, herpesvirus tipo 6, rubéola o parvovirus B19, la toxoplasmosis, las hepatitis virales en la fase anictérica, la faringitis estreptocócica, las borreliosis y algunas rickettsiosis. En ocasiones, una toxicodermia en el contexto del tratamiento antibiótico de otro proceso puede simular una infección aguda por el VIH. Ante estos casos el médico siempre debería preguntar al paciente si ha tenido algún episodio de exposición sexual en el último mes, sobre todo con parejas ocasionales y sin protección. En África, donde la prevalencia de la infección por VIH es muy elevada, en estudios en pacientes con ETS, la prevalencia de infección aguda por el VIH osciló entre 1,2 y 2,5%[13].

Diagnóstico de laboratorio

La clave del diagnóstico del episodio de infección aguda por el VIH se basa en la sospecha clínica y en realizar las pruebas de laboratorio en el momento adecuado, ya que la sensibilidad de cada método de diagnóstico varía en función del tiempo transcurrido desde el inicio de la infección. Como se ha comentado previamente, la mayoría de los pacientes no se diagnostican durante la fase aguda de la infección por varias razones: *a)* ya que desarrollan una infección aguda por el VIH de forma asintomática (10-20%); *b)* tienen síntomas pero no consultan (20-40%), o *c)* consultan por síntomas, pero el médico no piensa en la infección aguda por el VIH como causa de la sintomatología, o si lo hace no realiza las pruebas adecuadas (aproximadamente el 40% de los casos).

Algunos pacientes presentan una mayor exposición a situaciones de riesgo de transmisión, como el caso de personas con múltiples contactos ocasionales, prostitutas y ADVP que consumen en forma activa y comparten material de inyección. A estos individuos se les debería ofrecer la posibilidad de realizar un estudio serológico en forma periódica cada 6 meses con el fin de detectar episodios de seroconversión, además de interrogar sobre posibles cuadros clínicos compatibles con infección aguda por el VIH entre la última prueba negativa para sospechar posibles contagios recientes.

Debido a que las pruebas serológicas de diagnóstico habitual de infección por VIH se basan en la detección de anticuerpos, y como estas pruebas suelen ser negativas en los momentos iniciales de la infección (período ventana) la detección de una infección aguda requiere la demostración del virus en sangre, lo que puede hacerse con una prueba cuantitativa de la CV (ARN-VIH) o con la detección del antígeno del virus en sangre (antigenemia p24). Además se debe demostrar un perfil evolutivo de generación de anticuerpos, por la detección de anticuerpos frente al VIH que inicialmente eran negativos medidos con el análisis de inmunoabsorción ligado a enzimas (ELISA) (enzimoinmunoanálisis) o por la posterior positivización de una prueba de *Western blot* inicialmente negativa o indeterminada (tabla 2) (fig. 3).

Métodos para la detección del virus

Carga viral

Actualmente se considera la prueba de elección para la detección de un episodio de infección aguda por el VIH ya que su sensibilidad es superior al 99% a partir de la primera semana de exposición, y llega a ser mayor de 6 $\log_{10}$/ml a los 20 días de la infección. Es positiva desde los 7-10 días de la infección por el VIH, unos 3-5 días antes de que se detecte la antigenemia p24 y hasta 2-3 semanas antes que el EIA. Las desventajas son la posibilidad de falsos positivos con valores inferiores a 10.000 copias/µl[68]; el coste económico, que es tres a cinco veces mayor que el de la antigenemia p24; y que requieren un EIA negativo o un *Western blot* negativo o indeterminado en forma simultánea para descartar los casos de infección crónica.

Antígeno p24

Se basa en la detección del antígeno viral en sangre también por métodos de ELISA (EIA), se observa algunos días antes del inicio de los síntomas y desaparece con el aumento del nivel de anticuerpos en suero[69]. Tiene una especificidad comparable a la CV pero una sensibilidad menor (79%), por lo que no aporta datos adicionales a esta prueba. También puede positivizarse en fases avanzadas por lo que requiere la confirmación de un patrón evolutivo de anticuerpos medido por EIA o *Western blot*. Podría ser útil en lugares donde por problemas económicos no pudiera implementarse la detección rutinaria de CV.

Detección de anticuerpos

Técnicas de ELISA

La detección de anticuerpos con EIA de tercera generación detecta anticuerpos contra los virus VIH-1 y VIH-2. En el momento de los síntomas son negativos, pero se detectan relativamente pronto (a las 4-6 semanas de la infección). Al tiempo en el que el resultado es negativo se denomina "período de ventana" y se ha ido acortando con las nuevas generaciones de EIA, desde los 3 meses en las iniciales hasta un mes con las actuales[70]. Los EIA de cuarta generación se diseñaron para disminuir más esta ventana y combinan la detección de anticuerpos con la del antígeno. Aunque algunas pruebas logran detectar la infección 4-6 días antes con respecto a los EIA de tercera (Vidas VIH DUO ultra, Cobas Core HIV Combi) los resultados son muy variables entre las diferentes presentaciones comerciales. Se han comunicado casos de infección aguda por el VIH con reactividad para EIA de tercera generación, pero negativas con el EIA de cuarta (Virinostika HIV Uni-Form IIAg/Ab). El problema depende de los antígenos utilizados

TABLA 2. **Sensibilidad y especificidad de los métodos diagnósticos de la infección aguda por el VIH**

Método	Sensibilidad (IC 95%)	Especificidad (IC 95%)
Antígeno p24 (Abbott)	70 (60-92)	99 (97 a 100)
Carga viral-Branched ADN (Bayer)	100 (88-100)	95 (91-98)
Carga viral VIH método PCR (Roche)	100 (85-100)	97 (90-100)
Carga viral VIH método TMA (Gen-Probe)	100 (82-100)	98 (95-100)
EIA Combi-test (Abbott)	79 (60-92)	97 (93-99)

VIH: virus de la inmunodeficiencia humana; IC 95%: intervalo de confianza del 95%; PCR: reacción en cadena de la polimerasa; TMA: amplificación mediada por transcripción; EIA: enzimoinmunoanálisis.

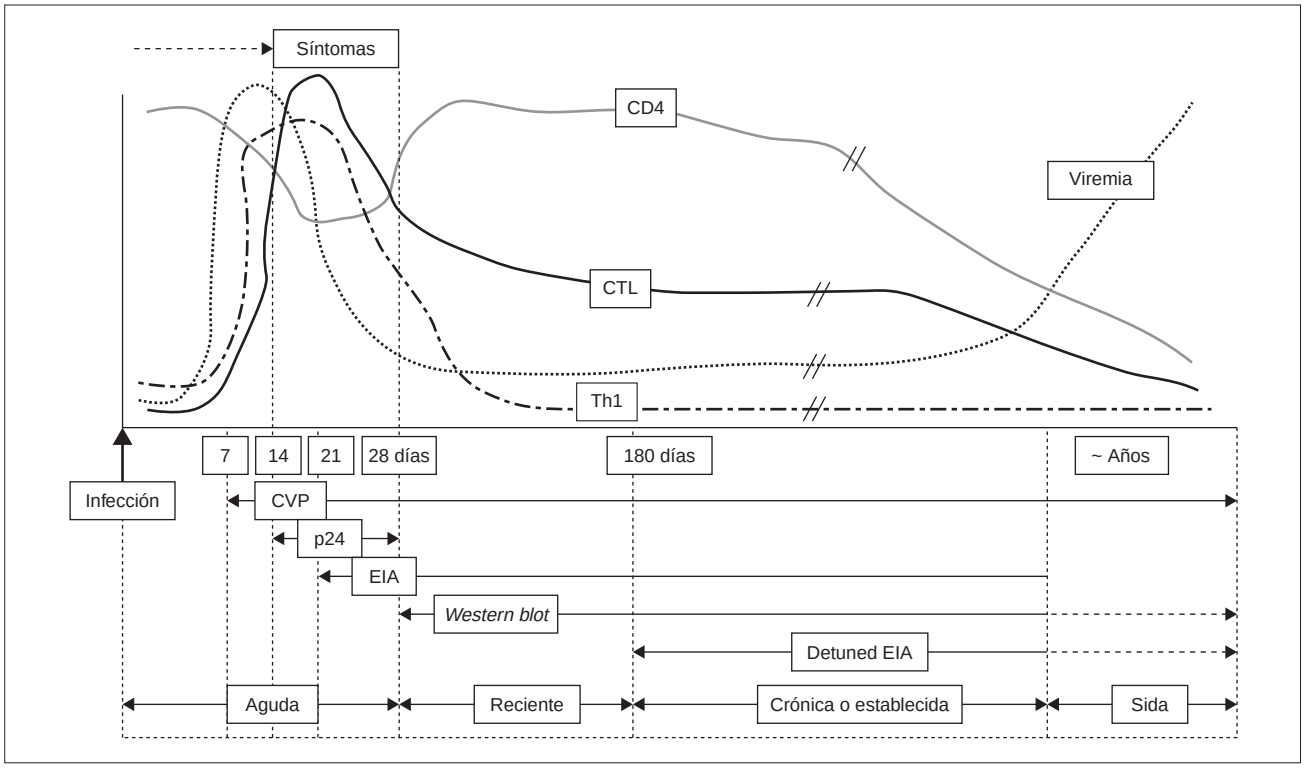

Figura 3. Esquema que muestra la correlación entre la evolución clínica, inmunológica y virológica en la infección aguda, la infección reciente y la infección crónica por el VIH y los marcadores serológicos de la infección por el VIH. EIA: enzimoinmunoanálisis; CVP: carga viral plasmática; p24: antigenemia p24; Detuned EIA: ELISA dual o STARHS (véase texto); CTL: linfocitos T CD8+ citotóxicos; Th1: respuesta proliferativa linfocitos T CD4+.

y del umbral de detección del antígeno p24, por lo que algunos autores recomiendan continuar las pruebas para detectar antígeno o anticuerpos en forma separada[71-73].

Western blot (fig. 4)

El *Western blot* detecta anticuerpos contra proteínas específicas del VIH-1 y VIH-2 y de acuerdo con el CDC para ser considerados positivos requieren la presencia de al menos dos de cuatro bandas (incluyen gp160, gp120, gp41, gp24). Los resultados con una sola banda con o sin anticuerpos contra bandas adicionales se consideran indeterminados y requieren para ser positivos un estudio posterior con el mismo criterio. Es el método de confirmación estándar de la serología de VIH y durante la fase aguda suele ser negativo o indeterminado. A partir de las 4-8 semanas de la infección es positivo y durante las semanas posteriores se añadirán bandas adicionales. La banda gp31 suele aparecer a las 8-12 semanas y su ausencia hace sospechar una infección reciente[74].

Nuevas estrategias de diagnóstico serológico

Debido al estrecho margen del período de ventana, se han diseñado técnicas que permiten detectar en forma retrospectiva una infección reciente. La prueba EIA L/S, también conocida como Detuned, ELISA dual o STARSHS (de su sigla en inglés; *Serologic Testing Algorithm for Recent HIV Seroconversion*) consiste en la realización simultánea de dos EIA (uno de tercera generación que tiene una mayor sensibilidad y otro de primera generación). Las muestras con resultados positivos para ambos EIA se interpretan como reactivas mientras que las positivas para el EIA de tercera generación y negativas para el EIA de primera se interpretan como no reactivas y sugieren que la infección se ha adquirido recientemente (menos de 180 días). Esta estrategia tiene un valor muy importante a nivel poblacional, donde el conocimiento de la prevalencia de la infección aguda en diferentes grupos de individuos (embarazadas, homosexuales, ADVP, inmigrantes, etc.) permite conocer las características del crecimiento de la epidemia. Esta técnica ha reclasificado hasta el 17% de los casos positivos de una campaña de detección serológica, y ha demostrado el impacto que tiene la infección aguda en la transmisión[75-78].

En la figura 5 se puede ver el algoritmo diagnóstico de la infección aguda, reciente o crónica del VIH en función de la determinación de la CV en plasma y el ELISA dual. Otros autores han propuesto clasificar la infección aguda por el VIH en distintos estadios según la combinación de la CV del VIH, la antigenemia p24, el resultado del ELISA y del *Western blot* (negativo o indeterminado o positivo con o sin banda 31)[79]. Esta clasificación tiene importancia fisiopatológica y permite estratificar muy bien a los pacientes con fines terapéuticos, pero tiene poca utilidad clínica.

Transmisión de cepas de VIH resistentes

La prevalencia de la transmisión de resistencias a los fármacos antirretrovirales en una población determinada puede variar en función del tipo de prueba empleada (genotipo o fenotipo), de los valores de corte de los estudios fenotípicos, de las mutaciones utilizadas como indicadoras

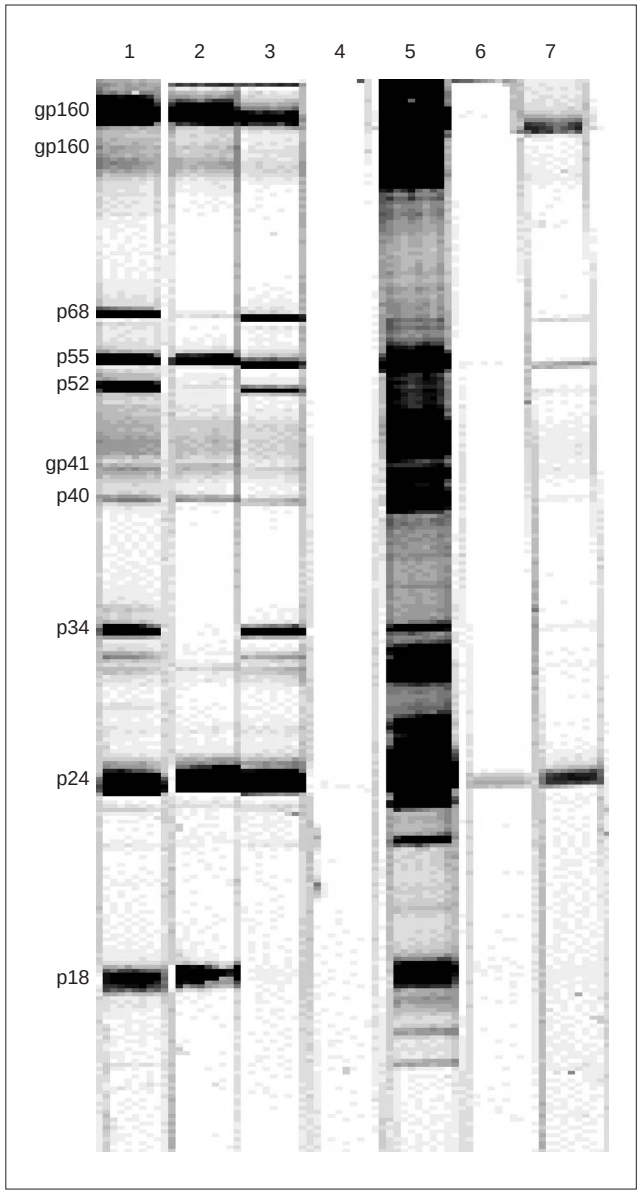

Figura 4. *Western blot* evolutivo de 2 pacientes (columnas 4 y 5 y 6 y 7) diagnosticados en la infección aguda por el VIH. Los resultados de las tiras 4 y 6 son negativos o indeterminados y los de las tiras 5 y 7, realizadas 6 meses después de la infección aguda por el VIH son positivos (véanse criterios en el texto).

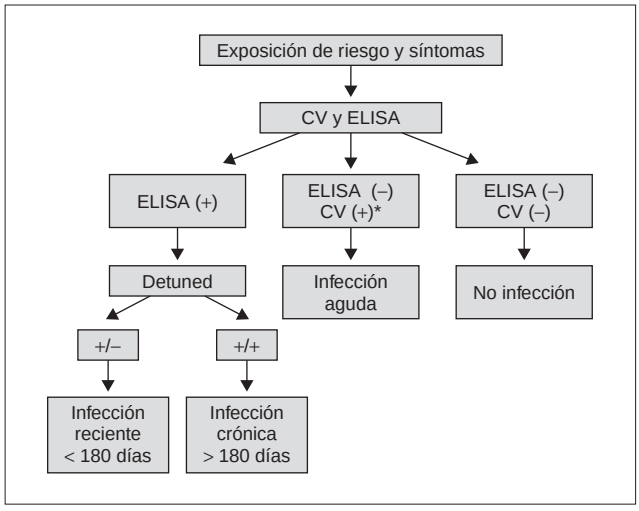

Figura 5. Algoritmo diagnóstico de la infección aguda, la infección reciente y la infección crónica por el VIH (véanse criterios en el texto). ELISA: análisis de inmunoabsorción ligado a enzimas. CV: carga viral del VIH en plasma.

de resistencia en las pruebas genotípicas, de las características epidemiológicas de la población estudiada y de la disponibilidad y las estrategias de implementación del TAR en un área o región determinada[80].

La primera evidencia de transmisión de una cepa de VIH resistente a la zidovudina se describió en 1993 y en la actualidad se ha descrito para todos los fármacos disponibles (tabla 3)[82-86,88-90]. La frecuencia de detección de resistencias en la infección aguda por el VIH aumentó con la introducción de los tratamientos antirretrovirales basados en mono o biterapia de inhibidores de la transcriptasa inversa análogos de nucleósidos (ITIAN). En 1996 la frecuencia de resistencias a estos fármacos alcanzaba un 20%. Desde 1998 hasta el año 2000 la resistencia a los ITIAN disminuyó en forma constante, con un aumento simultáneo hasta el 10% de la resistencia a los inhibidores de proteasa (IP) y un poco menor para los inhibidores de la transcriptasa inversa no nucléosidos (ITINN)[81-83]. En los últimos 3 años la frecuencia de resistencias a cualquier TAR se ha mostrado estable y de alrededor del 10% en la mayoría de los países de Europa[84-88], mientras que en Estados Unidos y también en el Reino Unido se han descrito valores algo superiores[89,90]. Por este motivo se recomienda en los pacientes con diagnóstico de infección aguda por el VIH realizar pruebas de resistencias a los antirretrovirales[91,92].

Otro aspecto que se debe resaltar, al contrario de lo que se creía[7], es que las mutaciones a las distintas familias de antirretrovirales pueden persistir en el tiempo y se pueden detectar cuando la infección por VIH es crónica, varios meses o años después[93,94]. Esta información es importante, ya que podría justificar la realización de pruebas de resistencia a los antirretrovirales en los pacientes diagnosticados con una infección crónica por el VIH. Finalmente, los estudios que se están efectuando para relacionar la capacidad replicativa del virus *(fitness)* con la transmisión de cepas resistentes y la progresión de la infección por VIH han demostrado que en los pacientes con cepas resistentes la capacidad replicativa del virus no aumenta con el tiempo y que aquellos pacientes con virus con baja capacidad replicativa tienen una mayor cifra de linfocitos CD4/μl[95].

Tratamiento antirretroviral en la infección aguda por el VIH

El inicio del TAR durante la infección aguda es un tema muy debatido que tiene sus ventajas e inconvenientes (tabla 4). Aunque el TAR durante la infección aguda por el VIH ha demostrado un efecto beneficioso sobre el sistema inmunitario y una rápida supresión de la replicación viral[96], el virus no se puede erradicar y no hay evidencias clínicas de que en la fase aguda de la infección el TAR aporte un beneficio clínico en términos de reducir la progresión a sida o muerte con respecto a iniciarlo en la fase crónica de

TABLA 3. **Prevalencia de mutaciones asociadas a resistencia a los antirretrovirales en estudios que incluyen pacientes con infección aguda o reciente por el VIH**

País	Número	1995-1998	1999-2000	2001-2002	Comentarios	Referencia
Estados Unidos	236	13 %	20 %	14 %	Las cepas resistentes que se transmiten tienen una capacidad replicativa similar a las sensibles	83
Estados Unidos	225	10 %	17 %	25 %	La proporción de cepas con resistencias a 2 familias de fármacos aumentó de 2,5 al 13%	82
Estados Unidos	301	8 %	23 %	NI	Hubo más fallo virológico y menor tiempo de supresión virológica en los pacientes con virus resistentes	89
Francia	251	9 %[a]	10 %	NI	[a]Publicación anterior del mismo grupo del período 1996-1999 (AIDS 2002;16:793)	84
Reino Unido	68	6	23 %	NI	Hay publicaciones con frecuencias menores de transmisión	
Italia	68	15 %[b]	NI	NI	[b]Datos desde 1996. La mayor frecuencia fue en 1997	90
España (Madrid)	57		15[c]		[c]En la cita 86 presentan los datos en los períodos 1996 a 1999 y de 2000 a 2001 que fueron del 26 % y del 4 %, respectivamente	85, 86
España (Barcelona)	52	8 %	12,5 %	8 %	Datos no publicados pertenecientes al Hospital Clínic de Barcelona	–

VIH: virus de la inmunodeficiencia humana; NI: no información.

TABLA 4. **Ventajas e inconvenientes de iniciar el TAR durante la fase aguda de la infección por VIH**

Ventajas

Virológicas
Suprimir la replicación viral
Disminuir la diversidad viral
Puede disminuir el reservorio*
Disminuir el riesgo de transmisión del VIH
Podría disminuir el *set point* en los pacientes que reciben TAR transitorio y cambiar la historia natural de la infección, pero no hay estudios al respecto

Inmunológicas
Preservar el sistema inmunitario
Preservar/mantener la respuesta inmunitaria específica frente al VIH: proliferativa (CD4) y citotóxica (CTL) que se pierden en la infección aguda por el VIH*

Clínicas
Disminuir la intensidad de los síntomas

Inconvenientes

Virológicos
No se erradica la infección
Riesgo de aparición de resistencias en pacientes con mala adherencia

Inmunológicos
Potencial tratamiento de LTNP

Clínicos
Disminución de la calidad de vida
Toxicidad (aguda/crónica)
Duración del tratamiento indefinida. Se desconoce la utilidad de pautas cortas de TAR
Coste económico

*Es más probable si se inicia el tratamiento antirretroviral (TAR) muy precozmente.
VIH: virus de la inmunodeficiencia humana; LTNP: pacientes que no progresan a largo plazo.

la infección. Además, en la actualidad y debido a la toxicidad aguda y crónica del TAR, existe un enfoque terapéutico mucho más conservador. Este fenómeno se ha visto reflejado en la práctica clínica. En la cohorte francesa PRIMO que recoge 291 pacientes con una infección aguda por el VIH diagnosticados en Francia entre 1996 y 2001, la proporción de pacientes que inició el TAR en la infección aguda por el VIH descendió del 92% en 1996 al 56% en 2001[97].

Sin embargo, no hay ninguna duda de que la infección aguda por el VIH es un escenario ideal para administrar TAR y estudiar cómo esta intervención terapéutica podría cambiar el equilibrio entre el VIH y el sistema inmunitario a favor de este último. El objetivo desde el punto de vista virológico e inmunológico sería reducir la diversidad viral y el reservorio y preservar o restaurar la inmunidad específica frente al VIH, tanto proliferativa (mediada por los linfocitos CD4+) como citotóxica (mediada por los linfocitos CD8+)[98-101]. Si se lograra este objetivo y esta respuesta fuera potente se podría tener el control inmunológico de la infección por VIH sin necesidad de continuar administrando el TAR. Ello permitiría cambiar la historia natural de esta infección evitando el deterioro inmunológico y la progresión clínica y convirtiendo a estos pacientes en supervivientes a largo plazo.

En la actualidad, ¿que evidencias se han recopilado para poder justificar la administración de TAR en la infección aguda por el VIH? Este tema ha sido recientemente revisado por los grupos de los Dres. Walker (Boston, Estados Unidos) y Cooper (Sydney, Australia)[102] y concluyen que no existen evidencias de que los pacientes con infección aguda por el VIH que recibieron TAR en la fase aguda tengan beneficio clínico adicional y que estaría plenamente justificado la realización de un ensayo clínico, probablemente multicéntrico y multinacional, con variables de análisis final clínicas (progresión a sida y/o muerte) en el que los pacientes sean aleatorizados a recibir un TAR precoz (antes de la seroconversión) frente a otro diferido. En este sentido, en la 11.ª Conferencia de Retrovirus del año 2004[103] se presentó un estudio de cohortes que no encontró diferencias en la evolución clínica, inmunológica ni virológica a los 3 años de iniciado el TAR entre un

grupo de pacientes que lo inició antes de la seroconversión y otro que lo hizo después de la misma (mediana: 164 días). Sin embargo, la evolución inmunológica y virológica de ambos grupos fue mejor que la de un tercer grupo de pacientes que no recibió TAR.

A continuación se revisan las distintas estrategias terapéuticas que se han realizado en pacientes con infección aguda por el VIH, que han recibido TAR antes de la seroconversión.

Ensayos clínicos y estudios de cohortes de TAR en la infección aguda por el VIH

Sólo se han realizado dos ensayos clínicos de TAR en la era pretratamiento antirretroviral de gran actividad (TARGA) con monoterapia con zidovudina (AZT), administrada durante 24 semanas (6 meses), en comparación con placebo que se publicaron en los años 1995 y 1998, respectivamente[104,105]. El seguimiento fue de unos 12 meses, evidentemente no se observó ningún beneficio virológico al final del seguimiento, aunque en ambos estudios se encontró un beneficio inmunológico (en términos de incremento, en un estudio, o menor pérdida, en el otro, de linfocitos CD4/μl) en la rama de zidovudina. Desde el punto de vista clínico, los pacientes que recibieron zidovudina en el primer ensayo clínico tuvieron menos eventos B que los que recibieron placebo. Sin embargo, una publicación posterior de este estudio[106] no se constató ningún beneficio virológico, inmunológico ni clínico a los 2 años de seguimiento.

Cuando en 1996 se iniciaron las potentes pautas de TAR con combinaciones de dos ITIAN y un inhibidor de la proteasas (IP) y posteriormente se introdujeron los ITINN, los pacientes con infección aguda por el VIH fueron tratados con estas combinaciones[107-109]. Numerosos estudios de cohortes, que incluyeron un escaso número de pacientes, demostraron que los resultados virológicos e inmunológicos fueron mucho mejores que en los pacientes que recibieron monoterapia o biterapia. Sin embargo, estos estudios ni fueron aleatorizados con un grupo placebo o sin TAR ni han tenido el suficiente seguimiento para poder valorar la progresión clínica de la infección por el VIH[102]. En general, el TARGA fue muy efectivo en suprimir la replicación viral, incluso de una forma más efectiva y sostenida que en pacientes con infección crónica por el VIH, lograba mejores parámetros de recuperación inmunológica y permitía la restauración de la respuesta proliferativa (mediana por los linfocitos CD4) y citotóxica (CTL) frente al VIH[98-101]. Sólo un estudio observacional encontró un beneficio clínico adicional a corto-medio plazo al comparar sus resultados con una cohorte histórica[110]. Por el contrario, otro estudio de cohortes comentado previamente no encontró diferencias virológicas, inmunológicas ni clínicas entre los pacientes que iniciaron el tratamiento antes o después de la seroconversión[103].

Al mismo tiempo se detectaron problemas de tolerancia y toxicidad crónica. En este sentido, hay que destacar que la prevalencia de lipodistrofia y dislipemia a los 12-24 meses de iniciado el TAR en la infección aguda por el VIH son similares a la de los pacientes con una infección crónica por el VIH[111-113]. Además, existe el riesgo de desarrollo de resistencias en los pacientes con mala adherencia a éste. Todo ello ha conducido a los investigadores a buscar otras estrategias terapéuticas o a no recomendar de forma sistemática el TAR de la infección aguda por el VIH.

TAR en la infección aguda por el VIH de duración limitada

Una estrategia que se ha evaluado en algunos estudios ha sido la de administrar el TAR durante un período de tiempo limitado con el fin de poder modificar la historia natural de esta infección y evitar la toxicidad crónica del TAR. Así, en los pacientes tratados se podría inducir, después de parar el TAR, un nivel de reaparición de la CV *(set point)* menor que en los no tratados y por tanto podrían tener un mejor pronóstico. Aparte de los dos ensayos clínicos con monoterapia con zidovudina comentados previamente, esta estrategia se ha evaluado en la era del TARGA en tres estudios en los que la duración del TAR y las pautas administradas fueron completamente diferentes.

En el primero estudio, Girard et al[114] administraron durante un año a 9 pacientes una combinación triple de ITIAN: zidovudina, didanosina y lamivudina. Después de suspender el TAR, la CV del VIH reapareció en todos los casos, y sólo en 5 permaneció por debajo de 12.395 copias/ml a los 18 meses de la suspensión. En el segundo, Markowitz et al[115] también observaron que la CV en plasma reapareció en los 16 casos al suspender el TARGA con IP que habían recibido durante una media de 3 años. La CV del VIH en plasma se estabilizó por debajo de las 5.000 copias/ml en sólo 4 casos (25%), siendo la distribución de CV similar a la cohorte americana de pacientes con infección aguda por el VIH sin tratamiento[116]. Además, en aquellos pacientes que tenían una CV en plasma mayor de 5.000 copias/ml, la cifra de linfocitos CD4/μl volvió a su nadir en un año, demostrando que el beneficio inmunológico generado se perdía aproximadamente al año de la suspensión del TAR. En el tercer estudio, Fidler et al[117] administraron TAR basado en ITINN (nevirapina o efavirenz) a 37 pacientes durante 3 meses o hasta que tuvieran una CV < 50 copias/ml. Al finalizar el TAR, las respuestas inmunitarias específicas frente al VIH (proliferativa y citotóxica) se habían preservado. Sin embargo a los 12 meses de interrumpir el TAR, estas respuestas habían desaparecido y los valores de CV fueron comparables a los de las cohortes europeas de pacientes con infección aguda por el VIH sin tratamiento[118].

TAR intermitente. El concepto de "autovacunación" en la infección aguda por el VIH

Esta estrategia busca, en aquellos pacientes tratados desde la infección aguda por el VIH con una supresión mantenida de la replicación viral en plasma, que las paradas periódicas del TAR (ciclos alternos de TAR y parada del mismo) originen una reaparición de la viremia, permitiendo una exposición de los antígenos del VIH al sistema inmunitario ("autovacunación"), con el fin de inducir y mantener una respuesta inmunitaria específica frente al VIH que permitiera, en una segunda fase, el control de la replicación viral en ausencia de TAR.

Esta estrategia se inició tras la publicación del "paciente de Berlín", un paciente que, tratado desde la infección aguda por el VIH, debió suspender en dos ocasiones el TAR por infecciones intercurrentes, seguidas de la retirada definitiva y en el que el virus permaneció suprimido durante 2 años[119]. Posteriormente se publicaron datos, tanto de series retrospectivas como prospectivas, que demostraron que los pacientes que controlaban la replicación

viral presentaban una respuesta citotóxica anti-VIH mejor, y que era posible mejorar esta respuesta con ciclos de suspensiones repetidas. Sin embargo, la inmunidad específica frente al VIH sólo se ha restaurado cuando el TAR se ha administrado en los primeros días de la infección aguda, antes de la seroconversión. Un estudio piloto realizado por el grupo de Walker et al[122] y que incluyó 8 pacientes que iniciaron el TAR durante la primera semana de inicio de los síntomas, mostró posteriormente un buen control a corto y medio plazo de la infección por VIH en ausencia de TAR en cinco de ellos[120]. Este fenómeno también se ha observado en modelos animales donde el TAR de la infección aguda por SIV permitió, tras varias interrupciones del mismo, el control inmunológico o de la infección sin TAR[121]. Sin embargo, esta estrategia terapéutica no parece tener tan buenos resultados como inicialmente parecía. El mismo grupo de Walker[122] presentó en la 11.ª Conferencia de Retrovirus del año 2004, los datos a los 2 años de seguimiento de los 14 pacientes que habían incluido en este estudio y sólo tres de ellos (21%) permanecían sin TAR, lo que indica que la duración del control inmunitario de la replicación viral es limitada en el tiempo. El estudio francés PRIMOSTOP (ANRS 100)[123] se presentó en la misma conferencia. Sólo 7 de los 26 pacientes (27%) tratados desde la infección aguda con didanosina, estavudina, nelfinavir e hidroxiurea que se incluyeron en un programa de tres ciclos de interrupción del TAR mantuvieron una CV en plasma menor de 1.000 copias/ml a los 6 meses de suspender el tratamiento. En la mitad de los pacientes se debió suspender la administración de hidroxiurea por los efectos tóxicos. Por otra parte, si el TAR se inicia inmediatamente después de la seroconversión, los resultados tampoco son alentadores. En un estudio piloto efectuado por nuestro grupo[124] con 12 pacientes que recibieron TAR en los primeros 3 meses desde el inicio de los síntomas, sólo cuatro (33%) tuvieron un adecuado control inmunológico de la infección tras realizar cuatro ciclos de interrupciones del TAR. La adición de interleucina 2 en dosis bajas (700.000 U/m^2 al día por vía SC) durante 24 semanas a 6 de los 12 pacientes en el cuarto y último ciclo de TAR[125] fue segura pero no mejoró la respuesta inmunitaria específica frente al VIH ni la proporción de pacientes que controló la replicación viral.

Por otra parte, esta estrategia no está exenta del riesgo de desarrollo de resistencias[126]. Esto que obliga a buscar otras alternativas antes de recomendar este tipo de tratamiento en pacientes con infección aguda por el VIH.

Tratamiento inmunomediado

Dado que con el TAR actual es imposible erradicar el reservorio y que con el TAR precoz el control inmunológico de la infección por VIH es transitorio, los esfuerzos se han dirigido a diseñar nuevas estrategias terapéuticas que puedan mejorar la respuesta inmunitaria específica frente al VIH y/o a controlar la activación inmunológica, que es la responsable de la mayor parte de la pérdida de linfocitos CD4.

La adición de citostáticos como la hidroxiurea o de inmunosupresores como la ciclosporina ha demostrado ser efectiva para reducir la activación inmunitaria. La hidroxiurea se ha usado en pacientes con infección aguda por el VIH asociada a suspensiones del TAR, tanto en animales como en seres humanos, demostrando efectos beneficiosos al mejorar los parámetros inmunológicos, en particular el cociente CD4/CD8[127]. Sin embargo, se debe tener en cuenta el riesgo de mayor toxicidad, sobre todo cuando se asocia a determinados fármacos antivirales como la didanosina o la estavudina y, además, en un ensayo clínico aleatorizado en pacientes con una infección reciente que recibieron didanosina, estavudina y nevirapina con/sin hidroxiurea, el grupo de pacientes que recibió hidroxiurea tuvo una mayor progresión a eventos B o C del CDC en los 12 meses de observación[128]. La ciclosporina asociada durante las primeras 8 semanas al TAR se asoció con un aumento significativo y persistente de linfocitos CD4 probablemente al disminuir el reservorio de linfocitos activados en tejido linfático susceptibles de ser infectados por el VIH entre otras funciones[129].

También se ha especulado que la adición de determinadas citocinas al TAR podría colaborar a disminuir (o eliminar) el reservorio viral o a mejorar la respuesta inmunitaria. La forma pegilada del interferón alfa acelera el descenso de la CV pero no ha demostrado otros beneficios además de tener una frecuencia relativamente elevada de efectos adversos[130]. La utilización de la interleucina 2 (IL-2), cuando se utiliza en forma de ciclos, no mejora las respuestas inmunitarias específicas frente al VIH pero, al igual que en los pacientes con infección crónica, se asoció a aumento importante de los CD4 y un descenso del ADN proviral[131,132].

Actualmente se están desarrollando protocolos de estudios para valorar el efecto de otras citocinas como IL-12 o factor estimulante de colonias de granulocitos y macrófagos (GM-CSF) asociado a otras intervenciones durante la fase aguda y de otras estrategias como la inmunoactivación, que propone lograr una activación de todas las células infectadas en estado de latencia con el fin de facilitar su lisis e intentar la erradicación, para ello se utilizan fármacos o moléculas como OKT3 combinado con la IL-2[133].

En general, todas estas estrategias han sido útiles para el control de la replicación viral y la restauración inmunológica. En cualquier caso, la toxicidad de las citocinas y los inmunosupresores y la falta de datos con respecto al beneficio clínico a medio y largo plazo, hace que sólo se recomiende su uso en el contexto de ensayos clínicos.

Vacunas terapéuticas

Otra estrategia que se está evaluando también en este escenario es la eficacia de las vacunas terapéuticas con el fin de restaurar/potenciar la respuesta inmunitaria específica frente al VIH en pacientes que reciben el TAR desde la infección aguda por el VIH. En estos pacientes, la supresión de la replicación viral y la restauración del sistema inmunitario inducida por el TAR hace que sean los más indicados para inducir o potenciar una fuerte respuesta inmunitaria específica frente al VIH con vacunas terapéuticas. En una segunda fase, se evaluaría si tras la suspensión del TAR existe o no un control inmunológico de la replicación viral. Resultados preliminares en modelos animales con algunas de estas vacunas han sido satisfactorios[134,135]. Sin embargo, los primeros resultados en humanos han sido desalentadores. En la 11.ª Conferencia de Retrovirus del 2004 se han presentado los resultados de dos ensayos clínicos vacunas terapéuticas en este escenario. Ambas se administraron en el contexto de ensayos clínicos controlados con placebo. En el estudio QUEST[136], 79 pacientes fueron aleatorizados a recibir TAR con placebo, TAR con ALVAC-HIV (vCP1452) y TAR con ALVAC-HIV

(vCP1452) y Remune. Posteriormente se suspendió el TAR. Los dos grupos que recibieron vacunas terapéuticas fueron analizados conjuntamente. A los 6 meses de haber parado el TAR, la proporción de pacientes que tenía una CV < 1.000 copias/ml fue del 22% en el grupo placebo y del 17% en los pacientes que recibieron vacunas terapéuticas. En el segundo estudio[137], un total de 30 pacientes fueron aleatorizados a recibir placebo o cuatro dosis de una vacuna terapéutica que utilizaba como vector el virus de la viruela aviar *(Avipox)* en el que se habían insertado los genes del VIH gag/pol con o sin el gen del interferón gamma (IFN-γ). No hubo diferencias, en términos de respuesta virológica o inmunológica (CTL), entre el grupo placebo y el que recibió la vacuna terapéutica sin el gen del IFN-γ. En el grupo de pacientes que recibieron la vacuna con los genes del VIH gag/pol y el del IFN-γ también reapareció la carga viral, sin embargo, se observó una reducción significativa de la carga viral de 0,8 $\log_{10}$/ml en comparación con los otros dos grupos de pacientes, lo que significa que esta nueva aproximación terapéutica puede dar mejores resultados. En ambos estudios, la vacunación fue segura.

Otro aspecto negativo en este campo ha sido la descripción de casos de superinfección[138] en pacientes que tenían un buen control inmunológico de la replicación por VIH en función de alguna de las estrategias terapéuticas comentadas previamente. El mensaje es que la inmunidad específica frente al VIH generada no es cruzada con otras cepas de VIH. Finalmente, este escenario puede complicarse aún más, ya que se han descrito casos de infección por dos cepas distintas del VIH (infección dual)[139]. En estos pacientes la progresión a sida fue muy rápida. El estudio de estos nuevos escenarios tiene implicaciones importantes para el desarrollo de vacunas frente al VIH.

Recomendaciones de TAR en la infección aguda por el VIH

En la actualidad, y en función de lo previamente comentado, las recomendaciones de expertos españoles, europeos y americanos[140-143] sugieren incluir a estos pacientes en ensayos clínicos o en estudios para obtener una mayor información de los efectos del TAR sobre el VIH y el sistema inmunitario. Si los pacientes no se pueden incluir en ensayos clínicos, se aconseja tratar los casos graves o que tienen una duración prolongada de los síntomas o a los pacientes que lo deseen, siempre y cuando se haya discutido, las ventajas e inconvenientes de iniciar el TAR en esta fase de la enfermedad (tabla 4). Si se inicia el TAR, se pueden utilizar las mismas pautas de TAR que en los pacientes con una infección establecida. La elevadísima CV de la infección aguda por el VIH y la baja barrera genética de algunos de los inhibidores de la transcriptasa inversa (lamivudina, efavirenz, nevirapina) hacen que se hayan utilizado con más frecuencia pautas con IP. Sin embargo, estudios recientes han demostrado la eficacia de pautas con ITINN en este escenario[97,109]. En cualquier caso, si se quiere iniciar TAR debe efectuarse antes una prueba de resistencias. La duración del TAR se desconoce, pero desde el punto de vista teórico debería ser indefinido, ya que no erradica el VIH. Los controles que se deben efectuar son los mismos que en la infección crónica por el VIH[142,144]. Si un paciente tratado quisiera parar el TAR, esta decisión debería analizarse de manera cuidadosa, sobre todo si presenta una buena respuesta al TAR y no ha presentado efectos secundarios. Siempre se debería tener presente que la viremia reaparecerá al suspender el tratamiento, que algunos de estos pacientes pueden presentar un nuevo episodio clínico de síndrome retroviral agudo y que el beneficio inmunológico alcanzado se perderá con el tiempo.

En los pacientes no tratados se recomienda reevaluar los criterios de TAR a partir de los 6 meses, cuando la infección es crónica.

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NOTA

Los artículos publicados en la sección "Formación Médica Continuada" forman parte de grupos temáticos específicos (antibiograma, antimicrobianos, etc.). Una vez finalizada la publicación de cada tema, se irán presentando al Sistema Español de Acreditación de la Formación Médica Continuada (SEAFORMEC) para la obtención de créditos.

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ANEXO 1. **Análisis en el diagnóstico y tratamiento de la infección aguda por el VIH-1**

1. **Con respecto a la importancia de detectar la primoinfección en pacientes infectados por el VIH, señale la afirmación falsa:**
   a) Es importante para disminuir la transmisión de la infección, ya que en este período las tasas son mayores.
   b) Es importante para identificar e iniciar tratamiento en los pacientes con serología positiva para VHC ya que son los que progresan a sida más rápido.
   c) Permite estimar la prevalencia de resistencia a fármacos antivirales en la comunidad.
   d) Permite explorar *in vivo* los mecanismos inmunopatológicos involucrados en la fisiopatología de la infección por VIH.
   e) Es importante para evaluar la incidencia de nuevas infecciones y reforzar medidas preventivas en los grupos más afectados.

2. **¿Cuál es la afirmación correcta?**
   a) El 60% de los pacientes con infección aguda por VIH presentan un síndrome mononucleósido típico (fiebre, faringitis y adenopatías).
   b) Los pacientes con mononucleosis infecciosa tienen con mucha frecuencia un resultado falsamente positivo para VIH.
   c) Más del 60% de los pacientes con infecciones aguda por VIH tienen un resultado falsamente positivo para VEB.
   d) Aunque un monotest puede dar falsamente positivo en un paciente con infección aguda esto sucede en menos del 2% de los casos, y estos pacientes presentan serología anti-VCA IgM negativa.
   e) La afección faringoamigdalar en la infección aguda por VIH es exclusivamente exudativa e imposible de distinguir de la mononuclesosis infecciosa.

3. **¿De las siguientes combinaciones de síntomas cuál tiene la mayor especificidad con una sensibilidad de alrededor del 50% para detectar una primoinfección por VIH?**
   a) Adenopatías inguinales y exantema.
   b) Fiebre, diarrea y úlceras orales.
   c) Fiebre y úlceras genitales.
   d) Fiebre y exantema.
   e) Fiebre y meningitis aséptica.

4. **¿Qué método o combinación de métodos diagnósticos es más útil para detectar una infección aguda por VIH en un paciente con controles serológicos periódicos para VIH, con resultados siempre negativos, el último hace un mes, y que presenta fiebre de 4 días de evolución, astenia marcada que lo obliga a estar en cama, faringitis y un discreto exantema en tronco y que refiere una exposición sexual sin protección, la última hace 2 semanas?**
   a) Carga viral y antígeno p24.
   b) Carga viral.
   c) Antígeno p24 y *Western blot*.
   d) Carga viral y ELISA para VIH.
   e) Detuned.

5. **Con respecto a la pregunta anterior: ¿qué resultado sugiere una infección aguda de menos de 4 semanas de duración?**
   a) ELISA no reactivo y *Western blot* negativo.
   b) Antígeno p24 positivo y *Western blot* positivo.
   c) ELISA no reactivo y CV detectable con valores mayores de 10.000 copias/ml.
   d) ELISA reactivo y CV detectable con valores mayores de 10.000 copias/ml.
   e) ELISA no reactivo y Detuned reactivo.

6. **Entre las siguientes afirmaciones ¿cuál es la incorrecta?**
   a) La prueba de ELISA se positiviza habitualmente al mes de la exposición.
   b) La prueba de *Western blot* se positiviza habitualmente entre 45 y 90 días de la exposición.
   c) El antígeno p24 aparece en forma transitoria habitualmente entre la primera y segunda semana de la exposición.
   d) La CV se positiviza habitualmente entre la cuarta y la séptima semana de la exposición.
   e) El Detuned es no reactivo los primeros meses de la infección.

7. **Con respecto a la transmisión de resistencias durante la infección por VIH, señale la afirmación incorrecta:**
   a) La prevalencia de resistencia en pacientes con infección aumenta en forma constante desde el año 1999.
   b) Con algunas diferencias geográficas menores, la prevalencia de resistencia en pacientes con infección aguda en Europa se mantiene estable desde la aparición del TARGA.
   c) La prevalencia de resistencia en pacientes con infección aguda en España se sitúa en los últimos años en cifras entre el 10 y el 15%.
   d) La resistencia genotípica puede persistir durante varios meses después de la transmisión.
   e) En los pacientes con diagnóstico de infección aguda o reciente se recomienda realizar un genotipo para VIH.

8. **Las recomendaciones actuales indican:**
   a) Iniciar tratamiento en todos los pacientes con una infección por VIH de menos de un año de duración.
   b) Iniciar tratamiento a los pacientes con síntomas graves.
   c) Ofrecer tratamiento a los pacientes con infección aguda en el contexto de ensayos clínicos.
   d) Sólo tratar los pacientes que demuestren que no tienen resistencia a fármacos antirretrovirales.
   e) b) y *c)* son correctas.

9. **¿Cuál de las siguientes afirmaciones sobre las potenciales ventajas de iniciar TAR en fase aguda es incorrecta?**
   a) Podría reducir los reservorios virales.
   b) Disminuye la posibilidad de transmisión.
   c) Disminuye las respuestas específicas inmunitarias anti-VIH.
   d) Detiene el deterioro inmunitario.
   e) Disminuye los síntomas.

10. **Con respecto a los estudios de tratamiento de paciente con infección aguda por VIH señale la afirmación correcta:**
    a) Varios estudios aleatorizados demuestran que es mejor iniciar el tratamiento en fase aguda en comparación a iniciarlo en la fase crónica.
    b) En estudios aleatorizados, un año de tratamiento seguido de suspensión ha sido eficaz para controlar la infección sin tratamiento a largo plazo.
    c) Los programas de paradas estructuradas permiten el control inmunológico de la replicación viral en ausencia de tratamiento en la mayoría de pacientes de forma sostenida.
    d) La frecuencia de aparición de efectos adversos en estos pacientes es mucho menor que en los pacientes con infección crónica.
    e) La evidencia disponible hasta el momento no permite recomendar tratamiento antirretroviral a todos los pacientes con infección aguda.

Comparison of Two Serological Tests for the Identification of Recent HIV Infection: Vironostika HIV-1 Microelisa and BED Capture Enzyme Immunoassay

Comparación de dos tests serológicos para la identificación de infecciones recientes por VIH-1: microelisa Vironostika HIV-1 y enzimoinmunoensayo de captura BED

To the Editor:

Identification of recent human immunodeficiency virus (HIV) infection is an important tool for monitoring HIV transmission. During the last few years, several serological assays have been developed for this purpose and used in cross-sectional studies.[1] The serological testing algorithm for recent HIV seroconversion (STARHS) was developed in 1998[2] and used with the Abbott HIVAB 3A11 assay (Abbott Laboratories, Abbott Park, Chicago, Illinois, USA) and with the Vironostika HIV-1 Microelisa System (bioMérieux SA, Marcy l'Etoile, France). The sensitivity of both assays was lowered in order to obtain a negative result in specimens with low antibody titers, such as those from individuals with a recent infection. These assays are no longer available, and laboratories have turned to new methods. The BED assay (Calypte Biomedical Corporation, Portland, Oregon, USA) measures anti-HIV IgG titers[3] and includes a calibrator to ensure comparability of results. Furthermore, the BED assay is included in an external quality program offered by the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia, USA).

In Catalonia, the STARHS was introduced using the Vironostika assay as part of the enhanced HIV/STI surveillance program in 2003. As the Vironostika assay has no longer been available since 2007, our laboratory changed to the BED assay in 2008. Our aim was to assess whether the results obtained by both techniques were comparable.

Table 1
Performance of both assays according to clinical information.

Group	Total no. of patients	No. of recent infections according to assay		
		Vironostika	BED	Both assays
Known recent HIV infection	15	14	14	13
AIDS	45	12	9	7
Long-standing HIV infection	3	0	0	0
Unclassified[a]	38	9	11	9
Total	101	35	35	29

[a] Patients with no clinical criteria for AIDS, without evidence of recent infection, neither long-standing infection.

A total of 101 serum specimens from HIV-1–positive individuals were selected from a previous study.[4] The selection criteria were sufficient sample volume and minimum available clinical and laboratory information ($CD4^+$ T-cell count, HIV viral load, HIV infection stage, previous antiretroviral treatment). Patients were classified into three groups. The first group included 15 recently infected individuals (sera were drawn no more than 6 months after seroconversion in nine patients, and the rest had a diagnosis of acute HIV infection). The second group included three patients with long-standing infection (subjects infected for >12 months) and 45 patients with a diagnosis of AIDS (clinical criteria or $CD4^+$ T-cell count under 200 cells/μl). Finally, 38 specimens were unable to be classified as either recent infections, long-standing infections, or AIDS.

The agreement between Vironostika and BED assays was good (κ=0.738, P<.005), which is consistent with the results of two published studies.[5,6] Sensitivity to detect recent infection was 93.3% (95% CI: 68.1 – 99.8) for both the Vironostika and the BED assays. Specificity for detecting long-term infections was 75.0% (95% CI: 60.4 – 86.4) using the Vironostika, and 81.3% (95% CI: 67.4 – 91.1) using the BED. Positive predictive values were 60.9% (95% CI: 38.5 – 80.3) using the BED, and 53.8% (95% CI: 33.4 – 73.4) using the Vironostika. Negative predictive values were 97.5% (95% CI: 86.8 – 99.9) using the BED, and 97.3% (95% CI: 85.8 – 99.9) using the Vironostika. Table 1 shows the samples identified as recent infections by both BED and Vironostika according to their clinical characteristics and laboratory information.

The BED assay correctly classified a greater proportion of recent infections and patients with AIDS than Vironostika. These results are similar to those of a previous study,[6] in which the Vironostika kit also tended to misclassify more individuals with long-standing infections or AIDS as recently infected in a comparison with the avidity index method.[7] The misclassification of patients with AIDS or $CD4^+$ T-cell counts $\leq$200 cells/μl is explained by the low anti-HIV IgG titers. Hence the importance of excluding those samples belonging to patients fulfilling these criteria from STARHS testing when this information is available.

The BED assay offers several advantages over the Vironostika: i) it has been reported to have better reproducibility, since it is based on the HIV IgG/non-HIV IgG ratio and uses a simple 1:100 dilution;[8] ii) the BED assay can also be automated providing more precise results than the Vironostika assay, which is performed manually; and iii) the window period of the Vironostika assay differs for B and non-B HIV-1 subtypes, whereas these differences are less pronounced with the BED.[9] For all those reasons, the BED assay offers a good alternative to the discontinued Vironostika assay.

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Appendix.

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Recently acquired HIV infection in Spain (2003–2005): introduction of the serological testing algorithm for recent HIV seroconversion

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ABSTRACT

Background: Identification of recent HIV infections provides a description of the current pattern of HIV transmission and, consequently, can help to design better preventive interventions. Our study shows the first implementation in Spain of the Serologic Testing Algorithm for Recent HIV Seroconversion (STARHS) strategy. We assess the viability of introducing STARHS in our setting and describe the frequency and epidemiological characteristics of recent infections (RIs).

Methods: Between 2003 and 2005, HIV-positive blood samples drawn for diagnostic purposes were collected from 28 Spanish laboratories to be tested using STARHS. Samples from patients with a previous HIV diagnosis, age <18 years, <200 CD4 cells/μL or clinical AIDS criteria were excluded from the analysis.

Results: A total of 660 (19.2%) samples were classified as RI. Most people identified with RI were male (79.8%) with a median age of 33.1 years, and 62.5% occurred among men who have sex with men (MSM). Immigrants made up 26.5% of individuals identified with RIs, with 48.7% coming from South America. Among the individuals with RI, at least 16.5% had reported another sexually transmitted infection (STI) during the year before the HIV diagnosis.

Conclusion: The study shows that the implementation of STARHS in our setting is feasible and has highlighted important features of the local HIV epidemic, such as the ongoing spread of HIV among MSM, the potential role of STIs in RIs and the vulnerability of immigrants as a new target population.

HIV infection remains a major public health problem in Europe and there is evidence of increasing transmission among vulnerable populations in different countries.[1] Spain has the fifth highest incidence rate of AIDS in Europe, with 36.0 cases per million people.[2] Since the introduction and widespread use of highly active antiretroviral therapy (HAART) in 1996, both the incidence of AIDS and HIV-related mortality rates have decreased dramatically. Therefore, AIDS data are less indicative of the underlying trends in HIV infection and many European countries have introduced HIV reporting as part of their surveillance systems. Data from eight Spanish regions with HIV reporting systems show a shift from parenteral to sexual transmission of HIV infection.[3] In Catalonia, Spain, data generated by the Integrated AIDS/HIV/STI Surveillance System (SIVES) suggest an increase in the incidence of HIV among men who have sex with men (MSM) and immigrants.[4] Nevertheless, since new diagnoses of HIV can include infections that occurred many years ago, they may not reflect the magnitude or characteristics of recent infections (RIs). Identification of RIs provides an accurate description of current epidemic patterns and enables us to better tailor preventive interventions. The Serological Testing Algorithm for Recent HIV Seroconversion (STARHS) was first described in 1998 as a tool to differentiate RIs from long-standing infection (LSI).[5] STARHS has been used in different settings to estimate incidence[6–11] and describe RIs.[12 13] In Spain, HIV testing is offered free at national health hospitals, primary health centres (PHC), sexually transmitted infection (STI) units and several community-based voluntary counselling and testing sites (CBVCTS), which offer free anonymous counselling and testing services regardless of the user's health insurance or residence status. Current policies promote testing in vulnerable populations and young adults on a voluntary basis. The objectives of this study are to assess the viability of introducing STARHS in our setting and to describe the frequency and epidemiological characteristics of people with RI.

METHODS

Study design

Three-year cross-sectional study (2003–2005).

Inclusion criteria

Confirmed HIV-positive test samples from patients aged 18 years or over without a previous confirmed HIV diagnosis during the 6 months before extraction.

Settings

Samples were collected from 28 laboratories: 19 at hospital outpatient clinics, one from a STI clinic, 7 laboratories covering 163 PHCs and 1 STI unit, and 1 laboratory collecting samples from 8 CBVCTS. Centres were selected on a voluntary basis. Twenty-two of these laboratories were from Catalonia, three from Madrid and three from Andalusia, La Rioja, and the Basque Country.

Specimen collection

Residual volumes of serum specimens collected for diagnostic purposes were sent by the participating laboratories to the coordinating centre (Centre d'Estudis Epidemiològics sobre les ITS i la Sida de Catalunya, CEEISCAT). All personal identifiers were removed from the samples, which were sent to the coordinating centre with a study number. Only the recruitment centre had access to personal identifiers. Specific testing was performed at the Microbiology Service of the Hospital Universitari Germans Trias i Pujol.

Laboratory methods

Samples were additionally tested for HIV by a conventional sensitive enzyme immunoassay (EIA) (Anti-HIV TETRA Elisa, Biotest, Dreieich, Germany) to double-check that all the samples were HIV positive. Positive specimens were then tested using a modified version of the Vironostika HIV-1 EIA (bioMérieux, Durham, North Carolina, USA) in which sample dilution and sample and conjugate incubation times were modified to render it less sensitive. Standard optical density (OD) was calculated as follows:

sample OD − median negative control OD/median calibrator OD − median negative control OD.

Specimens with a standard OD <2.0 were retested in triplicate. Duration of infection was defined as recent for retested specimens with a standard OD <1.0 and longstanding for those with a standard OD ⩾1.0. People with RI can be presumed to have seroconverted within the past 170 days (95% CI 144 to 200).[14]

Since 2000, CEEISCAT and the microbiology service of the Hospital Universitari Germans Trias i Pujol have been participating in an international STARHS quality control programme established by the Centers for Disease Control and Prevention (FDA BB-IND # 8193).

Interpretation of results

An infection was considered recent if the sample reacted with the sensitive EIA but not with the modified less sensitive EIA (that is, had low HIV antibody titres). Since antibody titres may fall in advanced stages of HIV infection, patients whose samples did not react and who presented clinical criteria for AIDS or a CD4+ T cell count <200 cells/μL were considered as having LSI.

Data collection

A data collection form, including CD4+ T cell counts, HIV viral loads and previous HIV testing, was filled out by the laboratory staff. A second data collection form, compiled by the patient's physician or a designated person, with demographical and clinical-epidemiological variables (sex, date of birth, country of origin, date of arrival in Spain, sexual orientation, use of injected drugs, STI diagnoses, HIV infection status at the time of diagnosis and use of antiretrovirals) was used only for diagnosis of RI.

The total number of HIV tests performed and the positive results were recorded for each centre.

Statistical analysis

The normal approximation 95% CI was calculated, as was the exact CI when appropriate. Differences in the distribution of several characteristics between diagnosis sites were assessed using Pearson's χ^2 test with a Bonferroni correction for multiple comparisons.

RESULTS

The number of HIV tests performed at recruitment sites was higher in hospitals, but STI clinic and CBVCTSs had the highest prevalence of HIV. We studied 59% of all the positive samples from each centre between January 2003 and December 2005. The samples studied as a percentage of all positive samples per type of centre were distributed as follows: hospitals 50%; PHCs 71%; STI clinic 77%; CBVCTSs 89% (table 1).

We collected 4172 samples, of which 728 did not meet the inclusion criteria and were excluded from the analysis. Out of the remaining 3444 specimens, 713 were non-reactive by the less sensitive assay, but 53 samples were from patients with <200 CD4+ T cells/μL or clinical criteria for AIDS and were considered to be LSIs. Therefore, 660 (19.2%) samples were considered RIs (fig 1).

Of the samples, 50.2% (n = 1728) were from hospitals, 37.3% (n = 1285) from PHCs, 10.2% (n = 351) from STI clinic and 2.3% (n = 80) from CBVCTSs. The RI percentage differed with the type of centre: 16.5% (n = 285; 95% CI 14.7 to 18.2) at hospitals, 21.5% (n = 276; 95% CI 19.2 to 23.7) at PHCs, 23.4% (n = 82; 95% CI 18.9 to 27.8) at STI clinic and 21.3% (n = 17; 95% CI 12.9 to 31.8) at CBVCTSs. The percentage of RI in hospitals was significantly lower than in PHC and STI clinic ($p \leqslant 0.001$) (table 1).

The characteristics of individuals with RI are shown in Table 2. Most were male (79.8%), median age was 33.1, MSM was the main HIV transmission group (62.5%) and 38.8% reported at least one STI within the 12 months before HIV diagnosis. Country of origin was known in 426 cases and 26.5% were immigrants, mainly from South America (48.7%). The date of arrival in Spain was collected in only 71 cases; 59 (83%) of which arrived more than 12 months before the time of HIV diagnosis (median 39 months; interquartile range 21–60). We found statistically significant differences by gender, origin, transmission group and coinfection with other STIs according to the site of diagnosis.

DISCUSSION

To our knowledge, this is the first time the STARHS technique has been used in Spain. The percentages of HIV diagnosis vary between the centres. The number of samples from Catalonian

Table 1 Distribution of HIV tests performed by centre (2003–2005)

	Total	Hospitals	PHCs	STI clinic	CBVCTSs
HIV tests performed*	478 932	250 398	210 359	14 962	3213
Positive HIV tests†	5800 (1.2%)	3455 (1.4%)	1805 (0.9%)	451 (3.0%)	89 (2.8%)
Samples collected*	4172	2305	1404	374	89
Samples studied‡	3444 (59.4%)	1728 (50.0%)	1285 (71.2%)	351 (77.8%)	80 (89.9%)
Recent infections¶	660 (19.2%)	285 (16.5%)	276 (21.5%)	82 (23.4%)	17 (21.3%)

*Number—this number does not represent single persons; †number of positive HIV tests performed and prevalence (percentage) of positive HIV tests—this number does not represent single persons. Out of 5800 positive HIV tests, 1628 were not collected for the study (mostly because of insufficient volume). Prevalence was calculated as follows: number of HIV tests performed divided by the total number of HIV tests performed; ‡number of samples included in the study and percentage of studied samples over the total of positive HIV tests by each centre—this number represents single persons; ¶number and percentage of recent infections—this number represents single persons. Percentages of recent infections by site of diagnosis were computed over the total number of samples with the inclusion criteria studied from each centre.
CBVCTSs, community-based voluntary counselling and testing sites; PHCs, primary health centres; STI, sexually transmitted infection.

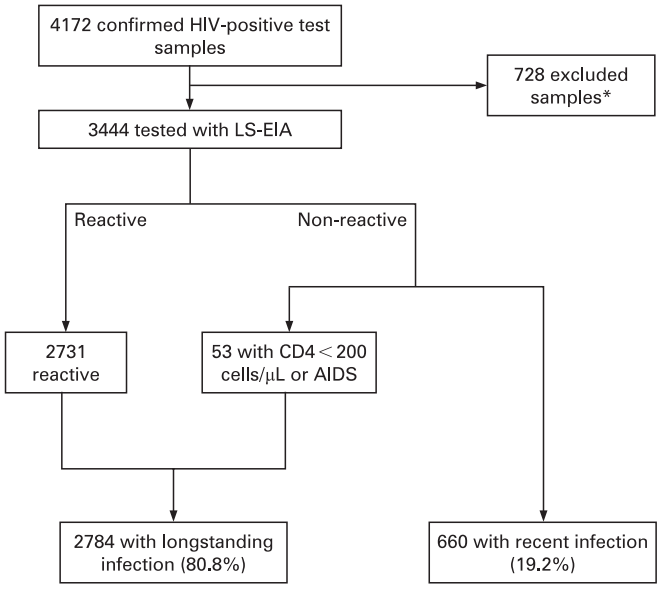

Figure 1 Algorithm for the identification of recently infected samples. *Of the 728 samples excluded, 75.0% belonged to patients whose HIV infection was diagnosed more than 6 months before sample collection, 11.1% were duplicate samples, 9.2% were HIV-negative samples, 2.7% did not have enough volume, 1.4% were negative by Western blot and 0.5% were from patients aged under 18 years.

centres—when compared with the HIV reporting system of Catalonia—represents 74% of all HIV cases reported during the same period. Therefore, although not representative, we consider the diagnoses of HIV in the study population to be indicative of those currently made in our setting. According to our results, 19.2% of new diagnoses had acquired the infection during the 6 months before the test and this is consistent with the results of a recently published study from 10 United States cities.[12] The percentage of RIs varied according to the type of recruitment centre and the differences were statistically significant. Although a lower percentage of positive samples from hospitals were included in the study, the fact that this group had the lowest percentage of RIs suggests that PHCs, STI clinic and CBVCTSs receive a high number of RIs because they are the first line of referral and, therefore, much closer to the community. Given the increasing evidence that people who are diagnosed with HIV reduce risk behaviour and, thus, HIV transmission,[15 16] this finding clearly supports the need to improve the offer of HIV testing and reinforce the activity of CBVCTSs, including implementation of rapid HIV testing.[17]

Data from regional HIV reporting systems in Spain show that 46% and 31% of all new diagnoses correspond to heterosexual individuals and MSM, respectively.[3] The high proportion of MSM among RIs in our study (62.5%) may be attributable to several factors. Although a potential selection bias may have occurred because the STI clinic in Madrid, which also has a voluntary counselling and testing service, attends a high proportion of patients who are MSM, even if this centre is removed from the analysis, MSM account for 56% of all individuals identified as having RI. Therefore, other factors should be considered. First, concern among the gay community about infection means that they test more often and sooner after a potential exposure. In Spain, 39.4% of the general population had taken an HIV test once,[18] whereas for MSM this percentage was 81.0%.[4] Accordingly, MSM is the group with the lowest percentage of diagnoses made with <200 CD4 cells/μL.[4] Second, the incidence of sexual risk practices and STIs are increasing among MSM in our setting,[4 19–21] suggesting that the incidence of HIV is higher among this group and that the likelihood of diagnosing infection at an earlier stage could increase. Our results also support that the HIV epidemic is growing again among the local gay community.

Almost 39% of all individuals with RI reported at least one STI diagnosed over the previous 12 months, the highest percentage being at PHCs (75%). This finding should be interpreted with caution because of the high number of missing values for this variable. While there was information on co-infection for all RIs identified at STI clinics, this was available for only 53.0%, 13.0% and 70.6% of RIs from hospitals, PHCs and CBVCTSs, respectively. Moreover, one laboratory receiving samples from PHCs in Barcelona also serves as a STI unit and this may contribute to the high percentage of co-infection at this site. However, even assuming that all patients from whom we have no information on STI co-infection were not infected, the percentage of patients with a RI and a STI during the year before HIV diagnosis would be 17% (table 2). Consistent with those of other studies,[4 20 21] our results suggest that STI may play a role in the transmission of HIV in our setting.

Although we only had information on the country of origin for 65% of the individuals with RI, more than a quarter of those infected were immigrants. The proportion of immigrants with RI was much higher (78.6%) at CBVCTSs than at the other sites. Since we have no information on LSI, it is difficult to interpret this data; however, if we assume a similar proportion of immigrants among the LSI, it may reflect a preference for non-official sites that offer anonymous voluntary counselling and testing to this population. Otherwise, it could reflect a higher incidence of HIV infection among this group. To ascertain whether these infections were imported or locally acquired, we recorded the date of arrival in Spain. Of 71 immigrants with RI for whom this variable was available, 59 (83%) arrived in Spain more than 1 year before they were diagnosed with HIV. Although the finding cannot be extrapolated, it does suggest that in Spain most HIV infections among immigrants are locally acquired. This is consistent with the fact that in our setting, unlike the UK, immigrants come mainly from countries with a low prevalence HIV, such as Morocco, Romania and Latin America.[22]

A possible limitation of our study was the difficulty in retrieving clinical-epidemiological data from all the patients whose samples were studied. On the one hand, we could not systematise the collection of additional data from the samples studied and had to limit it to RI. On the other, samples from laboratories corresponding to outside clinics could not be linked to clinical records because of confidentiality issues. Therefore, the characteristics of individuals with RI were not compared with the characteristics of individuals with LSI and this has resulted in a relatively high percentage of missing values in some variables from patients with RI. Another limitation was the differences in sample recruitment between the different centres. Whereas some centres collected 90% of all positive samples, others collected 50%. Nevertheless, our results are consistent with other studies and with the epidemiological trends identified using other information sources.[4]

The limitations of STARHS are well-known, especially misclassification of advanced disease stage, AIDS or patients on antiretroviral treatment as RIs. Nevertheless, access to clinical and laboratory information allowed us to detect and exclude misclassifications.[23] The Vironostika HIV-1 EIA is based on subtype B antigens. Therefore, when used to detect RI, this assay performs differently on B and non-B subtypes,[24] with a longer window period on non-B subtypes. Although few studies

Table 2 Characteristics of people with recent infection (2003–2005) by site of diagnosis

	Total n (%)	Hospitals n (%)	PHCs n (%)	STI clinic n (%)	CBVCTSs n (%)	p Value§
Gender (n = 639)*						<0.001**
Men	510 (79.8)	227 (80.2)	188 (73.2)	78 (95.1)	17 (100.0)	
Origin (n = 426)*						<0.001††
Spain	313 (73.5)	180 (73.2)	73 (83.0)	57 (73.1)	3 (21.4)	
Immigrants	113 (26.5)	66 (26.8)	15 (17.0)	21 (26.9)	11 (78.6)	
South and Central America	62 (54.9)	33 (50.0)	8 (60.0)	13 (61.9)	7 (63.6)	
Western and Central Europe	18 (15.9)	7 (10.6)	4 (26.7)	7 (33.3)	0 (0.0)	
Middle East and North Africa	11 (9.7)	6 (9.1)	1 (6.7)	1 (4.8)	3 (27.3)	
Sub-Saharan Africa	10 (8.8)	9 (13.6)	1 (6.7)	0 (0.0)	0 (0.0)	
Eastern Europe and Central Asia	8 (7.1)	7 (10.6)	0 (0.0)	0 (0.0)	1 (9.1)	
Other	4 (3.5)	4 (6.1)	1 (6.7)	0 (0.0)	0 (0.0)	
Age, y† (n = 623)*	32.9	33.2	33.0	31.8	30.1	0.741
<30	225 (36.1)	105 (38.2)	83 (33.1)	29 (36.3)	8 (47.1)	
30–40	274 (44.0)	113 (41.1)	116 (46.2)	38 (47.5)	7 (41.2)	
40–50	93 (14.9)	40 (14.5)	42 (16.7)	10 (12.5)	1 (5.9)	
>50	31 (5.0)	17 (6.2)	10 (4.0)	3 (3.8)	1 (5.9)	
Transmission group (n = 389)*						<0.001‡‡
MSM	243 (62.5)	125 (54.8)	41 (62.1)	73 (90.1)	7 (50.0)	
Heterosexual	90 (23.1)	64 (28.1)	15 (22.7)	7 (8.6)	1 (7.1)	
Injection drug users	56 (14.4)	39 (17.1)	10 (15.2)	1 (1.2)	6 (42.9)	
STI‡ (n = 281)*	109 (38.8)	54 (35.8%)	27 (75%)	27 (32.9%)	1 (8.3%)	<0.001§§
Non-specified syphilis¶	52 (47.7)	27 (50.0)	18 (66.7)	7 (25.9)	0 (0.0)	
Gonococci	10 (9.2)	4 (7.4)	3 (11.1)	3 (11.1)	0 (0.0)	
Herpes	5 (4.6)	2 (3.7)	1 (3.7)	2 (7.4)	0 (0.0)	
Chlamydia	3 (2.7)	2 (3.7)	0 (0.0)	1 (3.7)	0 (0.0)	
Other/non-specified	39 (35.8)	19 (35.2)	5 (18.5)	14 (51.9)	1 (100.0)	

*The number of samples with available information for each variable. Stratified subtotals may not match the total number of RI because missing values were excluded from each variable; †median; ‡having a diagnosis of a sexually transmitted infection (STI) within 12 months before study inclusion; §p value, Pearson's χ^2 test; ¶the diagnosis of syphilis was self-reported by the patient, during the last 12 months, before study inclusion; **differences between hospitals and STI clinic and primary health centres (PHC); ††differences between community-based voluntary counselling and testing sites (CBVCTSs) and each of the other sites; ‡‡differences between STI clinics and each of the other sites; §§differences between PHCs and each of the other sites.

have analysed the distribution of HIV subtypes in Spain, only 7.6% of all clinically diagnosed acute HIV infections may be non-B subtypes.[25] However, given that most immigrants come from South America,[22] it is unlikely that non-B HIV subtypes have significantly affected the results of this study. In any case, they would have produced a slight overestimation of the percentage of RI. Nevertheless, since 2008 our laboratory has been using the BED assay, which has a similar window period for HIV subtypes A to E[26 27] and better reproducibility since it is based on the HIV IgG to non-HIV IgG ratio and uses a simple 1:100 dilution, which performs better in subtypes B, E and D.[26 27]

In summary, the study showed that implementing STARHS in our setting is feasible and, more importantly, that in order to improve the use of the data collected, it is necessary to implement these studies in settings where more complete clinical-epidemiological data are available. Consequently, since 2006, STARHS has been included in the Integrated AIDS/HIV/STI Surveillance System in Catalonia by systematically testing all new HIV diagnoses made in 14 hospitals, nine of them participating in an open cohort (PISCIS) of people who are HIV positive,[28 29] as well as throughout the CBVCTS network of the region.[4] Eventually, this should enable us to collect data from patients with LSI and to estimate incidence rates. This study highlights important epidemiological features of local HIV infection, such as the ongoing spread of HIV among MSM, the potential role of STIs in new HIV infections and the vulnerability of immigrants as a new target population, which should be taken into account in national HIV and STI prevention strategies.

Key messages

- Serologic Testing Algorithm for Recent HIV Seroconversion (STARHS) is feasible and it helps to identify local epidemiological features.
- Since 2006, STARHS is included in the HIV/STI Integrated Surveillance System of Catalonia, Spain.

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Recently acquired HIV infection in Spain (2003–2005): introduction of the serological testing algorithm for recent HIV seroconversion

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Env-Specific IgA from Viremic HIV-Infected Subjects Compromises Antibody-Dependent Cellular Cytotoxicity

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ABSTRACT

Elucidating the factors that modulate HIV-specific antibody-dependent cellular cytotoxicity (ADCC) will help in understanding its role in HIV immunity. The aim of this study was to determine whether IgA could modify the magnitude of ADCC in HIV infection, abrogating its protective role. Plasma samples from 20 HIV-positive (HIV^+) subjects enrolled during primary HIV infection (PHI), 10 chronically infected subjects (chronic), and 7 elite controllers (EC) were used. ADCC was determined by using a fluorometric ADCC assay, before and after removal of plasma IgA. Data were analyzed by using nonparametric statistics. ADCC was documented in 80% of PHI enrollment samples and in 100% of PHI 12-month, chronic, and EC samples; it peaked after acute infection, reached a plateau in chronic infection, and decreased after initiation of antiretroviral treatment (ART). Significant associations between ADCC and disease progression were found only after removal of plasma IgA from 12-month PHI samples: the magnitude of ADCC not only increased after IgA removal but also correlated with $CD4^+$ T-cell preservation. This work provides evidence that gp120-specific IgA was capable of modifying ADCC responses during natural HIV infection for the first time and adds to similar evidence provided in other settings. Furthermore, it underscores the complexity of the ADCC phenomenon and will help in an understanding of its underlying mechanisms.

IMPORTANCE

Although the induction of ADCC-mediating antibodies in HIV-infected subjects has been extensively documented, the association of these antibodies with protection from disease progression is poorly understood. Here, we demonstrate that plasma IgA is a factor capable of modifying the magnitude of IgG-mediated ADCC in HIV infection, mitigating its beneficial effect. These results help in understanding why previous studies failed to demonstrate correlations between ADCC and disease progression, and they also contribute to the notion that an HIV vaccine should stimulate the production of ADCC-mediating IgG antibodies but not IgA.

Despite the success of antiretroviral treatment (ART), human immunodeficiency virus (HIV) still represents a major public health concern (1), and a vaccine is urgently needed. One major advance of the RV144 trial was the achievement of an estimated efficacy of 31.2% (2). Subsequent correlate analyses showed that Env-specific IgG antibodies correlated inversely with infection risk (3). Moreover, antibody-dependent cellular cytotoxicity (ADCC), broadly induced by this vaccine regimen (4), was found to be a correlate of protection in vaccinees with low levels of Env-specific IgA (3). These findings suggested that the modest protection induced by the RV144 vaccine regimen might be attributed to humoral immunity and, more specifically, to ADCC.

Besides the RV144 trial, there are many other reasons to reexamine the mechanisms of ADCC during the natural course of HIV infection. The induction of ADCC-mediating antibodies in plasma (5–11), cervicovaginal fluids (12, 13), and breast milk (14) from HIV-infected subjects has been extensively documented. However, their association with protection from disease progression is less unequivocal. Cohort studies performed with elite controllers (ECs) showed that these individuals had higher ADCC than viremic subjects (9). One early report by Baum et al. (5) established that ADCC was associated with disease progression in terms of $CD4^+$ T-cell counts, but later, other studies on recently and chronically infected subjects failed to demonstrate definitive and conclusive associations (7, 8, 11, 15–20). More recently, passively acquired ADCC activity in infants born to HIV-infected mothers was not associated with protection but was associated with reduced mortality (21).

Many factors could have influenced the dissimilar results and precluded the drawing of definite conclusions, including the use of different models to assay ADCC, inclusion criteria to enroll

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TABLE 1 Characteristics of HIV$^+$ subjects enrolled per study group

Group and patient	Sex[i]	Age (yr)	Infection stage[a]	No. of days postinfection[b]	VL[c,d]		Viral set point[f] (log_{10} HIV-1 RNA copies/ml plasma)	CD4$^+$ T-cell count[cec,e] (cells/μl)	CD4 set point[f] (cells/μl)	Presence of <350 CD4 cells/μl during 1st yr[g]	Treatment with HAART[h]
					HIV-1 RNA copies/ml plasma	Log_{10} HIV-1 RNA copies/ml plasma					
PHI (n = 20)											
NP01	M	45	Stage V	60	2,707	3.4	3.9	902	600	No	No
NP02	M	40	Stage VI	60	8,613	3.9	4.3	851	758	No	No
NP03	M	47	Stage V	45	112	2.0	2.5	743	679	No	No
NP04	M	43	Stage V	45	19,522	4.3	4.3	525	442	No	No
NP05	F	37	Stage V	60	9,532	4.0	3.6	590	587	No	No
NP06	M	32	Stage VI	150	256,051	5.4	4.7	797	943	No	No
NP07	M	33	Stage V	45	21,986	4.3	3.3	609	658	No	No
NP08	F	26	Stage VI	60	17,420	4.2	4.1	685	402	No	No
NP09	F	34	Stage V	24	>500,000	>5.7	4.3	603	490	No	No
NP10	M	73	Stage VI	120	13,962	4.1	4.6	577	544	No	No
NP11	M	30	Stage V	45	455,417	5.7	5.2	698	685	No	No
P01	F	31	Stage V	30	>500,000	>5.7	5.4	374	342	Yes	No
P02	F	41	Stage VI	90	3,662	3.6	3.9	302	281	Yes	No
P03	M	43	Stage V	45	98,684	5.0	4.9	256	256	Yes	No
P04	M	40	Stage V	70	1,147	3.1	3.5	568	424	Yes	No
P05	M	25	Stage VI	60	242,199	5.4		161		Yes	Yes
P06	M	36	Stage VI	60	>500,000	5.7		475		Yes	Yes
P07	M	26	Stage VI	60	>500,000	5.7		446		Yes	Yes
P08	M	45	Stage V	60	>500,000	5.7		259		Yes	Yes
P09	F	28	Stage VI	30	>500,000	5.7		222		Yes	Yes
Chronic (n = 10)											
C01	F	21	Chronic		85,947	4.9		25			No
C02	M	22	Chronic		18,580	4.2		511			No
C03	M	49	Chronic		43,436	4.6		479			No
C04	M	28	Chronic		555	2.7		555			No
C05	M	36	Chronic		1,288	3.1		431			No
C06	F	27	Chronic		1,817	3.2		689			No
C07	M	47	Chronic		11,026	4.0		585			No
C08	F	38	Chronic		22,475	4.3		143			No
C09	F	29	Chronic		14,784	4.3		139			No
C10	F	24	Chronic		253,167	5.4		5			No
EC (n = 7)											
EC01	M	54	Chronic		<50	<1.7		566			No
EC02	F	41	Chronic		<50	<1.7		912			No
EC03	M	36	Chronic		<50	<1.7		456			No
EC04	F	60	Chronic		<50	<1.7		570			No
EC05	F	43	Chronic		<50	<1.7		817			No
EC06	M	39	Chronic		<50	<1.7		595			No
EC07	F	37	Chronic		<50	<1.7		344			No

[a] PHI subjects were stratified according to Fiebig stages (31).
[b] Number of days from the presumed date of infection to the date at which the enrollment sample was obtained.
[c] For PHI subjects, data correspond to enrollment samples. For chronically infected and EC subjects, data correspond to samples obtained at enrollment.
[d] Performed by using a Versant HIV-1 RNA 3.0 assay (Siemens). Lower and upper detection limits are 50 and 500,000 HIV RNA copies/ml, respectively (1.7 and 5.7 log_{10} HIV RNA copies/ml, respectively).
[e] Flow cytometry double platform (FACSCanto; BD Biosciences).
[f] Applicable only to PHI subjects who did not start highly active antiretroviral therapy during the first year postinfection (see Materials and Methods).
[g] Indicates whether the CD4$^+$ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not, thus defining subgroups where the PHI counts were <350 and >350 cells/μl (see Materials and Methods).
[h] Regarding PHI subjects, data illustrate if subjects started highly active antiretroviral therapy (HAART) during the first year postinfection. For those subjects who started highly active antiretroviral therapy, enrollment samples were obtained before treatment initiation. Data indicate whether chronically infected and EC subjects had ever been on highly active antiretroviral therapy.
[i] F, female; M, male.

TABLE 2 Summary of clinical data corresponding to HIV$^+$ subjects enrolled per study group[g]

Group	Median no. of days postinfection (IQ range)[a]	Viral load[c,d]		Viral set point[f] (mean $\log_{10}$ HIV RNA copies/ml ± SD)	Median CD4$^+$ T-cell count[d,e] (cells/μl) (IQ range)	Median CD4 set point[f] (cells/μl) (IQ range)
		Median HIV RNA copies/ml (IQ range)	Mean HIV RNA $\log_{10}$ copies/ml ± SD			
PHI						
All ($n = 20$)[b]	60 (45–60)	60,335 (8,843–488,854)	4.6 ± 1.07	4.1 ± 0.7	572 (320–695)	544 (402–679)
PHI>350 ($n = 11$)	60 (45–60)	17,420 (8,613–256,051)	4.2 ± 0.74	4.2 ± 1.7	685 (590–797)	600 (490–685)
PHI<350 ($n = 9$)	60 (37,50–65)	242,199 (51,173–500,000)	4.9 ± 0.9	4.4 ± 0.87	302 (239–460)	311 (262–403)
Chronic ($n = 10$)		16,682 (1,685–54,064)	4.1 ± 0.83		455 (110–562)	
EC ($n = 7$)		<50	<1.7		570 (456–817)	

[a] Number of days from the presumed date of infection to the date at which the enrollment sample was obtained.
[b] In this group, 5 subjects initiated highly active antiretroviral therapy during the first year postinfection.
[c] Versant HIV-1 RNA 3.0 assay (Siemens). Lower and upper detection limits are 50 and 500,000 HIV RNA copies/ml, respectively (1.7 and 5.7 $\log_{10}$ HIV RNA copies/ml, respectively).
[d] For PHI subjects, data correspond to enrollment samples. For chronic and elite controller subjects, data correspond to samples obtained at enrollment.
[e] Flow cytometry double platform (FACSCanto; BD Biosciences).
[f] Set points were not calculated for subjects who initiated highly active antiretroviral therapy during the first year postinfection ($n = 5$) (see Materials and Methods).
[g] IQ, interquartile.

study subjects, and definitions of progression, but also, the putative existence of mitigating plasma factors interfering with ADCC has been proposed as a factor. In other words, if the potential protective role of ADCC-mediating antibodies was mitigated by any factor during natural infection, it would not be surprising to find any associations between ADCC and progression. Remarkably, this has not been extensively studied yet, highlighting that the field deserves further research. The IgG1 and IgG3 subclasses were shown to be potent inducers of anti-HIV ADCC (19, 21–23). Conversely, the role of the IgA isotype is controversial (24). Correlate analysis from the RV144 trial suggested that vaccine-induced plasma IgA might block IgG binding, interfering with its effector function (23). However, whether such an effect might occur in HIV-infected subjects has not been elucidated yet.

The aim of this study was to determine if IgA was a factor capable of modifying the magnitude of IgG-mediated ADCC in HIV infection, abrogating its protective role. The results indicated that the magnitude of ADCC after removal of IgA was higher than that in nondepleted plasma and correlated directly with the percentage of CD4$^+$ T cells in viremic subjects, thus supporting the hypothesis presented. To our knowledge, this is the first study demonstrating that the beneficial effect of ADCC is mitigated by gp120-specific IgA during natural HIV infection.

MATERIALS AND METHODS

Study subjects. The following study groups were enrolled (Tables 1 and 2): 8 healthy HIV-seronegative donors (HIV^{neg}), 10 chronically infected subjects (chronic) (infected for >3 years, detectable viral load [VL], and ART naive), 7 ECs (infected for >5 years, undetectable VL [<50 HIV RNA copies/ml plasma], ART naive, and no record of opportunistic and/or AIDS-related diseases), and 20 subjects enrolled within 6 months from the presumed date of infection (primary HIV infection [PHI]). PHI subjects were enrolled by the Grupo Argentino de Seroconversión Study Group. Inclusion criteria, time from infection, and set point calculations were reported previously (25–27). Briefly, a PHI enrollment sample was obtained in the case of detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western blot assay result or a positive Western blot result with a negative test within the previous 6 months. Chronically infected individuals were defined as subjects infected for >3 years who had a detectable VL (>50 HIV RNA copies/ml plasma) and were ART naive, and ECs were defined as subjects infected for >5 years who had an undetectable VL (<50 HIV RNA copies/ml plasma), were ART naive, and had no record of opportunistic and/or AIDS-related diseases. The study was reviewed and approved by the Comité de Ética Humana, Facultad de Medicina, Universidad de Buenos Aires. All participants provided written informed consent.

Plasma VL (branched DNA) (Versant HIV-1 RNA 3.0 assay; Siemens Healthcare, United Kingdom), CD4$^+$ T-cell count (flow cytometry double platform) (BD FACSCanto; BD Biosciences, USA), cellular immune activation (HLA-DR and CD38 expression on peripheral blood mononuclear cells [PBMCs] as determined by flow cytometry [26]), and plasma soluble factors (39-plex Milliplex multi-analyte panel human cytokine/chemokine kit; Millipore, USA) were assessed as described previously (26–28).

Samples. Blood samples were collected and centrifuged to separate plasma. For ADCC assays, plasma was first diluted (10-fold in RPMI medium), passed through a 0.2-μm-pore-size filter, and heat inactivated (1 h at 56°C). PBMCs from one HIV-negative donor were isolated by Ficoll-Hypaque density centrifugation (Amersham, Sweden), cryopreserved, and used as effector cells in ADCC assays. Cells from the same donor were used in all assays to avoid bias from donor to donor.

RFADCC assay. A rapid fluorometric ADCC (RFADCC) assay was performed as described previously (29). Briefly, CEM-NK^R target cells (AIDS Research and Reference Reagent Program) (30) were double stained with PKH26 red fluorescent cell linker (Sigma-Aldrich, USA) and CFSE (carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, USA) and coated with a recombinant gp120 protein derived from the HIV-1 BaL strain (recombinant $gp120_{BAL}$, obtained from the AIDS Research and Reference Reagent Program, DAIDS, NIAID). After 1 h, cells were dispensed in U-bottom 96-well plates (5,000 cells/well) together with different dilutions of inactivated plasma, in triplicates. After 15 min at room temperature, effector cells (PBMCs thawed and rested overnight) were added at an effector-to-target cell ratio of 50:1. Plates were centrifuged and incubated for 4 h at 37°C. Cells were washed, fixed, acquired on a FACSCanto flow cytometer (BD Biosciences, USA), and analyzed by using FACSDiva v6.1.3 software (BD Biosciences). Target cells were initially gated on a forward-scatter (FSC)-versus-side-scatter (SSC) plot and subsequently gated on an SSC-versus-PKH26 plot. Next, a PKH26-versus-CFSE plot was generated to determine the percent target cell killing (%ADCC killing), which was calculated as the proportion of cells that remained PKH26^{high} but that had lost the viability dye (CFSE^{neg}). Results are presented as the medians of data from experiments performed in

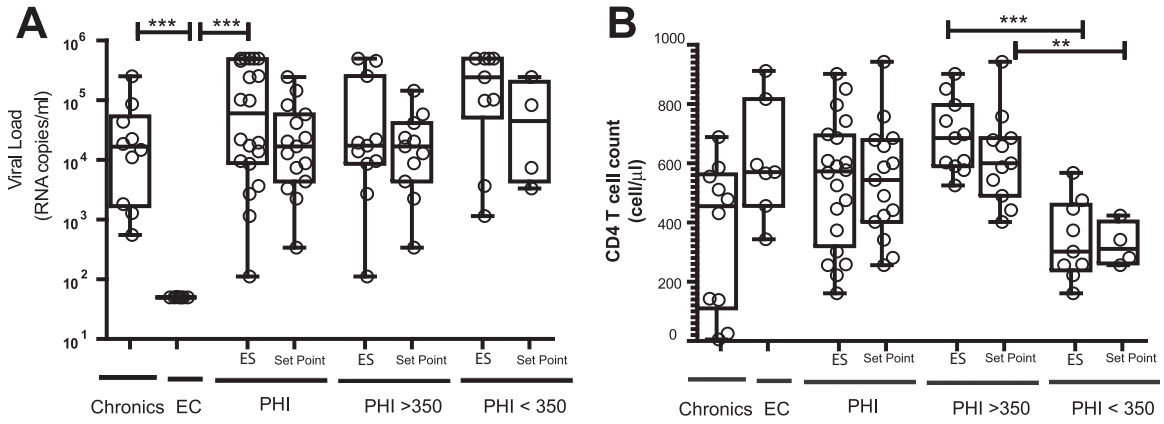

FIG 1 Viral loads (A) and CD4 T-cell counts (B) of enrolled HIV$^+$ subjects per study group. For PHI subjects, values corresponding to both baseline and the set point are shown. Also, PHI subjects are shown as a whole group (PHI) and split into subgroups, PHI>350 and PHI<350, dependent on whether their CD4$^+$ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not. For PHI subjects, viral and CD4 set points were calculated as the geometric means of determinations obtained between 6 and 12 months after the presumed date of infection. Set points were not calculated for those subjects who started ART during the first 12 months of infection ($n = 5$). Inclusion criteria for subjects and time from infection were reported previously (25–27). The 350-cells/μl endpoint was chosen based on national and international recommendations for initiation of ART from 2010, when most of these individuals were already enrolled (25). Horizontal lines stand for median values. ES, enrollment samples. *P* values were calculated by using a Mann-Whitney U test. Asterisks denote different *P* values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

triplicate and with the background subtracted (%ADCC killing for uncoated target cells). The threshold for positive responses for a given plasma dilution was defined as the mean of the background plus 3 standard deviations (SD). The endpoint ADCC titer was defined as the highest plasma reciprocal dilution at which the %ADCC killing was greater than or equal to the cutoff value.

Coating and saturation of target cells were verified by flow cytometry with anti-gp120 monoclonal antibody 2G12 (AIDS Research and Reference Reagent Program) followed by staining with anti-human IgG-allophycocyanin (APC).

ELISA for Env-specific plasma IgG and IgA. The gp120-specific IgG concentration and gp120-specific IgA titers were determined by an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well, flat-bottomed, half-area plates (GreinerBio-One, Germany) were coated with 25 ng/well of $gp120_{BAL}$. For gp120-specific IgG quantitation, 25 μl/well of plasma dilutions (initially 1/500 for PHI enrollment samples and 1/10,000 for the other groups) was dispensed in triplicates. A standard curve was constructed, consisting of 2-fold serial dilutions of anti-gp120 monoclonal antibody 2G12 starting at 24 ng/ml. IgG detection was performed by using an anti-human IgG antibody labeled with horseradish peroxidase (HRP; Sigma-Aldrich, USA) and developed with tetramethylbenzidine (TMB)-ELISA solution (BD Biosciences, USA). The absorption at 450 nm was read on a Multiskan EX microplate reader (Thermo/Labsystems). The IgG concentration was extrapolated from the standard curve and multiplied by the dilution factor. gp120-specific IgA levels were determined by endpoint titration. Twofold serial dilutions of plasma were prepared, starting at a 1:20 dilution. The secondary antibody was an anti-human IgA-HRP (Sigma-Aldrich, USA). Plates were developed and read as described above for IgG. The endpoint IgA titer was defined as the reciprocal of the highest plasma dilution at which the average optical density (OD) value was ≥2-fold the average OD value for control wells. Sera from HIV-negative subjects were tested as controls.

Depletion of plasma IgA. Bulk IgA was removed from plasma by using the Pierce immunoprecipitation kit (Thermo Scientific, USA). Briefly, 1 mg of goat anti-human IgA antibody (Sigma-Aldrich, USA) was immobilized onto columns. Plasma samples diluted 1/10, filtered, heat inactivated, and supplemented with protease inhibitors (Pierce) were added to the columns, incubated overnight at 4°C, and eluted by centrifugation. The flowthrough was collected and stored for further analysis. Plasma samples passed through columns coupled with an isotype-matched control antibody were used as controls (nondepleted plasma). To confirm IgA (but not IgG) depletion, dot blots developed by using either HRP-labeled goat anti-human IgA or IgG antibodies were performed. Additionally, levels of gp120-specific IgA and IgG were requantitated by an ELISA after IgA depletion, as described above for whole plasma. Additionally, IgG binding to gp120-coated cells in IgA-depleted and nondepleted plasma samples was evaluated by flow cytometry using anti-human IgG-APC as a secondary antibody. The mean fluorescence intensity (MFI) was recorded.

Data analysis. Statistical analyses were performed by using GraphPad Prism 5 software. Data are expressed as median values with interquartile ranges (25% to 75%) and were analyzed by nonparametric methods unless otherwise stated. Mann-Whitney or Kruskal-Wallis tests were used to compare two or multiple intergroup variables, respectively. A Wilcoxon test was used to compare paired variables. Correlations were determined by using Spearman's rank test. All tests were considered significant when the *P* value was <0.05.

RESULTS

Description of study subjects. Plasma samples from 37 HIV-infected subjects were used: 20 PHI subjects enrolled within 6 months of infection, 10 chronically infected subjects, and 7 ECs (shown in Table 1 and summarized in Table 2). Enrollment samples for PHI subjects were obtained at a median of 60 days from the presumed date of infection and corresponded to Fiebig stages V and VI (31). The median VL and CD4$^+$ T-cell counts for the PHI group at enrollment were 60,335 HIV RNA copies/ml and 572 cells/μl, respectively (Table 2 and Fig. 1). For the chronic group, median VL and CD4$^+$ T-cell counts were 16,682 HIV RNA copies/ml and 455 cells/μl, respectively. ECs had undetectable VL and a median of 570 CD4$^+$ T cells/μl. In the PHI group, 5 subjects initiated ART during the first year postinfection (median of 90 days postinfection) following medical indication (Table 1). However, enrollment samples were always obtained before ART instauration. Data obtained by using samples obtained when the subject was on ART were analyzed separately. For certain analyses, PHI subjects were further divided into two subgroups (Tables 1 and 2 and Fig. 1): those whose CD4$^+$ T-cell counts dropped below

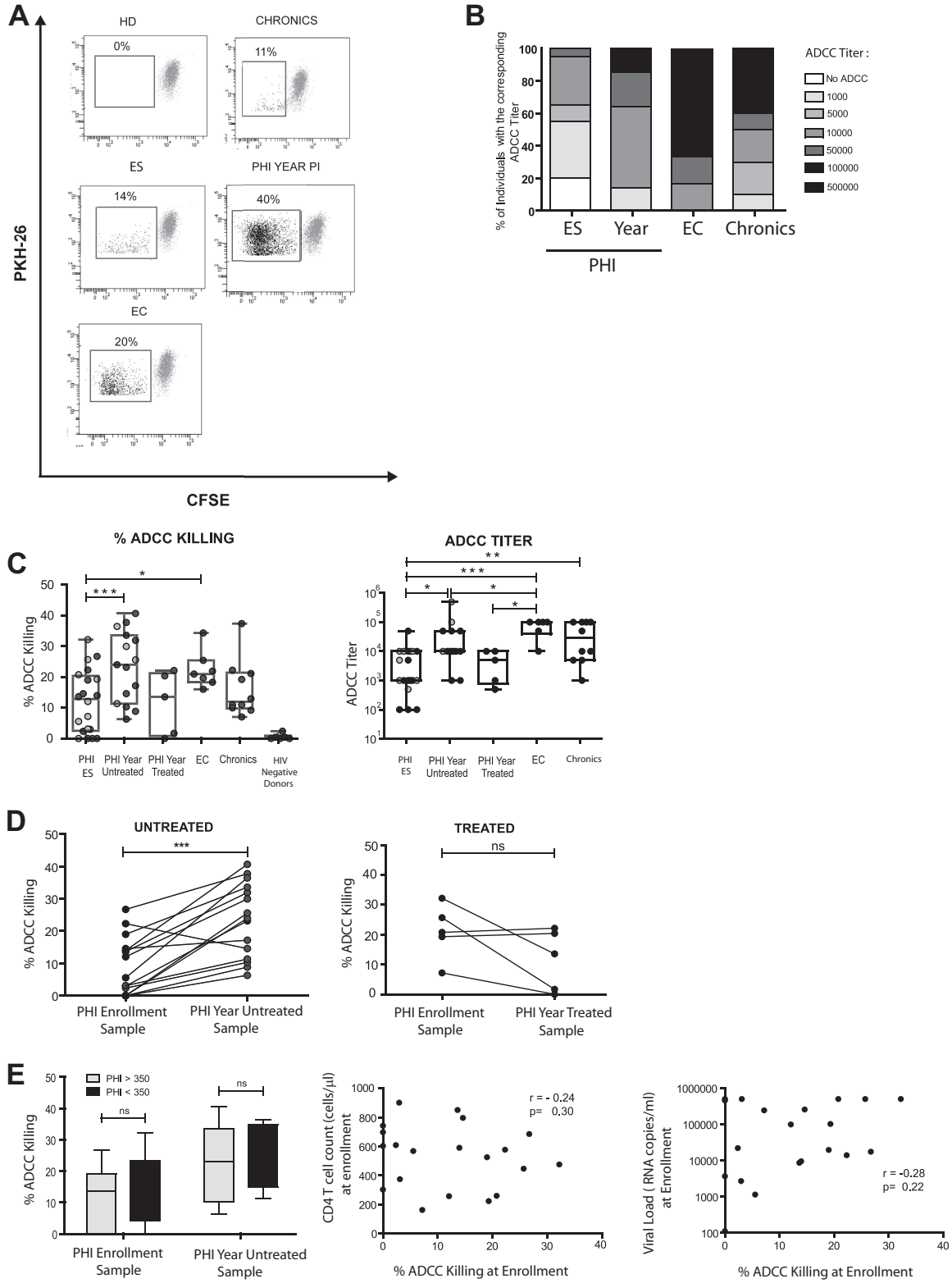

FIG 2 ADCC responses in PHI subjects, chronically infected individuals (chronics), and elite controllers (EC) measured by an RFADCC assay.(A) Dot plots for one representative subject per study group. A PKH26-versus-CFSE plot was generated to determine the percentage of ADCC killing, defined as the proportion of cells that remained PKH26^{high} but lost the viability dye (CFSE^{neg}). Percentages shown in each plot correspond to results obtained after calculating and subtracting the background (%ADCC killing for uncoated target cells) from the media of triplicate conditions. Initially, gating was performed with an FSC-versus-SSC plot. Target cells were gated on an SSC-versus-PKH26 plot. (B) Proportion of individuals with the corresponding ADCC titers. To calculate ADCC titers, the threshold for positive responses was defined as the mean background %ADCC killing plus 3 SD. The endpoint ADCC titer was defined as the reciprocal of the higher plasma dilution at which the %ADCC killing was greater than or equal to the threshold. (C)

350 cells/μl at any time during the first year postinfection (PHI<350) (rapid progressors) and those whose $CD4^+$ T-cell counts did not (PHI>350) (typical progressors). By doing this, we aimed to differentiate subjects with more rapid or aggressive progression of early infection and to investigate the association of this pattern with the magnitude of ADCC responses. Regarding chronically infected subjects, the individuals enrolled in this study included subjects with preserved immune status as well as subjects with advanced immune deterioration (Table 1), reflecting the natural heterogeneity of such an HIV-positive (HIV^+) population.

ADCC responses emerge early after infection and increase during the first year postinfection but not in subjects receiving ART. First, we aimed to revisit previously reported ADCC findings by determining gp120-specific antibodies capable of mediating ADCC in plasma samples from all study subjects (for PHI, enrollment and 12-month samples were used) by using an RFADCC assay. Representative dot plots are shown in Fig. 2A. The results indicated that 80% of PHI subjects had detectable ADCC responses (defined as a titer of ≥1,000) at enrollment, with this number increasing to 100% by 12 months postinfection. Chronically infected subjects had ADCC titers of ≥1,000, and all ECs had titers of ≥10,000 (Fig. 2B). When comparing PHI enrollment and 12-month samples from ART-naive subjects, both the %ADCC killing and ADCC titers significantly increased over time (median %ADCC killing of 12.85% versus 24% [$P = 0.0003$]; median titer of 1,000 versus 10,000 [$P = 0.0206$]) (Fig. 2C and D, left). Conversely, subjects who started ART during the course of the study showed a decrease or stability of both ADCC titers and %ADCC killing with the 12-month samples, compared to samples from untreated PHI subjects, chronically infected subjects, and ECs (Fig. 2C and D, right). The %ADCC killing and ADCC titers did not differ significantly between chronically infected and EC subjects. Only a minor, but still significant, difference was observed in comparisons of EC and PHI 12-month ADCC titers.

Overall, gp120-specific antibodies capable of mediating ADCC arise early after infection, and their levels increase over time. This was further noticed when PHI subjects were segregated according to their Fiebig stage at enrollment: samples corresponding to Fiebig stage VI had significantly higher ADCC than did Fiebig stage V samples by analysis of either %ADCC killing (medians of 3.1% and 19.33%, respectively; $P = 0.02$) or ADCC titers (medians of 1,000 and 10,000, respectively; $P = 0.01$), thus reinforcing the notion that the magnitude of the ADCC antibody response rapidly increases over time after infection. A plateau is then eventually reached, since no significant difference was observed between PHI 12-month samples and samples from the chronically infected group. Although ECs tended to have higher ADCC than chronically infected individuals, no statistically significant difference was observed, indicating that high titers of ADCC-mediating antibodies are maintained despite the absence of detectable viral replication in standard VL assays (50 HIV RNA copies/ml). The results also illustrated that initiation of ART during the first year postinfection modifies the kinetics of the ADCC response.

No association between the magnitude of the ADCC response and early disease progression. We then sought to investigate if the magnitude of the early ADCC response impacts subsequent disease progression. First, ADCC responses in samples from the PHI<350 and PHI>350 groups at both enrollment and 12 months were compared. No significant differences were observed between the PHI<350 and PHI>350 groups for either enrollment or 12 month samples (Fig. 2E, left [%ADCC killing]; ADCC titers not shown). No correlations were found between %ADCC killing at enrollment and enrollment VL (Fig. 2E, middle), $CD4^+$ T-cell counts (right), levels of cellular immune activation, or levels of soluble plasma biomarkers of immune activation (not shown). More relevant, no correlations were observed between ADCC at enrollment (evaluated as either %ADCC killing or ADCC titer) and viral set point, immune set point, $CD4^+$ T-cell decay slope, or 12-month VL and $CD4^+$ T-cell counts as alternative markers of disease progression (not shown). Finally, there were no observed correlations between 12-month ADCC and concurrent VL or CD4 T-cell count. Overall, these analyses showed that the magnitude of the early ADCC response has no impact on subsequent disease progression during the time frame investigated.

HIV-1 Env-specific IgG levels and IgG/IgA ratios (but not IgA levels) correlate with ADCC responses. Given the lack of observed correlations between ADCC and protection from disease progression, and considering previous reports attributing a protective role to ADCC-mediating antibodies in humans (5, 8, 9), we decided to investigate the presence of putative mitigating factors in the plasma of infected subjects by determining gp120-specific IgG and IgA levels and IgG/IgA ratios in subjects' plasma samples.

Levels of Env-specific IgG in PHI enrollment samples were significantly lower than those in PHI 12-month samples and in samples from the chronic and EC groups and increased over time in untreated infection but decreased in those subjects that initiated ART (Fig. 3A). No significant difference was observed between PHI 12-month, chronic, and EC samples. Conversely, gp120-specifc IgA titers declined over the course of infection except in ECs, who maintained significantly higher gp120-specifc IgA titers than those in 12-month PHI and chronic samples (Fig. 3B).

In order to exclude that declining gp120-specific IgA concur-

%ADCC killing corresponding to a plasma dilution of 1/1000 (left) and ADCC titers (right), corresponding to each group. %ADCC data correspond to background-subtracted values. Of note, the indicated differences in %ADCC killing were also statistically significant at all plasma dilutions tested. For HIV-negative donors, %ADCC killing corresponded to a 1/100 plasma dilution, which was the only dilution tested. (D) ADCC responses in PHI enrollment and 12-month postinfection samples from subjects who remained ART naive (left) ($n = 15$) or not (right) ($n = 5$) during the first year postinfection. Each line represents one subject. Results correspond to a plasma dilution of 1/1,000. (E) PHI subjects were further divided into two subgroups depending on whether their CD4 T-cell count dropped below 350 cells/μl at any time during the first year postinfection (PHI<350) or not (PHI>350). (Left) Comparison of %ADCC killing between the PHI<350 and PH>350 groups in both enrollment and 12-month samples (only ART-naive subjects). Results correspond to a 1/1,000 plasma dilution. (Middle and right) Correlations between ADCC responses evaluated in PHI subjects at enrollment versus enrollment $CD4^+$ T-cell counts (middle) and enrollment viral loads (right). For box-and-whisker plots, the horizontal line represents the median value, the boxes represent the interquartile range, and the whiskers extend from minimum to maximum values. Intra- and intergroup differences were analyzed by using Wilcoxon and Mann-Whitney tests, respectively. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. In panel B, black and gray dots denote PHI>350 and PHI<350 subjects, respectively. In correlation analyses, r and P values were determined by Spearman's test, and all P values were nonsignificant (ns). ES, enrollment samples; PI, postinfection; HD, healthy HIV-negative donors.

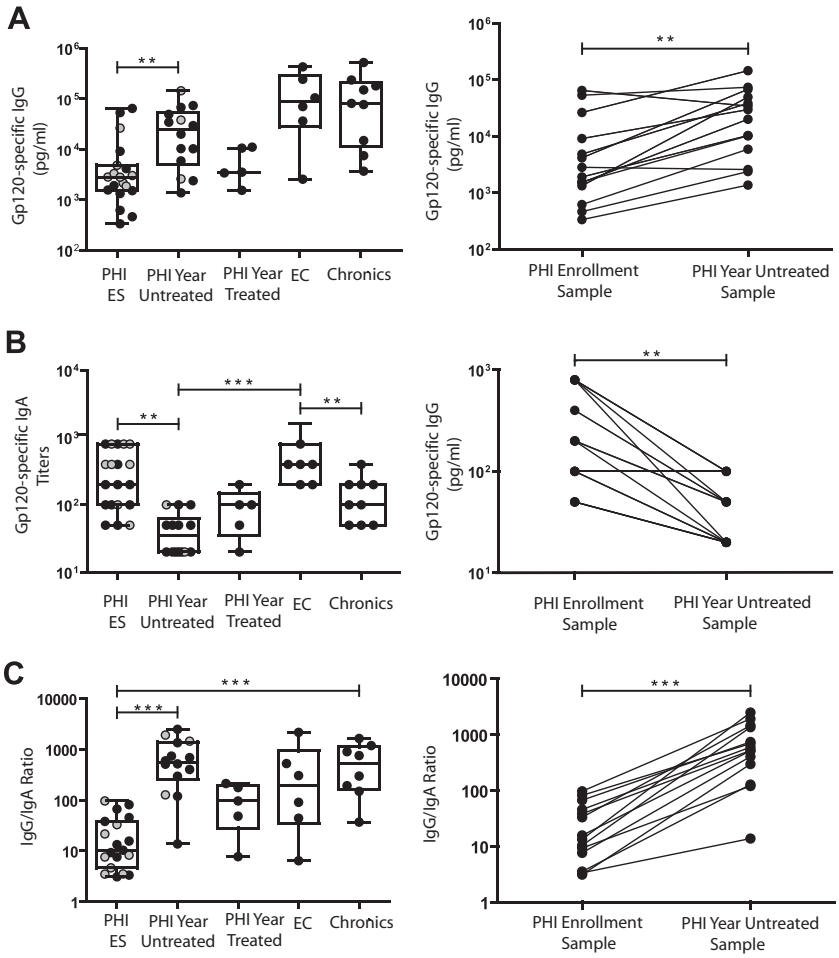

FIG 3 gp120-specific IgG concentrations, IgA titers, and IgG/IgA ratios among PHI, chronic, and EC subjects. To determine gp120-specific IgG antibodies and gp120-specific IgA titers, ELISAs were performed. (A) gp120-specific IgG antibodies (picograms per milliliter). (B and C) gp120-specifc IgA titers (B) and IgG/IgA ratios (C). (Left) Distribution of gp120-specific antibodies and IgG/IgA ratios in all study groups. (Right) Subject-by-subject comparison of gp120-specific antibodies in PHI enrollment versus 12-month samples (ART-naive subjects only). Each line represents one subject. In the left panels, the horizontal lines in box-and-whisker plots represent the median values, the boxes represent the interquartile ranges, and the whiskers extend from the minimum to the maximum values. Intra- and intergroup differences were analyzed by using Wilcoxon and Mann-Whitney tests, respectively. ES, enrollment sample. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. Black and gray dots denote PHI>350 and PHI<350 subjects, respectively.

rent with increasing gp120-specific IgG titers could be an artifact introduced by IgG outcompeting IgA, IgG was depleted in five plasma samples, and gp120-specific IgA was reanalyzed by an ELISA. Identical titers were obtained, excluding the above-mentioned possibility (not shown). IgG/IgA ratios increased over time during PHI, being significantly higher in chronic and in PHI 12-month samples than in PHI enrollment samples (Fig. 3C). No significant difference was observed among 12-month PHI, chronic, and EC samples.

Afterwards, correlations between ADCC and gp120-specific antibodies were studied. For this purpose and since no differences in IgG, IgA, and IgG/IgA ratios between 12-month PHI and chronic samples were observed, data from these groups were merged. To do this, we also took into account that samples from subjects with PHI at 12 months postinfection and from chronically infected subjects did not differ in their virological and immunological parameters (contrary to ECs) (Fig. 1). As reported previously (12, 14, 17, 20), gp120-specific IgG levels directly correlated with ADCC in all study groups ($r = 0.476$ and $P = 0.039$ for PHI enrollment samples [not shown] [shown in Fig. 4A for the rest of the groups]). Conversely, no correlations between gp120-specific IgA and ADCC responses were observed (Fig. 4B). Interestingly, gp120-specific IgG/IgA ratios showed strong direct correlations with ADCC magnitude in the group of 12-month PHI and chronic subjects and in the EC group (Fig. 4C), providing initial support to the notion that gp120-specific IgA antibodies might mitigate the capacity of specific IgG to mediate ADCC.

Depletion of IgA from plasma of viremic HIV-infected subjects enhances the magnitude of ADCC. According to our initial hypothesis, and based on the significant correlations between IgG/IgA ratios and ADCC, we depleted IgA from plasma samples of six randomly selected PHI subjects (12-month samples) and four ECs and reevaluated ADCC responses in both IgA-depleted and non-depleted (passed through an isotype control-coupled column) plasma samples. Figure 5A shows results from one representative PHI subject: IgA was successfully and specifically depleted, as shown by dot blotting (left). In all cases, the gp120-specific IgA titer was <10 after depletion. The ADCC measured in IgA-de-

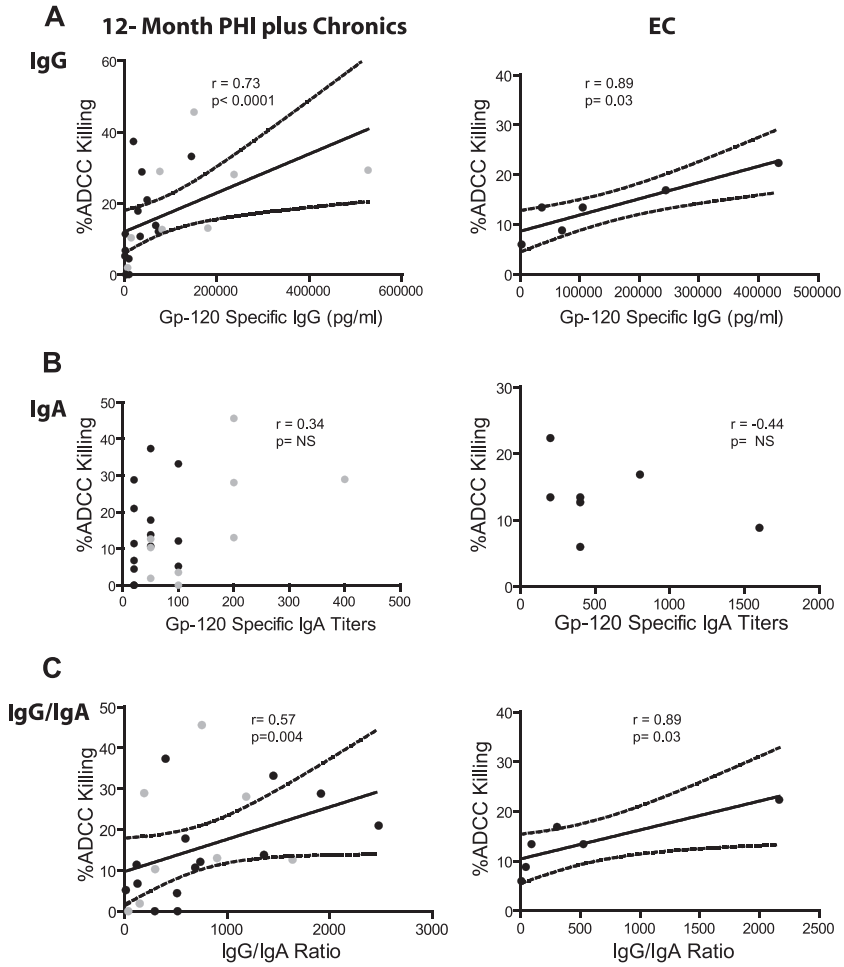

FIG 4 Magnitude of ADCC correlates with gp120-specific IgG and the IgG/IgA ratio but not with gp120-specific IgA. Shown are correlations between ADCC and plasma gp120-specific IgG concentrations (A), plasma gp120-specific IgA titers (B), and gp120-specific IgG/IgA ratios (C) in PHI 12-month plus chronic (left) (%ADCC killing tested at a 1/5,000 plasma dilution) and EC (right) (%ADCC killing tested at a 1/10,000 plasma dilution) samples. In the right panel, black and gray dots denote PHI and chronic subjects, respectively. *r* and *P* values were determined by Spearman's test. Regression lines and 95% confidence intervals are shown.

pleted plasma was significantly higher than that in nondepleted plasma from the same subject (Fig. 5A, right). In this particular example, the ADCC response was negative in nondepleted plasma but became positive after IgA depletion. Moreover, the increment in the magnitude of the ADCC response correlated with a higher level of IgG binding to cells coated with gp120 (Fig. 5, right). This finding was then extended to the other five PHI subjects tested, except for one (Fig. 5B, left). Moreover, the increment was statistically significant when grouped (Fig. 5B, right). ADCC was not modified by IgA removal in ECs (Fig. 5C). In line with this observation, the ADCC ratio of IgA-depleted over nondepleted plasma was significantly higher in subjects with PHI than in ECs (Fig. 5D). Altogether, these results show that plasma IgA modulates ADCC in the context of natural viremic infection, probably mitigating its protective function.

ADCC responses in IgA-depleted plasma correlate with the percentage of CD4$^+$ T cells. Finally, correlation analyses between clinical parameters and %ADCC killing observed in IgA-depleted or nondepleted plasma were performed. No correlations between ADCC evaluated in nondepleted plasma and CD4$^+$ T-cell percentages were observed (Fig. 6, left). Conversely, a strong, positive, nearly significant correlation between the percentage of CD4$^+$ T cells and the magnitude of ADCC obtained with IgA-depleted plasma was found (Fig. 6, right), indicating that eliminating IgA-mediated interference strengthens the association between ADCC and the percentage of CD4$^+$ T cells (higher *r* coefficient) and also the significance (lower *P* value) of this association. These results, together with those presented above, where higher ADCC values were obtained after IgA removal, indicate that IgA might be interfering with the ADCC mechanism, as measured under our experimental conditions. In turn, this would impede the finding of an association between ADCC and disease progression, which becomes evident after the removal of the IgA component.

DISCUSSION

Understanding the role of ADCC-mediating antibodies in the modulation of HIV disease progression is a field of ongoing intense research and renewed interest. In line with the description that vaccine-elicited Env-specific IgA antibodies block gp120 binding of ADCC-mediating IgG (23), evidence of a similar effect occurring in natural viremic HIV infection is

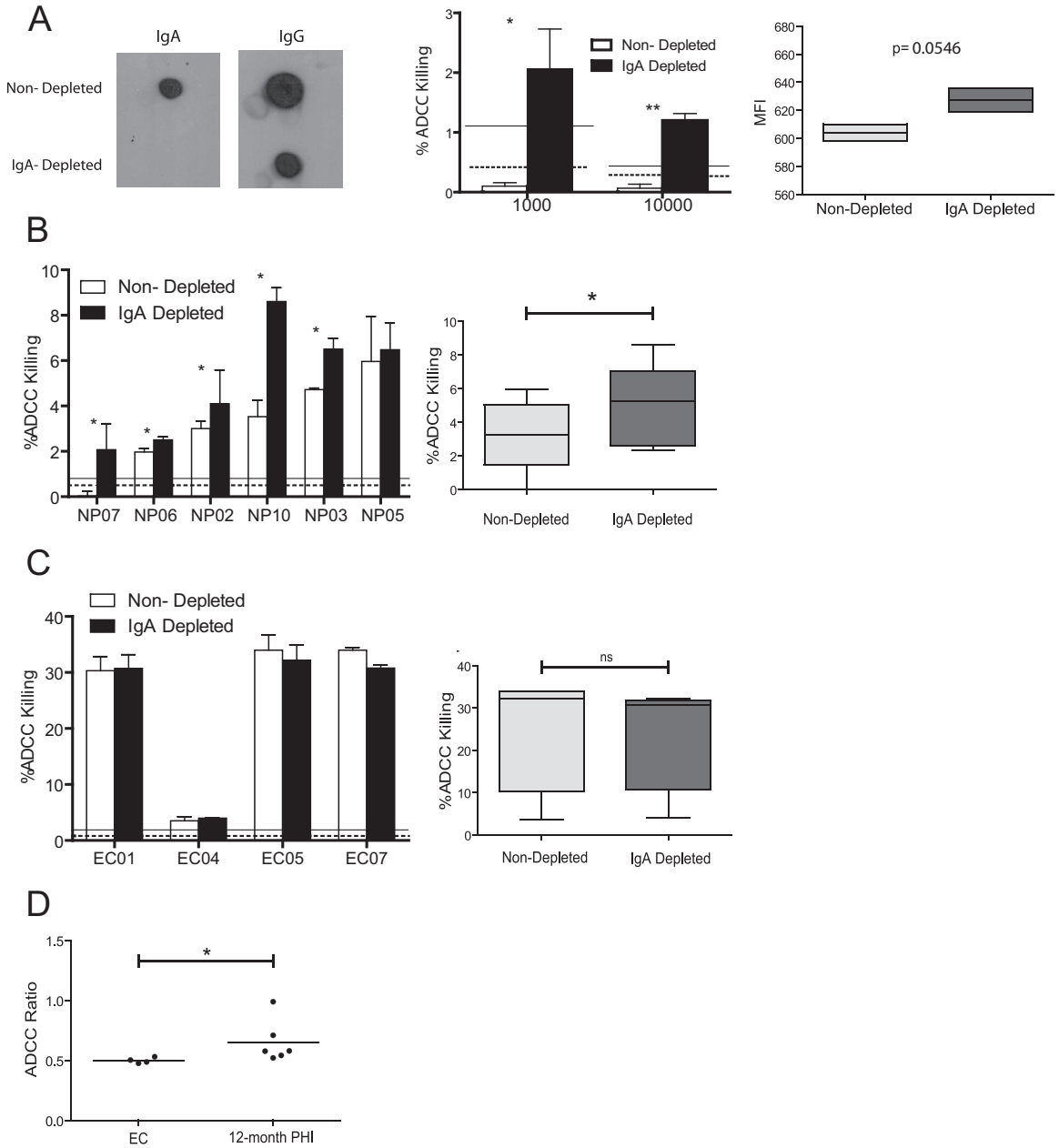

FIG 5 Evaluation of ADCC responses after IgA plasma depletion in HIV-infected subjects. An immunoprecipitation procedure was performed to deplete plasma IgA from 6 randomly selected PHI subjects (12-month samples) and 4 ECs. (A) Results corresponding to one representative subject. After confirming that IgA was successfully and specifically depleted (left), %ADCC killing was evaluated in IgA-depleted and nondepleted plasma samples at 1/1,000 and 1/10,000 dilutions (middle). Horizontal lines represent cutoff values (calculated as described in Materials and Methods) under nondepleted (dashed lines) and IgA-depleted (continuous lines) conditions. IgG binding to cells coated with gp120 was evaluated with a 1/1,000 dilution of IgA-depleted and nondepleted plasma (left) by flow cytometry. Mean fluorescence intensity (MFI) was recorded as a measure of IgG binding. (B) The magnitude of ADCC measured in IgA-depleted plasma is significantly higher than that in nondepleted plasma for 5 out of 6 12-month PHI samples (left), and this increment is statistically significant when grouped (right). (C) The magnitude of ADCC is not modified by IgA removal from plasma samples of ECs (D). The ADCC ratio [%ADCC killing in nondepleted plasma/(%ADCC killing in nondepleted + IgA-depleted plasma)] was significantly higher in 12-month PHI samples than in EC samples. Each assay was performed three times, each in triplicates. In panels B and C, horizontal lines represent mean cutoff values under nondepleted (dashed lines) and IgA-depleted (continuous lines) conditions. In these panels, all responses (except the response observed in nondepleted plasma from subject NP07) are positive according to the criteria described in Materials and Methods. NP, nonprogressor; EC, elite controller. For box-and-whisker plots, the horizontal line represents the median value, the boxes represent the interquartile range, and the whiskers extend from the minimum to the maximum values. Intra- and intergroup differences were analyzed by using Wilcoxon and Mann-Whitney tests, respectively. *, $P < 0.05$; **, $P < 0.01$.

presented here for the first time. Moreover, ADCC measured in IgA-depleted plasma correlated with $CD4^+$ T-cell preservation in viremic subjects.

One major achievement of this work was the simultaneous evaluation of ADCC in three groups of HIV-infected subjects at different disease stages (acute versus chronic) and with different clinical outcomes (controlled versus noncontrolled infection). Moreover, clinical follow-up of subjects identified during PHI

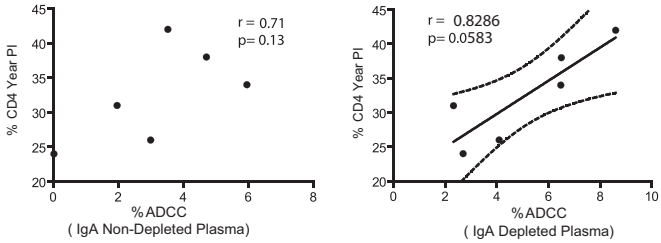

FIG 6 ADCC responses evaluated in IgA-depleted plasma are correlated with the percentage of $CD4^+$ T cells. Shown are correlations with the magnitude of ADCC evaluated in nondepleted plasma (left) and in IgA-depleted plasma (right) and the percentage of $CD4^+$ T cells at a year postinfection. *r* and *P* values were determined by Spearman's test. The regression line and 95% confidence intervals are shown in the right panel.

allowed us to correlate the magnitude of the ADCC response observed at enrollment with subsequent disease progression. We demonstrated that ADCC activity peaks very early after infection, reaches a plateau in chronic infection, and decreases after the instauration of ART, confirming data from previous reports (5–17, 19, 32). The results that we initially obtained using whole plasma supported the notion that ADCC has no beneficial role in slowing disease progression, despite ECs showing slightly higher ADCC than the other groups. This is because no association between ADCC and disease progression (defined by several means) after acute infection in terms of VL, $CD4^+$ T-cell count, soluble factors, and cellular immune activation was found, as shown in Fig. 2. Because results obtained between the PHI>350 and PHI<350 groups were so similar and because the correlations were so weak, we considered that the same results would be obtained even if the sample size was increased. Also, although PHI follow-up was at 1 year, we believe that if ADCC was truly a strong correlate of protection from progression, differences should be observed in the short term after infection, as in the case of cellular immunity (26, 27). The discrepancy between our data and the sizable evidence supporting the idea that ADCC responses could contribute to defense against HIV in the course of natural infection (5, 8, 9) made us reason that it was quite probable that a there is beneficial effect of this effector function in our study but that it is being mitigated by some plasma factors. Thus, we went one step further and provided evidence regarding gp120-specific IgA acting as such a mitigating factor in the context of natural HIV infection, as has been reported in the RV144 vaccine setting (3, 23).

We demonstrate that the level of gp120-specific IgG was associated with the capacity to mediate ADCC in all study groups, which is in agreement with data from previous studies (12, 14, 17, 19, 20), and that there was no correlation between gp120-specific IgA titers and ADCC responses, as also reported previously (12, 14). New to this study is the fact that gp120-specific IgG/IgA ratios are associated with ADCC activity, providing the first evidence of the effect of the IgG/IgA ratio on the magnitude of the ADCC response. One point to consider is that recombinant monomeric gp120 was used in both ADCC analyses and ELISAs. Although a correlation between the magnitude of ADCC and gp120-specific IgG binding in ELISAs was observed here and in other reports, as mentioned above, it is important to note that the gp120 epitopes exposed in each assay differ largely. It has been specifically demonstrated that gp120 epitopes exposed in the RFADCC assay as performed here are those exposed in the gp120 protein after adopting the CD4-bound conformation (33). Moreover, previous reports have shown that exposure of ADCC-specific epitopes in infected cells occurs only after conformational changes associated with CD4 binding occur and were mapped to the gp120 C1 region (34–36). This is particularly relevant in the context of our results: it indicates that antibodies that are able to recognize the gp120 protein bound to CD4 molecules and subsequently trigger ADCC are present in HIV-infected subjects. Thus, therapeutics taking advantage of this mechanism and enhancing this recognition are desirable, as proposed by Richard et al. (37).

Finally, direct evidence of IgA interference in the magnitude of ADCC in natural viremic infection is presented here for the first time, as the level of ADCC measured in IgA-depleted plasma was significantly higher than that in nondepleted plasma for 5 out of 6 subjects. Moreover, ADCC measured in IgA-depleted plasma correlated with $CD4^+$ T-cell preservation. It should be remarked that in the RFADCC assay, NK cells are the effector component (18), and although there are some controversies, it is accepted that NK cells are unable to perform IgA-mediated ADCC (38), highlighting that it is a proper assay to uncover the interfering role of IgA.

The presence of IgA mitigating the protective role of ADCC activity may be a suitable explanation for the discrepancy observed between studies where a correlation of ADCC with protection was reported and others (like ours) where it has been difficult to demonstrate such a correlation. The association of ADCC activity with protection reported previously was achieved by evaluating ADCC responses with the use of purified IgG instead of whole plasma (8, 19). Consequently, the role of the inhibitory effect of IgA in those cases was nonexistent. On the other hand, some early studies reported inhibition of ADCC activity at high plasma concentrations but reported a correlation with disease status using serial dilutions of serum (5, 13). By doing this, the inhibitory factor, in this case IgA, could have been diluted out. Finally, a recent report indicates that the relative ratios of the different IgG subclasses impact ADCC activity during the course of HIV infection. More specifically, the loss of ADCC activity was related to a loss of HIV-specific IgG3 levels and rising titers of subclasses with less functionality (19). Our work adds further evidence to the field demonstrating that IgA interferes with ADCC activity and, therefore, that higher gp120-specific IgG3 levels that are able to counteract the detrimental role of IgA would lead to protective ADCC responses.

Detrimental roles of IgA in other pathologies have also been described (discussed in references 23 and 38). Our results are limited to show that gp120-specific plasma IgA diminishes the magnitude of *in vitro* IgG-mediated ADCC in viremic HIV^+ subjects infected for 1 year, masking its association with the rate of disease progression. What happens in other body compartments, different from plasma, was unexplored, although it deserves consideration.

Finally, a particular picture is observed in ECs: a high magnitude of ADCC despite the absence of significant antigen stimulation, high plasma gp120-specific IgG levels but also high IgA levels, and a positive correlation of the gp120-specific IgG/IgA ratio with ADCC but no IgA interference in depletion experiments. Further insights into IgG and IgA epitope fine-mapping, structure (including glycosylation profiles), function, and affinity in ECs will be needed since dissecting the mechanisms governing HIV-specific IgG and IgA development in ECs, and differences from those in viremic subjects, will be instrumental for developing

strategies to elicit the best humoral immune profile. Specifically, we hypothesize that gp120-specific IgA is directed toward C1 conformational epitopes in subjects where IgA depletion has a considerable impact on ADCC activity (PHI viremic subjects), thus outcompeting C1-specific ADCC-mediating IgG (as reported in the RV144 vaccine setting by Tomaras et al. [23]). On the contrary, in ECs, where IgA depletion has no impact on ADCC activity, IgA specificity may be different from C1, so no IgA-versus-IgG competition occurs. Further investigations in this line will be needed to better understand the basis of ADCC mechanisms in viremic and aviremic subjects.

In summary, we consider that this study represents an important extension beyond the scope of previous reports dealing with ADCC in HIV infection. As initially suggested in the RV144 vaccine setting, gp120-specific IgA antibodies are now identified as factors interfering with the ADCC response in viremic infection, precluding its effect on slowing disease progression. This further emphasizes the notion that vaccine strategies may be more beneficial if they induce IgG-biased antibody responses. Also, much remains to be learned regarding the factors that shape the complex humoral responses in both typical HIV-infected progressors and ECs as well as about the complex interactions between the virus itself and host immunity. As development of an effective vaccine and novel immunotherapies progresses, the data presented here underscore the importance of continuously improving our understanding of the functionality of antibodies elicited either during natural infection or by candidate vaccines.

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Th17 and Th17/Treg ratio at early HIV infection associate with protective HIV-specific CD8$^+$ T-cell responses and disease progression

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The aim of this study was to analyze Th17 and Treg subsets and their correlation with anti-HIV T-cell responses and clinical parameters during (acute/early) primary HIV infection (PHI) and up to one year post-infection (p.i). Samples from 14 healthy donors (HDs), 40 PHI patients, 17 Chronics, and 13 Elite controllers (ECs) were studied. The percentages of Th17 and Treg subsets were severely altered in Chronics, whereas all HIV-infected individuals (including ECs) showed Th17/Treg imbalance compared to HDs, in concordance with higher frequencies of activated CD8$^+$ T-cells (HLA-DR$^+$/CD38$^+$). Better clinical status (higher CD4 counts, lower viral loads and activation) was associated with higher Th17 and lower Treg levels. We found positive correlations between Th17 at baseline and anti-HIV CD8$^+$ T-cell functionality: viral inhibitory activity (VIA) and key polyfunctions (IFN-γ^+/CD107$_{A/B}^+$) at both early and later times p.i, highlighting the prognostic value of Th17 cells to preserve an effective HIV T-cell immunity. Th17/Treg ratio and the IL-17 relative mean fluorescence intensity (rMFI of IL-17) were also positively correlated with VIA. Taken together, our results suggested a potential link between Th17 and Th17/Treg ratio with key HIV-specific CD8$^+$ T-cell responses against the infection.

Despite medical and scientific efforts made over the past 30 years, HIV infection continues to be a major global public health concern. The mechanisms and immune system components that contribute to the natural control of the infection and disease progression in some HIV-infected persons, in contrast to the vast majority of patients that undergo rapid progression, are not fully elucidated. The discovery of these key components and their interactions during HIV infection remains a major goal in the field that could allow the design of new approaches to control and perhaps even eradicate the disease.

Th17 cells are a CD4$^+$ T-cell subset, of a lineage different from Th1 and Th2[1,2]. They are characterized by interleukin 17 (IL-17) production and play key roles in protective inflammatory mucosal responses against bacteria and fungi, as well as in mucosal barrier integrity and homeostasis[3–6]. Recent studies have demonstrated that SIV and HIV infections lead to a selective depletion of Th17 cells in both blood and gastrointestinal lymphoid tissues that can predict disease progression[7,8]. Even more, several publications highlighted the importance of the Th17/Treg ratio in the progressive disease developed during HIV-1 and SIV infections[9]. During chronic infection it has been shown that the loss of Th17/Treg balance associates with disease progression in individuals with typical progression in contrast to ECs[10].

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Group	Sample time points, n° of subjects	Estimated dpi[a], median (IQR)	Age (years), median (IQR)	Log_{10}VL[b], mean± SD	CD4 counts[c], median n° of cells/μl (IQR)	CD8 counts[c], median n° of cells/μl (IQR)	CD4/CD8 ratio, median (IQR)
PHI cohort	Baseline (n = 40)	75 (50–120)	32 (23–37)	4.52 ± 1.10	577 (388–681)	1099 (767–1593)	0.50 (0.27–0.79)
	Year (n = 22)	330 (300–360)	–	4.27 ± 0.84	480 (396–660)	845 (594–1200)	0.57 (0.35–0.91)
PHI RPs	Baseline (n = 11)	55 (30–98)	37 (27–40)	4.98 ± 0.85	282 (222–379)	1324 (930–1771)	0.23 (0.12–0.33)
	Year (n = 5)	300 (230–370)	–	4.01 ± 1.14	316 (178–358)	961 (540–1219)	0.27 (0.25–0.41)
PHI TPs	Baseline (n = 29)	90 (60–120)	30 (23–36)	4.35 ± 1.14	603 (515–741)	1021 (634–1420)	0.60 (0.40–0.87)
	Year (n = 17)	330 (300–360)	–	4.34 ± 0.63	575 (443–680)	769 (592–1217)	0.70 (0.44–0.94)
ECs	≥5 years (n = 13)	–	37 (33–44)	<1.70	599 (559–894)	618 (287–802)	1.54 (0.61–2.38)
Chronics	≥3 years (n = 17)	–	34 (25–36)	4.71 ± 0.70	141 (11–395)	1009 (540–1265)	0.19 (0.03–0.37)

Table 1. Characteristics of HIV-infected individuals enrolled for the study. *PHI:* primary HIV infection. *RPs:* rapid progressors. *TPs:* typical progressors. *ECs:* elite controllers. *dpi:* days post-infection. *VL:* viral load. *IQR:* inter quartiles. *SD:* standard deviation. RPs and TPs distinguish PHIs that had CD4 counts below or above 350 cells/μl during the first year post-infection, respectively. [a]In symptomatic patients, estimated as 14 days before the onset of symptoms. In asymptomatic patients, estimated as the midpoint between the last negative and the first positive test or one month before the date of the indeterminate or negative Western blot assay[25]. [b]Versant HIV-1 RNA 3.0 assay, Siemens. Lower and upper detection limits are 50 and 500.000 RNA copies/ml, respectively (1.7 and 5.7 log_{10}). [c]Flow cytometry double plataform, FACSCanto, BD Biosciences.

All these previous studies indicate that both Th17 cells and the Th17/Treg ratio have a critical role during HIV-1 infection. However, an evaluation of the possible correlations between these parameters and the HIV-specific antiviral adaptive T-cell response is still needed.

In a previous study our group demonstrated that, during PHI, the early relative immunodominance of Gag-specific $CD8^+$ T-cells was associated with $CD4^+$ T-cell count preservation, in consonance with Gag immunodominance in ECs and "viremic controllers"[11], linking the antiviral $CD8^+$ T-cell response with the natural control of disease progression.

In this context, and in light of the evidence pointing to the relevance of Th17 and Treg subsets during HIV infection and AIDS progression, we hypothesized that preservation of the Th17 sub-population and Th17/Treg ratio are determinant immune factors that could impact the HIV-specific $CD8^+$ antiviral response, and hence disease progression. Therefore, the aim of the present study was to perform an in depth evaluation of the dynamics of Th17 cells and Th17/Treg ratio at different stages of HIV infection, and to investigate the correlations between these parameters and markers of disease progression and the antiviral $CD8^+$ T-cell functions previously associated with protection.

For the first time we demonstrated that, during PHI, higher Th17 levels directly correlate with more potent HIV antiviral T-cell responses associated with protection. Remarkably, we verified that baseline proportions of Th17 cells may have a possible prognostic value for the functional anti-HIV T-cell responses detected at later times p.i.

Results

Clinical characteristics of the HIV-infected individuals enrolled. The different groups of HIV-infected participants selected to perform the present study were: a group of 40 individuals diagnosed during PHI (HIV seroconversion and/or within 6 months from presumed date of infection, 95% of them corresponded to Fiebig stages V and VI[12]), 17 typical chronically infected patients (Chronics), and a group of 13 infected individuals defined as ECs. These two last groups were included as control groups in order to compare the different parameters to be evaluated in relation to those found in the PHI cohort. All the patients enrolled were ART naïve at the time of sample collection (detailed inclusion criteria for each group are defined in Materials and Methods).

A description of the clinical characteristics of the different HIV-infected groups is summarized in Table 1. For PHIs, the median estimated time p.i was 75 days (day at which baseline sample was obtained), whereas 330 days was the median day p.i. of the "one year" follow-up sample. For some analyses, PHIs were further divided into two sub-groups taking into account whether their CD4 counts dropped below 350 cells/μl, or not, at any time during the first year p.i, denoted as rapid (RPs) and typical

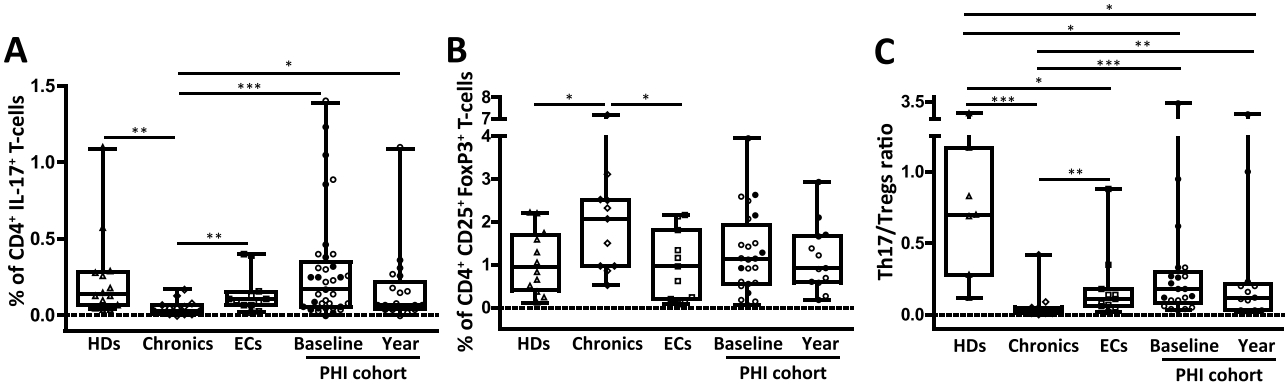

Figure 1. Alteration of Th17 and Treg subsets and Th17/Treg ratio at different stages of HIV infection. PBMCs were stimulated for 6 hours with anti-CD3/anti-CD28 or medium alone (background control) prior to intracellular staining. Background subtracted values of $CD3^+$ $CD4^+$ IL-17^+ producing cells (Th17) are shown (**A**). Intranuclear staining for detection of $CD3^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ cells (Tregs, **B**). Th17/Treg ratio was calculated for each patient (**C**). Boxes indicate median values with 25–75 percentiles and bars show the maximum and minimum values. Symbols represent individual patients: Healthy donors (Δ, HDs), Chronics (◊), Elite controllers (□, ECs) and primary HIV infection (PHI) cohort at baseline and one year p.i follow up (○, typical progressors or TPs with CD4 counts above 350 cells/μl during the first year p.i; •, rapid progressors or RPs with CD4 counts below 350 cells/μl during the first year p.i). The *p* values obtained are depicted as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

(TPs) progressors, respectively. Clinical differences between TPs and RPs were observed at baseline. Thus, significant higher CD4 counts ($p < 0.0001$) and CD4/CD8 ratios ($p < 0.0001$) were found in TPs. Also, mean Log_{10} viral load (VL) from RPs tended to be higher than that seen in TPs ($p = 0.0713$). These differences showed the same trend at one year p.i, although caution must be taken due the limitation imposed by the limited number of RP samples at this time point.

Total PHIs (at both baseline and one year p.i), showed significant higher CD4 counts and CD4/CD8 ratios in comparison with Chronics (*p* values < 0.001). As expected, ECs had preserved CD4 counts compared to Chronics ($p < 0.001$) and higher CD4/CD8 ratios compared to both Chronics ($p < 0.001$) and PHIs (baseline and on year p.i; *p* values < 0.03).

Th17/Treg ratio was diminished since early times post-infection whereas Treg and Th17 frequencies were severely altered at advanced stages of HIV progressive infection. The first aim of the study was to analyze the Th17 and Th17/Treg changes that occur during PHI infection, contrasting the variation of these parameters in PHI patients with those observed in HDs and in chronically infected persons defined as typical Chronics and ECs.

In Fig. 1A it can be seen that the median percentage (IQR) of Th17 cells present in PHIs (both at baseline and one year follow-up, medians of 75 and 330 days p.i, respectively) were significantly higher compared to Chronics [0.17% (0.06–0.35) at baseline and 0.07% (0.05–0.22) at one year p.i in PHIs vs. 0.03% (0.01–0.07) in Chronics; *p* values 0.0002 and 0.0145, respectively]. Within the PHI cohort, a trend to a decrease in Th17 levels was observed with the advancement of the infection [0.17% (0.06–0.35) at baseline vs. 0.07% (0.05–0.22) at one year; $p = 0.11$]; however, differences did not reach significance probably due to the high data dispersion. Interestingly, no significant differences were found between PHIs at baseline and both HDs [0.14% (0.07–0.28)] and ECs [0.11% (0.07–0.15)]. As expected, Chronics showed the lowest Th17 levels. When analysis was done taking into account the absolute Th17 counts (number of cells/μl) similar and enhanced differences between groups were observed (data not shown).

Conversely, the percentage of Treg cells found in Chronics was higher compared to ECs and HDs [2.07% (0.97–2.52) in Chronics vs. 0.96% (0.20–1.81) in ECs and 0.94% (0.41–1.70) in HDs; *p* values 0.0356 and 0.0392, respectively; Fig. 1B]. Despite PHIs showed Treg frequencies similar to those observed in HDs and ECs [1.13% (0.56–1.93) in PHIs at baseline and 0.92% (0.59–1.68) at one year p.i], no significant differences were observed compared to Chronics. When the Th17/Treg ratio was analyzed (Fig. 1C), all HIV-infected groups showed significant differences compared to HDs [0.70 (0.28–1.17); all *p* values < 0.02], including ECs. It is worth noting that data comparing Th17/Treg ratio of ECs versus (vs.) HDs is scaring in the literature.To highlight, the ratio between these two $CD4^+$ T-cell subsets was severely reduced in Chronics [0.02 (0.01–0.05)], compared to ECs [0.11 (0.06–0.18); $p = 0.0054$] and the PHI cohort at both baseline [0.18 (0.08–0.30); $p = 0.0004$] and one year p.i follow-up [0.12 (0.03–0.22); $p = 0.0058$].

The results described above indicated that HIV infection has a negative impact in the Th17/Treg ratio, despite natural HIV control (ECs). We also corroborated for the patient cohort of the present study that

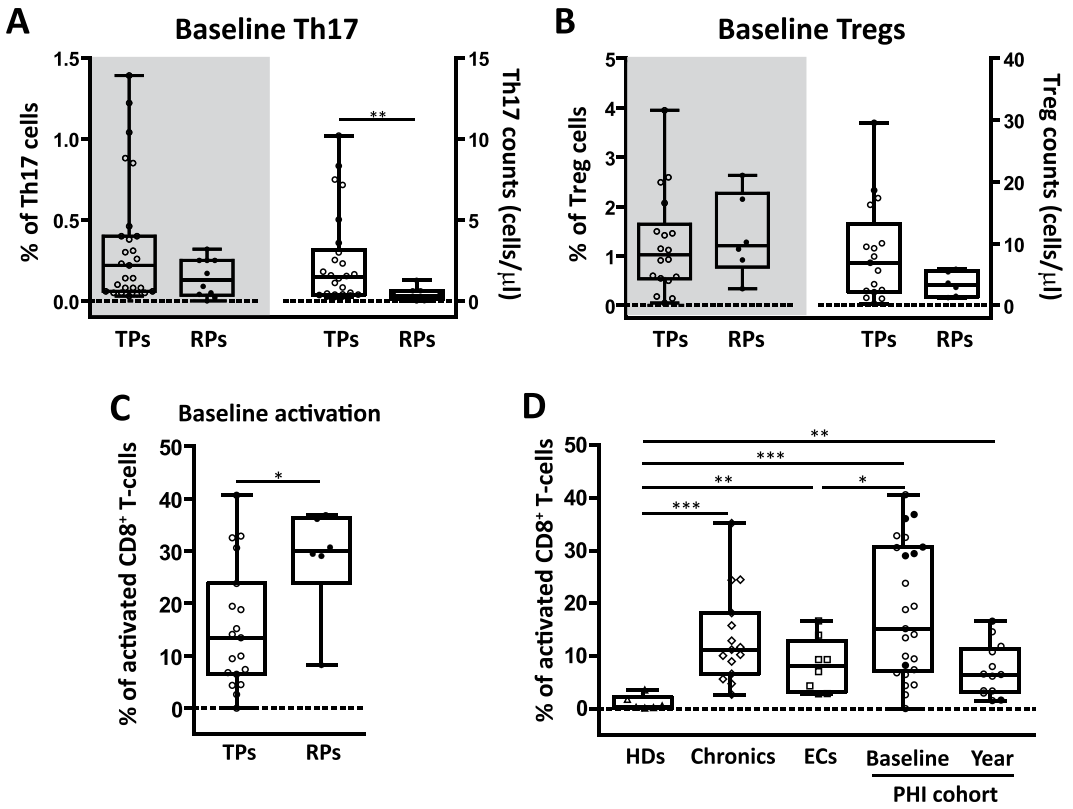

Figure 2. Th17 and Treg subsets among recently HIV-infected individuals that show different patterns of immune activation and disease progression. Comparison of baseline frequencies (right *y* axes) and absolute counts (left *y* axes) of Th17 (A) and Treg (B) cells between typical (TP, white symbols) and rapid (RP, black symbols) progressors. Baseline frequency of $CD3^+$ $CD8^+$ T-cells positive for $CD38^+$ and HLA-DR^+ activation markers was determined in TP and RP PHI sub-groups by flow cytometry (**C**). Frequency of activated $CD8^+$ T-cells was determined for all groups (**D**). Boxes indicate median values with 25–75 percentiles and bars show the maximum and minimum values. Symbols represent individual patients within each group. The *p* values obtained are depicted as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

percentage of Th17 and Treg cells were severely altered in chronically infected patients with a typical progression pattern.

Baseline Th17 cell levels were associated with immune T-cell activation and rates of disease progression. Immune activation constitutes a hallmark of HIV infection, and has been reported to occur at early times p.i[13,14], thus our next aim was to analyze its relation with Th17 and Treg subsets at early stages (75 days p.i) of HIV infection, comparing individuals with different rates of immune deterioration (denoted as RPs and TPs).

First, we compared both baseline proportions and absolute counts of Th17 cells that were present in PHIs with a rapid immune deterioration (RPs) compared to those that showed a more typical progression pattern (TPs). Thereby, a trend to lower percentages of Th17 cells was found in RPs [0.13% (0.04–0.25) vs. 0.22% (0.06–0.40) in TPs; $p = 0.13$; Fig. 2A left *y* axis], in which also a significant lower baseline median absolute number of Th17 cells was evident [0.33 (0.10–0.63) cells/μl vs. 1.47 (0.40–3.14) cells/μl in TPs; $p = 0.0040$; Fig. 2A right *y* axis]. When the same analysis was extended to the Treg compartment, no significant differences were found [i.e Treg counts: 6.92 (2.26–13.17) cells/μl in TPs vs. 3.27 (1.43–5.53) cells/μl in RPs; $p = 0.24$; Fig. 2B]. Importantly, when the levels of $CD8^+$ T-cell activation were analyzed in these two sub-groups, higher frequencies of activated $CD8^+$ T-cells were found in RPs [30.1% (23.8–36.3) vs. 13.5% (6.5–23.8) in TPs; $p = 0.0450$; Fig. 2C]. We also corroborated that, as expected, HIV infection resulted in higher levels of $CD8^+$ T-cell activation since early times p.i [15.2% (7.1–30.6) in baseline PHI samples] compared to both ECs [8.2% (3.2–12.8); $p = 0.0460$] and normal values found in HDs [0.38% (0.18–2.19); $p = 0.0007$; Fig. 2D]. And at one year p.i, although not significant, immune activation tended to decrease [6.5% (3.2–11.3) in one year p.i PHI samples]. Data from this part of the study indicated that, not only Chronics but also ECs showed higher immune activation levels than HDs (Fig. 2D), in concordance with their low Th17/Treg ratio (Fig. 1C).

Preservation of the Th17 subset positively correlated with improved clinical parameters of disease progression whereas Treg cells associated with higher viral loads and CD8$^+$ T-cell activation. Our next aim was to analyze the relationships between these T-cell sub-populations and variables of disease progression in the different groups of patients. In order to perform a more exhaustive evaluation of the Th17 subset, not only frequencies and absolute counts, but also IL-17 relative mean fluorescence intensity (rMFI) and plasma IL-17 levels (as parameters of Th17 functionality) were determined.

When we analyzed PHIs at baseline, a positive correlation between Th17 levels and CD4 counts ($p = 0.0091$ $r = 0.4288$; Fig. 3A) was observed, in concordance with a direct correlation between IL-17 plasma levels and CD4 counts ($p = 0.0308$ $r = 0.4960$; data not shown). Interestingly, a significant positive correlation was found between percentage of Th17 cells and plasma levels of the macrophage-derived chemokine (MDC; $p = 0.0101$ $r = 0.5747$; Fig. 3B), reinforced by another positive association of this chemokine with the rMFI of IL-17 ($p = 0.0360$ $r = 0.4960$; data not shown). Of note, MDC has been previously characterized to have HIV-suppressive activities[15]. On the other hand, the percentage of activated CD4$^+$ T-cells was found to be inversely correlated with the rMFI of IL-17 ($p = 0.0304$ $r = -0.5780$; Fig. 3C) and, in contrast, directly associated with the percentage of Treg cells ($p = 0.0246$ $r = 0.595$; Fig. 3D).

A similar scenario emerged at one year p.i for the PHI cohort, evidenced by the association of high Th17 levels and better immune status of the patients (CD4 counts; $p = 0.0070$ $r = 0.5963$; Fig. 3E), and the direct relation observed between Treg cells and CD8$^+$ T-cell activation ($p = 0.0333$ $r = 0.5914$; Fig. 3G). Notably, levels of Th17 present at later times p.i were inversely correlated with baseline plasma quantities of the inflammatory soluble mediator CD40 ligand (sCD40L; $p = 0.0165$ $r = -0.6484$; Fig. 3F), a biomarker associated with disease progression during HIV/AIDS[16].

When all chronically HIV-infected subjects were included in the analysis (Chronics, ECs and one year p.i samples from the PHI cohort), VL was found to be negatively correlated with Th17 counts ($p = 0.0041$ $r = -0.4294$; Fig. 3H) and directly associated to Treg levels ($p = 0.0174$ $r = 0.3995$; Fig. 3I). This last observation was in concordance with the associations of higher Treg frequencies with lower CD4 counts ($p = 0.0100$ $r = -0.4423$; Fig. 3J) and, also, with higher activated CD8$^+$ T-cell counts ($p = 0.0194$ $r = 0.4176$; data not shown).

Overall these results suggest that the preservation of the Th17 subset is positively linked with an improved clinical status of the patients, in terms of CD4 counts, VL, soluble markers of disease progression and immune activation. On the contrary, the Treg subset expansion is directly associated with increased immune activation, viral replication and lower CD4 counts.

Higher Th17 baseline levels were related to improved HIV-specific CD8$^+$ T-cell functionality. One of the major objectives of the present study was to analyze the potential impact that the proportions of Th17 and Tregs and alterations in their balance could have on the specific CD8$^+$ T-cell responses with documented relevance on the antiviral HIV immunity. Therefore, our next aim was to analyze if baseline Th17 levels were related with specifc CD8$^+$ T-cell functions previously associated with better clinical prognosis during HIV infection[11]. A significant positive correlation was observed between Th17 frequencies and proportions of HIV-specific $CD107_{A/B}{}^+$ IFN-γ^+ CD8$^+$ T-cells ($p = 0.0161$ $r = 0.6749$; Fig. 4A). Interestingly, in concordance with the hypothesis that a better T-cell immunity would be present in those patients with higher proportion of baseline Th17 cells,we found associations between the preservation of this subset with higher MIP-1β (T-cell chemokine associated with anti-viral properties; $p = 0.0165$ $r = 0.5563$; Fig. 4B) and also a trend to higher IL-2 plasma levels (T-cell homeostasis cytokine; $p = 0.0537$ $r = 0.4617$; Fig. 4C).

Remarkably, baseline proportions of Th17 cells suggested a possible prognostic value for the functional antiviral T-cell responses detected at later times p.i (year samples). Thus, baseline Th17 counts were positively correlated with the proportion of HIV-specific bifunctional CD8$^+$ T-cells (positive for IFN-γ and $CD107_{A/B}$) at later times ($p = 0.0370$ $r = 0.6055$; Fig. 4D). Although not significant, a trend was also found between baseline Th17 counts and the viral inhibitory activity (VIA) against *in vitro* HIV growth exerted by CD8$^+$ T-cells measured in one year samples ($p = 0.0655$ $r = 0.5727$; Fig. 4E). Significantly, we found that baseline levels of Th17 cells were negatively correlated with the percentage of HIV-specific CD8$^+$ T-cells that express the immunosuppressive PD-1 molecule (a marker of cell exhaustion) at later times ($p = 0.0440$ $r = -0.6150$; Fig. 4F). It is important to note that all these correlations were specific for the Th17 subpopulation as no relationship between CD8$^+$ T-cell responses and Th1 levels were found (Supplementary Fig. S1).

The results described in this section suggest a direct relationship between Th17 baseline levels and protective HIV-specific CD8$^+$ T-cell functionality (at both early as well as later times p.i), highlighting a potential impact of the maintenance of this CD4$^+$ T-cell subpopulation to preserve an effective anti-HIV immunity.

Th17 levels were also correlated with other T-cell effector subsets. After the preceding results indicating a correlation between Th17 cells and HIV-specific CD8$^+$ T-cells, we also considered interesting to evaluate the possible influence of Th17 levels on other potential T-cell effector functions. For this

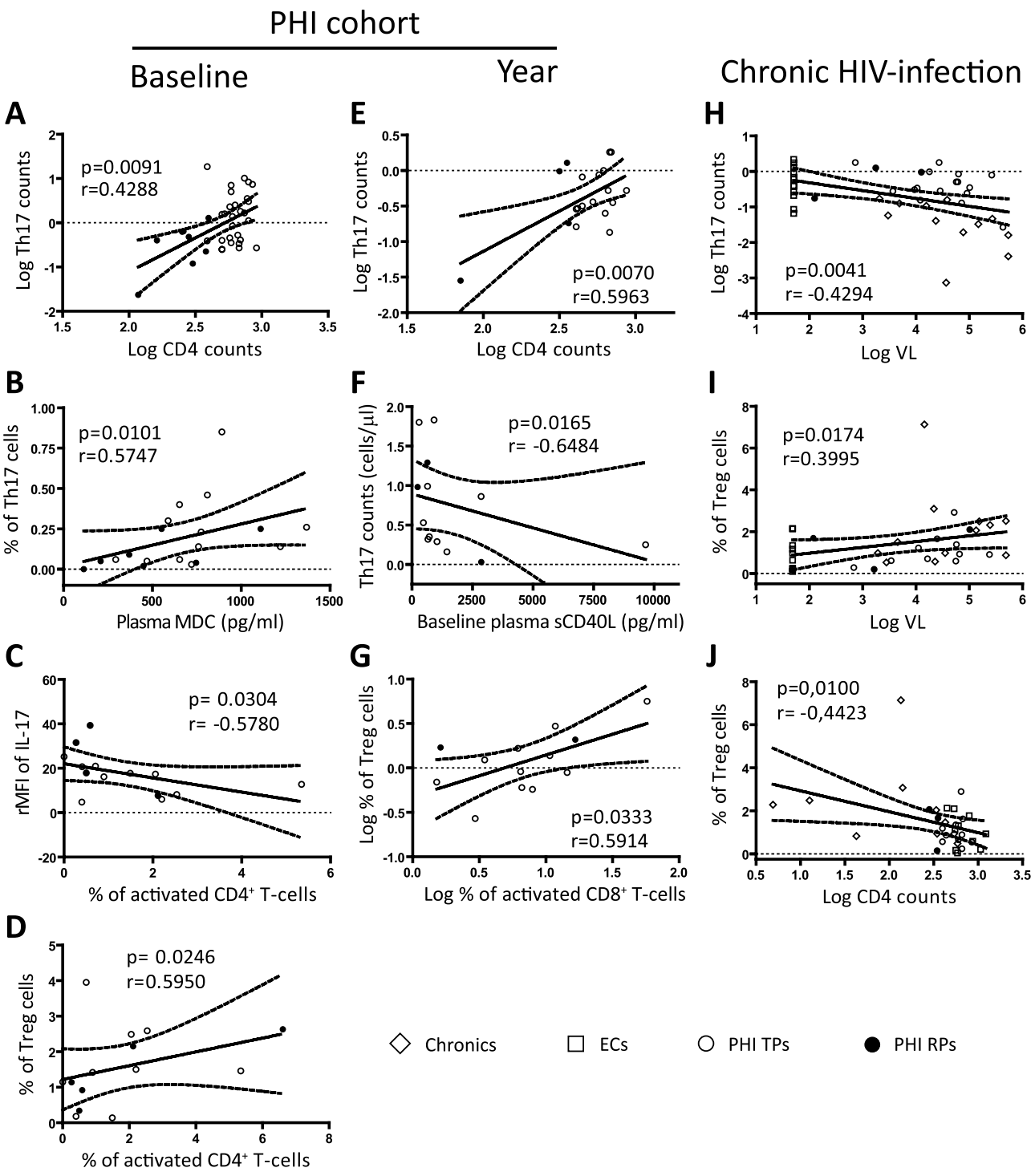

Figure 3. Correlations between Th17 and Treg subsets with clinical parameters of disease progression. Correlations found among PHIs at baseline: Log CD4 counts versus (vs.) Log Th17 counts (**A**); plasma macrophage-derived chemokine (MDC) levels vs. % of Th17 cells (**B**); % of activated CD4 T-cells vs. relative mean fluorescence intensity (rMFI) of IL-17 (**C**) and % of Treg cells (**D**). Correlations found among PHIs at one year p.i follow up: Log CD4 counts vs. Log Th17 counts (**E**); baseline plasma soluble CD40 ligand (sCD40L) levels vs. Th17 counts at one year p.i (**F**); Log % of activated CD8 T-cells vs. Log % of Treg cells (**G**). Relationships present at chronic stages of HIV infection (Chronic, EC and PHI one year p.i samples): Log VL vs. Log Th17 counts (**H**) and % of Treg cells (**I**); Log CD4 counts vs. % of Treg cells (**J**). Soluble plasma proteins were determined by Luminex, for details see Materials and Methods. Symbols distinguish individual patients from the different groups indicated in the figure. *ECs*: Elite controllers. *PHI*: primary HIV infection cohort. *TPs*: typical progressors. *RPs*: rapid progressors. All *r* and *p* values shown correspond to Spearman's correlations.

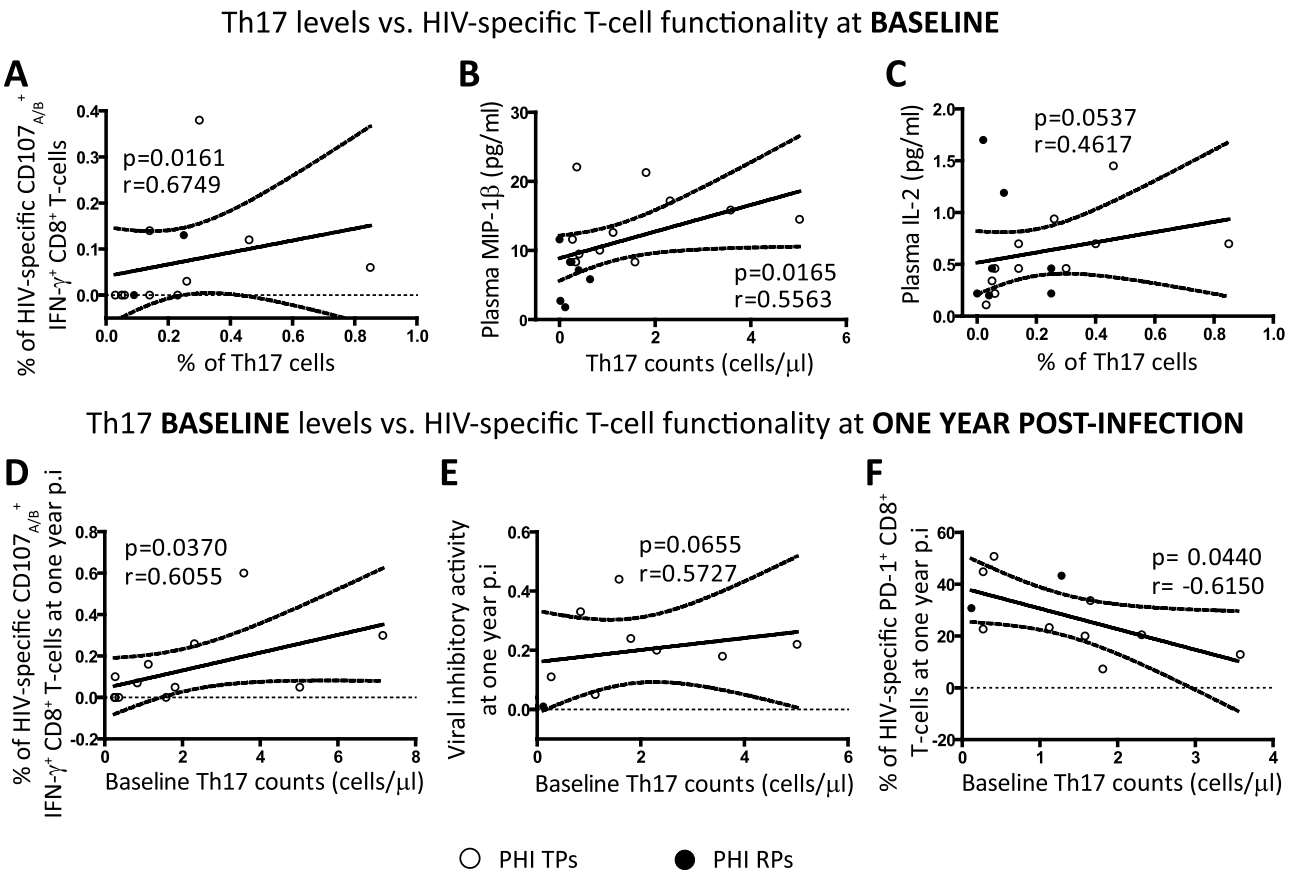

Figure 4. Correlations between Th17 baseline levels and HIV-specific CD8 T-cell responses previously associated with protection. Positive correlations found within PHI cohort at baseline: % of Th17 cells versus (vs.) % of HIV-specific $CD107_{A/B}^{+}$ IFN-γ^{+} CD8 T-cells (**A**) and plasma IL-2 levels (**C**); Th17 counts vs. plasma MIP-1β levels (**B**). Correlations between baseline Th17 levels and anti-HIV specific responses at later times p.i within PHI group: baseline Th17 counts vs. % of HIV-specific $CD107_{A/B}^{+}$ IFN-γ^{+} CD8 T-cells (**D**), viral inhibitory activity (**E**) and % of HIV-specific PD-1^{+} CD8 T-cells all at one year p.i (**F**). Soluble plasma proteins were determined by Luminex. HIV-specific CD8 functionality was determined with different assays: one of them allowed the evaluation of CD8 T-cells with the capacity to degranulate and simultaneously secrete IFN-γ upon HIV-peptides stimulation by flow cytometry (**A** and **D**), other measured the overall CD8 T-cell capacity to inhibit *in vitro* HIV-1 replication in autologous CD4 T-cells (**E**), and the last one measured the expression of the exhaustion marker PD-1 on HIV-specific CD8 T-cells determined upon HIV-peptides stimulation by flow cytometry (**F**). These assays are described in detail in our previous publications[11,48]. Symbols distinguish individual patients from the different sub-groups indicated in the figure. *PHI*: primary HIV infection cohort. *TPs*: typical progressors. *RPs*: rapid progressors. All *r* and *p* values correspond to Spearman's correlations.

purpose, effector T-cells were phenotyped, after TCR polyclonal stimulation, as Th1 ($CD4^{+}$ IFN-γ^{+}), Tc1 ($CD8^{+}$ IFN-γ^{+}) and Tc17 ($CD8^{+}$ IL-17^{+}) following the protocol described in Materials and Methods.

In Fig. 5 it can be seen that baseline Th17 frequencies in PHIs were directly correlated with Th1 counts ($p = 0.0085$ $r = 0.4504$; Fig. 5A) and also with proportions of Tc17 cells ($p = 0.0002$ $r = 0.5980$; Fig. 5B) both at baseline. When these analyses were performed in samples obtained at one year p.i, we also found a direct correlation between Th17 cells and frequencies of Th1 ($p = 0.0009$ $r = 0.7458$; Fig. 5C) and Tc1 ($p = 0.0169$ $r = 0.6047$; Fig. 5D) cells.

A further analysis revealed that the frequencies of Th1 and Tc17 cells tended to suffer a reduction during the first year p.i and, at this late time points, levels were significantly reduced compared to HDs. In contrast, Tc1 cells evaluated at one year p.i were not diminished neither in relation to HDs or baseline values (Supplementary Fig. S2). These results reinforce the concept that, during HIV infection, not only the Th17 but also other T-cell subsets with important effector functions are damaged.

In conclusion, the results described above indicated that the proportion of Th17 cells was correlated not only with HIV-specific $CD8^{+}$ T-cell responses but also with other T-cell effector functions exerted by different T-cell populations.

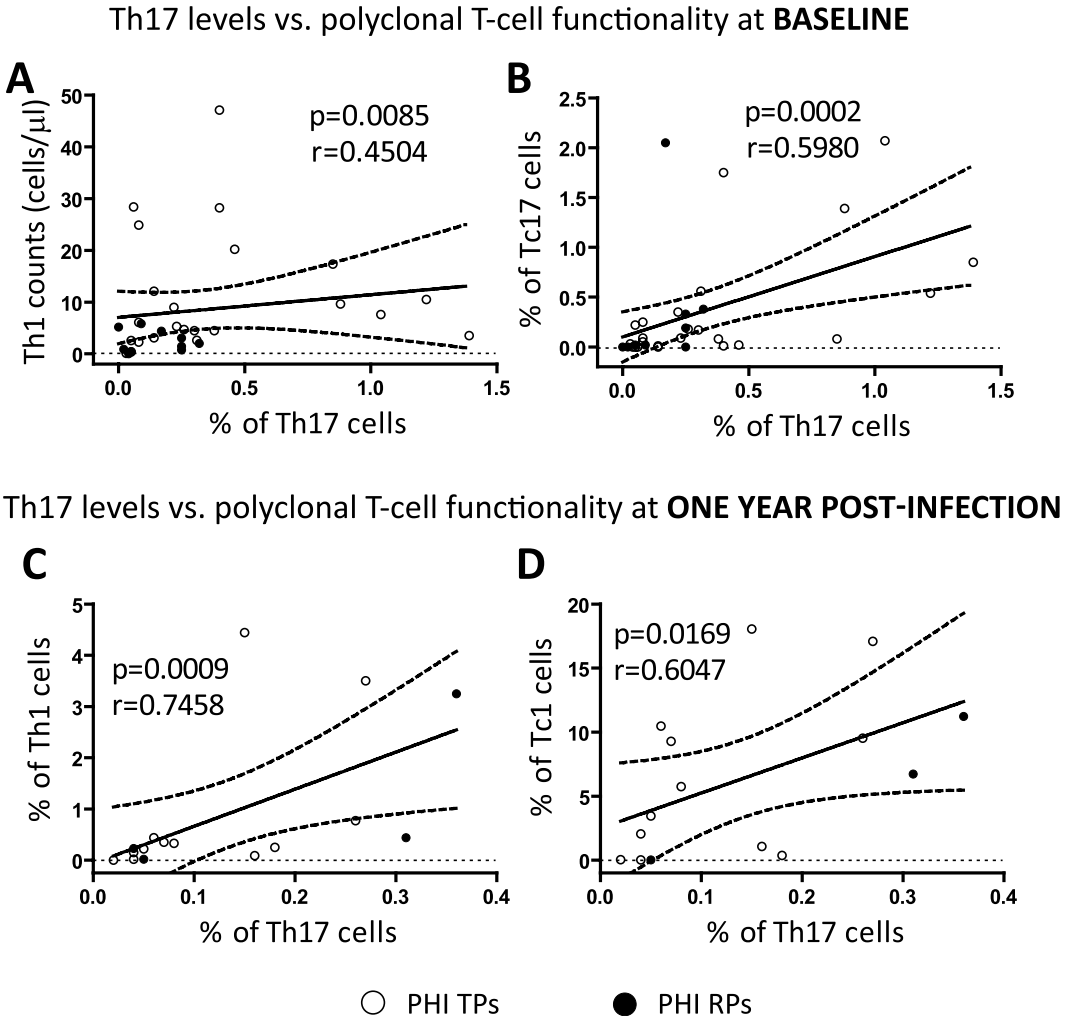

Figure 5. Correlations between the Th17 subset and other T-cell effector sub-populations measured upon polyclonal stimulation. Positive correlations between Th17 levels and polyclonal T-cell effector subsets found within PHI cohort: % of Th17 cells versus (vs.) Th1 ($CD4^+IFN\text{-}\gamma^+$) counts (**A**) and % of Tc17 ($CD8^+IL\text{-}17^+$) cells (**B**) both at baseline; % of Th17 cells vs. % of Th1 cells (**C**) and % of Tc1 ($CD8^+IFN\text{-}\gamma^+$) cells (**D**) both at one year p.i. Polyclonal T-cell effector sub-populations were determined by flow cytometry after 6 hours of stimulation with a mixture of anti-CD3/anti-CD28 antibodies as described in Materials and Methods. Symbols distinguish individual patients from the different sub-groups indicated in the figure. *PHI*: primary HIV infection cohort. *TPs*: typical progressors. *RPs*: rapid progressors. All *r* and *p* values correspond to Spearman's correlations.

Th17 functionality and Th17/Treg ratio at later times post-infection also influence the quality of the specific concurrent anti-HIV $CD8^+$ T-cell activities. Next, we proceeded with the analysis of the possible relationships between Th17 levels, Th17 functionality (rMFI of IL-17) and Th17/Treg ratio vs. relevant anti-HIV $CD8^+$ T-cell activities at later times p.i within the PHI cohort. The importance of evaluating the *ex vivo* VIA of $CD8^+$ T-cells is reflected by the fact that it was probed to be predictive of the rate of $CD4^+$ T-cell decline during HIV infection[17]. Importantly, when PHIs were sub-divided considering the magnitude of their $CD8^+$ T-cell VIA, we found that PHI subjects with higher baseline VIA (>25%) showed significantly higher CD4/CD8 ratio compared to PHIs with lower VIA (<25%) at both, baseline and one year p.i (Fig. 6A). These results highlight the prognostic value of this antiviral $CD8^+$ T-cell function as it was previously reported for other patient cohorts[17]. Then, it was interesting to find that Th17 functionality (evaluating rMFI of IL-17 as indicator of IL-17 production) was positively correlated with VIA ($p = 0.0062$ $r = 0.7636$; Fig. 6B). And in this case when samples were subdivided by their VIA levels (>25% or <25%), we detected significant differences between both groups, finding that in those patients with a higher VIA, higher rMFI of IL-17 was also detected [25.8 (21.8–31.5) for VIA>25% vs. 17.9 (12.9–19.8) for VIA<25%; $p = 0.0206$; Fig. 6C]. Finally, we also found a direct correlation between Th17/Treg ratio and VIA ($p = 0.0120$ $r = 0.8649$; Fig. 6D). Thus, PHI patients with a

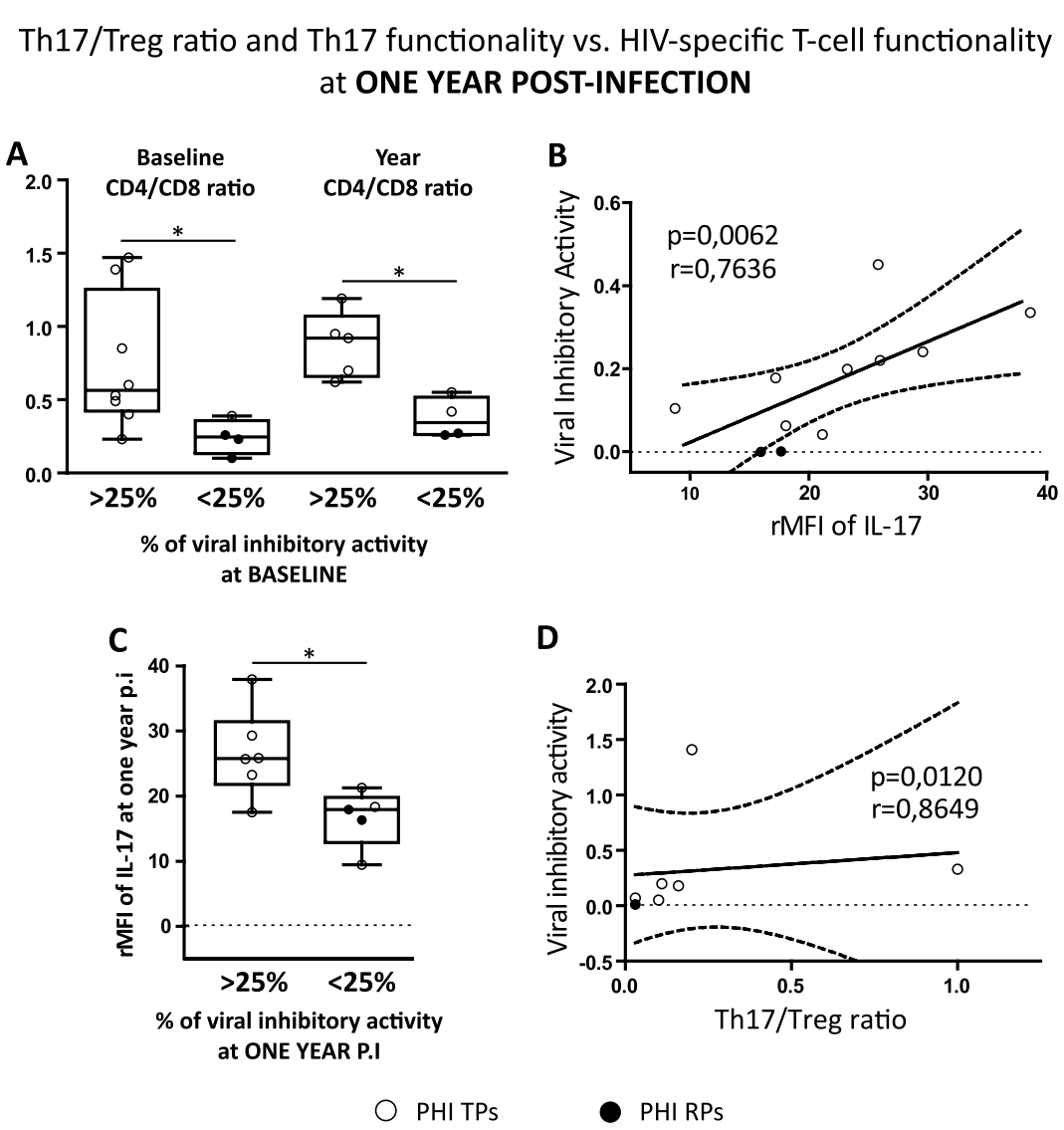

Figure 6. Influence of Th17 levels and Th17/Treg ratio on the quality of the HIV-specific CD8 T-cell responses at later times post-infection. Differences between PHI patients showing baseline viral inhibitory activity (VIA) higher or lower to 25 percent (> and <25%, respectively) regarding to their CD4/CD8 ratio at baseline and one year p.i (**A**). Correlation found between VIA versus (vs.) relative mean fluorescent intensity (rMFI) of IL-17 within PHI cohort at one year p.i (**B**). Comparison of PHI patients with VIA > and <25% at one year p.i regarding to their rMFI of IL-17 also at one year p.i (**C**). Correlation found between VIA versus (vs.) Th17/Treg ratio within PHI cohort at one year p.i (**D**). Symbols distinguish individual patients from the different sub-groups indicated in the figure. *PHI*: primary HIV infection cohort. *TPs*: typical progressors. *RPs*: rapid progressors. Boxes indicate median values with 25–75 percentiles and bars show the maximum and minimum values. The *p* values obtained are depicted as * $p < 0.05$ (**A** and **C**). All *r* and *p* values correspond to Spearman's correlations (**B** and **D**).

higher preservation of the Th17/Treg ratio showed HIV-specific $CD8^+$ T-cells with superior capacity to suppress HIV replication *in vitro*.

The results described in this section showed that at later times p.i both the functionality of Th17 cells and also the Th17/Treg ratio were related with the functional anti-HIV activity of the $CD8^+$ T-cells (VIA).

Discussion

In this study we analyzed the frequency of Th17 and Treg subsets, their ratio and correlation with clinical parameters and HIV-specific $CD8^+$ T-cell responses during PHI.

Noticeably we found that, compared to HDs, the Th17/Treg ratio was significantly reduced in all HIV-infected patients, and within the PHI cohort we found a rapid reduction in the Th17/Treg ratio (in baseline samples). This finding is relevant when interpreted in the context of a previous study performed

in the SIV model by Favre *et al.*[18]. That work showed that pathogenic SIV acute infection of pigtailed macaques was characterized by a rapid and marked selective depletion of Th17 cells and drop of the Th17/Treg ratio in blood and multiple tissues, indicating that an acute imbalance is related to SIV disease progression. The Th17/Treg ratio was significantly reduced even in ECs, and this reduction in the Th17/Treg ratio coincided with higher proportions of activated $CD8^+$ T-cells in this group (compared to the normal levels found in HDs, Fig. 2D). In contrast to the many recent studies showing evidences of an activated innate and T-cell immune response in ECs[19–21], we could only find one report that compared Th17/Treg ratio between ECs and HDs, in which a trend to lower Th17/Treg ratio in ECs can be appreciated[22]. In this context, our results suggest that the potential correlation between Th17/Treg imbalance and levels of immune activation that are sometimes present in ECs is a topic that merits further in-depth analyses.

Within the PHI cohort, we found a trend towards a Th17 reduction between baseline and one year p.i samples, however significant differences were not reached in line with previous studies[13,23]. However, at baseline we found a clear difference between RPs and TPs in relation to $CD8^+$ T-cell activation, VL and Th17 counts. The pattern observed in RPs is in line with previous data of advanced HIV infection[8,24] and more importantly, with the factors associated with severe symptomatic PHI previously described for the patients from the Grupo Argentino de Seroconversión study group[25] and also for other HIV infected cohorts[26]. At early times p.i, preservation of the Th17 subset (evaluated by different means such as plasma IL-17 levels, percentage of Th17 cells and rMFI of IL-17) correlated with lower levels of activated $CD4^+$ T-cells, and on the other hand with both higher CD4 counts and levels of MDC/CCL22. This β chemokine has been previously associated with HIV-protective activities[15,27], although still some controversy exist regarding its role in inhibition of HIV replication depending on the sources of the infected cells, virus strains and chemokines employed in the *in vitro* studies performed[28–30].

Afterwards, at later times p.i, higher Th17 levels remain positively correlated with clinical status (CD4 counts) and inversely correlated with baseline levels of the inflammatory sCD40L, a biomarker associated with disease during HIV/AIDS[16] (Fig. 3E-F). Summarizing, findings related to the first part of our study indicated a clear relationship between a better clinical status with higher Th17 and lower Treg levels. As this last T-cell subset is known to suppress T-cell responses, negative correlations with levels of immune activation could be expected, however we found direct correlations with $CD8^+$ T-cell activation and VL, and an inverse relationship with CD4 counts. These results are in line with recent studies which described direct correlations between Treg cells and T-cell activation[22] or disease progression[23], and inverse correlations with CD4 frequencies[22], thus supporting the hypothesis of a detrimental role for this subset or the expansion of non-functional Tregs during HIV infection.

The main novel contribution of the present work relies on the analysis of the potential impact that the alterations recorded in the proportions and balance of Th17 and Treg subsets could have on the HIV-specific $CD8^+$ responses with relevance on the antiviral HIV immunity during PHI. Our results revealed direct relationships between baseline Th17 levels and VIA against HIV and also with other key polyfunctional features (IFN-γ^+/$CD107_{A/B}{}^+$) of the HIV-specific $CD8^+$ T-cells at both early and later times p.i (Fig. 4), highlighting the potential prognostic value of the Th17 subset for the preservation of an effective HIV immunity. Importantly, we still detected positive correlations between Th17 functionality (rMFI of IL-17) and Th17/Treg ratio with VIA at one year p.i follow-up (Fig. 6).

In line with our findings, some previous reports also suggested the potential influence of the Th17 subset on the functionality of the HIV/SIV-specific T-cell responses. One of these works analyzed $CD4^+$ T-cell restoration in GALT of HIV-infected patients, finding that an effective restoration was associated with an enhanced proportion of Th17 cells and polyfunctional HIV-specific T-cell responses[31]. Another study performed in the SIV model not only demonstrated that SIV replication is limited by the preexisting Th17 cell compartment and that a reduction in the Th17/Treg ratio was associated with higher VL, but also that these variables could shape and interact with T-cell mediated responses against the virus[32]. Consequently, animals with higher levels of Th17 cells had the highest SIV-specific $CD4^+$ and $CD8^+$ T-cell responses, and more importantly, specific $CD4^+$ T-cell responses observed in animals with high Th17/Treg ratios were also more functional.

To further inquire into the possible relationship between Th17 levels and potential T-cell effector functions, we decided to also analyze other T-cell effector subsets after a polyclonal stimulation. Our results suggest that preservation of the Th17 cell compartment can be also important to preserve higher levels of other T-cell effector functions, potentially implicated with the T-cell capacity to confront other pathogen infections.

The Th17 and the Th17/Treg ratio preservation/deterioration observed in our study may have different consequences with relevant implications on the anti-HIV $CD8^+$ T-cell functions. One of them is the loss of $CD4^+$ T-cells that we found to be positively and negatively correlated with frequencies of Tregs and Th17 counts, respectively (Fig. 3). The progressive decline in the CD4 counts in HIV, as well as in other chronic viral infections, has been associated with the dysfunction of $CD8^+$ T-cells justified by the reduction in the $CD4^+$ T-cell help which is necessary for the $CD8^+$ T-cell secondary expansion[33,34]. Also, the impact that high VL may have on T-cell dysfunction is an important factor to take into account[34–36], as well as the presence of activated virus-specific $CD8^+$ T-cells without an effector function (exhausted) as a mechanism of viral immune evasion[37]. In relation to these two last important factors, in this study we found that while Th17 levels were inversely correlated with VL, the percentage of Treg cells positively

correlated with viral replication, in line with their direct relationship with higher frequencies of activated $CD8^+$ T-cells. On the other hand, higher proportions of exhausted HIV-specific $CD8^+$ T-cells (positive for PD-1) at later times p.i inversely correlated with baseline Th17 levels (Fig. 4F). Another plausible hypothesis which could explain the positive direct link between Th17 with $CD8^+$ T-cell functionality, is the action exerted by interleukin 21 (IL-21), which is a pleiotropic cytokine expressed at high levels by Th17 cells and other activated $CD4^+$ T-cells[38]. This cytokine exerts multiple functions, as to maintain long-term functional anti-viral $CD8^+$ T-cells[33,39–41] and an adequate pool of fully functional Th17 cells[42,43]. Thus, in a recently published study administration of IL-21 to SIV-infected macaques was accompanied by higher expression of perforin and granzyme B in total and SIV-specific $CD8^+$ T-cells (better functionality) and higher levels of intestinal Th17 cells[44]. These previous observations raise the possibility that our findings, showing a positive correlation between HIV-specific $CD8^+$ T-cell responses and Th17 levels and functionality (rMFI of IL-17), could be explained by inferring that higher levels of Th17 cells would translate into higher IL-21 production.

It must be notice that despite the novelty of our findings, one of the constraints of the present study was the limitation in the samples available to perform the multiple evaluations. Thus, although 40 PHI subjects were enrolled, not all the samples rendered sufficient cell numbers to perform the complete set of assays. On the other hand, another limitation is that all the analyses were performed in the peripheral blood compartment, and it must be taken into account that the Th17 T-cell subset exerts its main functions at mucosal level.

A relevant topic considered is the effect of ART on the reestablishment of Th17 proportions and Th17/Treg balance, as different previous studies have reported that after ART treatment the restoration of Th17 numbers and functionality[31,45] as well as the Th17/Treg balance may not be fully recovered[46]. In this context a recent important study highlighted the relevance of the initiation of ART during early acute HIV infection, demonstrating its benefits to preserve the mucosal Th17 function and to reverse the HIV-related immune activation[47]. Thus, considering these previous data and the results from the present study it will be important to evaluate in the future the potential impact of the ART treatment on Th17 and Th17/Treg balance restoration and its influence on the re-establishment of the quality of the CD8+ T-cell responses after the treatment.

In summary, the findings described in this study suggest for the first time that an interaction between Th17 and Th17/Treg balance with the functionality of the HIV-specific $CD8^+$ T-cell response may exist. Overall, to our knowledge this is the first report from a South-America cohort of HIV subjects to address the interplay of Th17/Treg cells with disease progression and specific $CD8^+$ T-cell functionality.

Materials and Methods

Study population. A total of 84 individuals participated in this study: 14 healthy HIV-seronegative donors [HDs; voluntary blood donors from the Sanatorio Dr. Julio Mendez blood bank (Buenos Aires, Argentina), all of them individuals older than 18 years that completed and passed a survey on blood donation and were screened for serological markers before being accepted as donors] and 70 HIV-infected patients, of whom 40 were enrolled during PHI, 17 were chronically infected (Chronics) and 13 were defined as ECs (Table 1). PHI subjects were enrolled by the Grupo Argentino de Seroconversión Study Group under the following inclusion criteria[25]: (i) detection of HIV-1 RNA or p24 antigen with a simultaneous negative or indeterminate Western blot (WB) assay or (ii) positive WB assay with a negative test within the previous 6 months. For some analyses, the PHI group was further sub-divided into 2 subgroups whether their CD4 counts dropped below 350 cells/μl during the first year p.i or not, denoted as rapid (RPs) and typical (TPs) progressors, respectively. Chronics were defined as individuals with established HIV-1 infection for more than 3 years, high VL (>10.000 HIV-1 RNA copies/ml plasma), and that were ART naïve at the time of enrollment. ECs were defined as persons HIV-infected for more than 5 years with undetectable VL (<50 HIV-1 RNA copies/ml plasma) without ART, CD4 counts >450 cells/μl and with no records of opportunistic infections and/or AIDS-related diseases. The study was reviewed and approved by two institutional review boards: *Comité de Bioética y Docencia e Investigación Hospital General de Agudos Juan A. Fernández (Buenos Aires, Argentina; protocolo CODEI 201115, 29/08/2011)* and *Comité de Bioética Fundación Huésped (Buenos Aires, Argentina).* All HIV-infected participants provided written informed consent accepting to participate in this study and the methods applied were carried out in accordance with the approved guidelines.

Human samples. Blood samples were collected at enrollment on tubes with EDTA and centrifuged to separate plasma, which was stored at −80 °C until use. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham, Sweden) and cryopreserved. For those PHI patients that remained ART naïve, additional samples were obtained at a median of 330 days p.i (see Table 1). For Chronics, ECs and PHIs, plasma VL (branched-DNA, Versant HIV-1 RNA 3.0 assay; Siemens Healthcare) and CD4/CD8 counts (flow cytometry double platform, BD FACSCanto; BD Biosciences) were determined.

Subsequent functional assays were performed, according to sample availability, using only thawed cells that showed >95% viability after overnight rest in complete RPMI medium [RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 2 mM L-glutamine (Sigma-Aldrich,

USA), 100 U/ml penicillin (Sigma-Aldrich, USA), 100 mg/ml streptomycin (Sigma-Aldrich, USA), and 10 mM HEPES (Gibco, USA)].

Phenotyping of Th17, Treg and other T-cell subsets of interest. Different subsets were evaluated by flow cytometry using thawed and overnight rested PBMCs dispensed in U-bottom 96-well plates (between 5×10^5 and 10^6 cells/well were used, depending on sample availability). All fluorochrome-conjugated, isotype and co-stimulatory antibodies (Abs) used in this study were obtained from BD Biosciences (USA).

Treg cells (defined as $CD4^+$ $CD25^+$ $FoxP3^+$) were evaluated in unstimulated PBMCs, stained for 30 minutes (min) at 4 °C with LIVE/DEAD Fixable NEAR-IR (Invitrogen), anti-CD3-PerCP, anti-CD4-FITC and anti-CD25-APC. For intranuclear staining, the Human FoxP3 Staining Kit (BD Biosciences) was used according to manufacturer's instructions. Briefly, after the fixation and permeabilization steps, cells were incubated for 30 min at 4 °C with anti-FoxP3-PE. Isotype-matched APC- and PE-conjugated non-specific Abs were used in each sample in order to accurately set FoxP3 and CD25 negative populations (see Supplementary Fig. S3).

Th17 cells (defined as $CD4^+$ $IL\text{-}17^+$) were identified by intracellular cytokine staining (ICS) after 6 hours (hs) of polyclonal stimulation at 37 °C with anti-CD3 and anti-CD28 Abs (10 ng/ml each) and monensin (0.7 μl/ml; GolgiStop, BD Biosciences). This assay also allowed the simultaneous detection of the following T-cell subsets: Th1 ($CD4^+$ IFN-γ^+), Tc17 ($CD8^+$ $IL\text{-}17^+$) and Tc1 ($CD8^+$ IFN-γ^+). Briefly, surface staining consisted of 30 min incubation at 4 °C with LIVE/DEAD Fixable NEAR-IR (Invitrogen), anti-CD3-PECy7, anti-CD4-PerCP and anti-CD8-APC. Then, ICS was performed following the instructions of the Cytofix/Cytoperm kit (BD Biosciences), incubating 30 min at 4 °C with anti-IL-17A-PE and anti-IFN-γ–FITC. Unstimulated controls (medium only) were included and frequencies presented correspond to background-subtracted results for each patient (see Supplementary Fig. S3). Samples with a background higher than 0.05% were retested using a new vial of frozen cells, when available.

Additionally, Th17 functionality was evaluated as the density of expression of IL-17 in Th17 cells (MFI values on a logarithmic scale) relative to the MFI of IL-17 in total $CD4^+$T-cells in each sample as follows: PE MFI_{Th17}/PE $MFI_{total\ TCD4+}$.

All samples were immediately acquired in a 2-laser, 6-color BD FACSCanto flow cytometer and analyses were performed using the BD FACSDiva software. Instrument settings and fluorescence compensation were performed for each day of testing using unstained and single-stained samples. The same initial gating strategy was applied in all flow cytometry assays (see Supplementary Fig. S3). First, small lymphocytes (between 250.000 and 80.000 events) were selected in a plot of forward scatter (FSC) vs. side scatter (SSC). Then, FCS area (FSC-A) vs. height (FSC-H) was constructed to remove doublets. Dead cells were then excluded by the LIVE/DEAD fluorescence. Subsequently, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ events were gated in a CD3 vs. CD4 or CD8 dot plot. Finally, for selection of Th17 (as well as Th1, Tc17 and Tc1) the corresponding CD4 (or CD8) vs. IL-17 (or IFN-γ) were constructed. For Treg evaluation, CD4 vs. CD25 and CD4 vs. FoxP3 dot plots were constructed and the "derived gate tool" was used to accurately and automatically determine the double positive $CD25^+$ $FoxP3^+$ population.

Immune activation. Immune activation was defined as the percent of $CD38^+$ HLA DR^+ T-cells (CD4 or CD8) and analyzed by flow cytometry. For this, thawed and overnight rested PBMCs were stained for 30 min at 4 °C with LIVE/DEAD Fixable NEAR-IR (Invitrogen), anti-HLA-DR-FITC, anti-CD4-PerCP, anti-CD38-APC, anti-CD3-PeCy7 and anti-CD8-PE (all Abs obtained from BD Biosciences). Data acquisition and analysis was performed using the BD FACSDiva software. Initial gating was performed as described above. Isotype-matched FITC- and APC-conjugated non-specific Abs were used in each sample to set HLA-DR and CD38 negative populations. The "derived gate tool" was used to accurately and automatically determine the double positive $CD38^+$ HLA-DR^+ population.

Virus inhibitory activity (VIA) and $CD8^+$ T-cell bi-functionality. The *ex vivo* ability of $CD8^+$ T-cells to inhibit HIV-1 replication in primary autologous $CD4^+$ T-cells (VIA) and the capacity of HIV-specific $CD8^+$ T-cells to simultaneously produce IFN-γ and degranulate (evidenced by $CD107_{A/B}$ mobilization) upon HIV-peptides stimulation were evaluated following the same protocols previously published[11]. Examples of the gating strategy applied to detect bi-functional cells by flow cytometry are illustrated in Supplementary Fig. S4.

Exhaustion of the HIV-specific $CD8^+$ T-cell response. The expression of PD-1 marker was evaluated by flow cytometry on HIV-specific $CD8^+$ T-cells as recently published[48].

Quantification of plasma soluble factors. Simultaneous determination of the following 39 cytokines and chemokines was performed in plasma samples from a subset of 20 PHI subjects (at baseline only) using Luminex technology (MILLIPLEX MAP Human Cytokine/ Chemokine; Millipore): EGF, eotaxin, FGF-2, Flt-3 ligand, fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, sCD40L, sIL-2Rα, TGF-α, TNF-α, TNF-β, VEGF. Samples were processed and analyzed as described by Giavedoni[49].

Data analysis. For PHI patients, the estimated time p.i was calculated as described (see[25] and Table 1). Statistical analyses were performed using GraphPad Prism 5 (Graph-Pad Software). All data except Log_{10} VL were analyzed using nonparametric statistics. Kruskal-Wallis and two-tailed Wilcoxon and Mann-Whitney tests were used to compare intra- and intergroup variables, respectively. Correlations were determined using Spearman's rank test. All tests were considered significant if the p value obtained was less than 0.05.

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Author Contributions

J.F. and M.M.G. planned experiments, interpreted the data, and wrote the manuscript. J.F., G.T. and M.M.G. planned experiments, analyzed data and corrected the manuscript. N.L., M.E.S., M.I.F., P.C., H.S. and O.S. recruited the donors and corrected the manuscript. J.F., G.T., Y.G., M.P.H., M.J.R. and C.M. collected clinical data and performed experiments L.D.G. performed experiments and corrected the manuscript

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Host Genetic Factors Associated with Symptomatic Primary HIV Infection and Disease Progression among Argentinean Seroconverters

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Abstract

Background: Variants in HIV-coreceptor C-C chemokine receptor type 5 (CCR5) and Human leukocyte antigen (HLA) genes are the most important host genetic factors associated with HIV infection and disease progression. Our aim was to analyze the association of these genetic factors in the presence of clinical symptoms during Primary HIV Infection (PHI) and disease progression within the first year.

Methods: Seventy subjects diagnosed during PHI were studied (55 symptomatic and 15 asymptomatic). Viral load (VL) and CD4 T-cell count were evaluated. HIV progression was defined by presence of B or C events and/or CD4 T-cell counts $<$ 350 cell/mm^3. CCR5 haplotypes were characterized by polymerase chain reaction and SDM-PCR-RFLP. HLA-I characterization was performed by Sequencing.

Results: Symptoms during PHI were significantly associated with lower frequency of CCR5-CF1 (1.8% vs. 26.7%, p = 0.006). Rapid progression was significantly associated with higher frequency of CCR5-CF2 (16.7% vs. 0%, p = 0.024) and HLA-A*11 (16.7% vs. 1.2%, p = 0.003) and lower frequency of HLA-C*3 (2.8% vs. 17.5%, p = 0.035). Higher baseline VL was significantly associated with presence of HLA-A*11, HLA-A*24, and absence of HLA-A*31 and HLA-B*57. Higher 6-month VL was significantly associated with presence of CCR5-HHE, HLA-A*24, HLA-B*53, and absence of HLA-A*31 and CCR5-CF1. Lower baseline CD4 T-cell count was significantly associated with presence of HLA-A*24/*33, HLA-B*53, CCR5-CF2 and absence of HLA-A*01/*23 and CCR5-HHA. Lower 6-month CD4 T-cell count was associated with presence of HLA-A*24 and HLA-B*53, and absence of HLA-A*01 and HLA-B*07/*39. Moreover, lower 12-month CD4 T-cell count was significantly associated with presence of HLA-A*33, HLA-B*14, HLA-C*08, CCR5-CF2, and absence of HLA-B*07 and HLA-C*07.

Conclusion: Several host factors were significantly associated with disease progression in PHI subjects. Most results agree with previous studies performed in other groups. However, some genetic factor associations are being described for the first time, highlighting the importance of genetic studies at a local level.

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Introduction

Research studies on primary HIV infection (PHI) are increasing worldwide to better understand the natural history of HIV infection and to identify the most important disease prognostic markers. As most of these studies were performed in other countries and due to genetic differences in the circulating virus and in the host, local studies are needed to better understand the particular characteristics of HIV infection dynamics [1].

In Argentina, an estimated 110,000 persons live with HIV (approximately 5,000 new cases per year) [2]. The first multicenter follow-up study of PHI (*Grupo Argentino de Seroconversión*) started in 2008. Retrospective and prospective data analyses allowed identifying factors associated with disease progression among untreated subjects. Symptomatic PHI, high VL (≥100,000

RNA copies/ml) or low CD4 T-cell count (≤350 cell/mm^3) at baseline were identified as relevant factors for faster progression during the first year follow-up [3]. Data comparisons with other PHI cohorts revealed that VL at baseline in the Argentinean cohort was higher than those found in developed countries [4–5], closer to African and Asian levels [6–7]. Globally, 50–90% of subjects diagnosed during PHI are symptomatic [8–10], reaching 74% in the mentioned Argentinean cohort [3].

Previous studies demonstrated extensive variability in host susceptibility to HIV infection and disease progression [11–13]. Several host genetic factors affecting HIV infection and pathogenesis were identified, like chemokine receptors and HLA alleles [14–17]. Multiple variations were described in the CCR5 gene, in particular the 32 base-pair deletion (CCR5-Δ32). This deletion provides protection against HIV-1 infection with CCR5 tropic viruses in homozygotes and delays progression in heterozygous subjects [16,18–19]. Seven Single Nucleotide Polymorphisms (SNPs) were defined in the *cis*-regulatory region between −2761 and −1835 of the CCR5 gene: −2733, −2554, −2459, −2135, −2132, −2086 and −1835 (GenBank accession number AF031236 and AF031237) [20]. Based on these variations and on the CCR2-V64I polymorphism, nine polymorphisms, called CCR5 Human Haplotypes (HH)-A, -B, -C, -D, -E, -F (F*1 and F*2), and –G (G*1 and G*2) were defined [15,20–21]. One of the largest studies in the subject demonstrated that the frequency and effect of CCR5-HH differ among different ethnic groups. CCR5-HHA was associated with disease retardation among African-Americans, whereas CCR5-HHC did so among European-Americans. In the same study, specific sequences of CCR5-HHE were associated with higher transcriptional activity, surface expression and HIV/AIDS susceptibility [21]. Another factor associated with disease progression is the dose of the gene encoding CCL3L1 (MIP-1α), a natural ligand of CCR5. A previous study found an association between lower gene dose and disease progression, and this susceptibility is even greater in individuals with CCR5 genotypes associated with disease progression [22].

The HLA system has an impact on several aspects of HIV infection such as transmission, progression and therapeutic response [23–24]. HLA class I molecules are involved in peptide presentation to CD8 cytotoxic T lymphocytes (CTLs), which play a key role in reducing viral replication. HIV specific CD8 T-cell response emerges along with the control of viremia and resolution of clinical symptoms, which varies from person to person and constitutes a strong predictor of disease progression [25–26]. Heterozygosis at HLA class I region is considered to be a selective advantage because those individuals are able to present a greater range of antigenic peptides to CTLs than homozygotes, deferring the emergence of escape mutants and prolonging the period before the development of AIDS [18]. Even when several HLA alleles were associated with disease progression, HLA-B*27 and HLA-B*57 alleles showed a particularly strong association with delayed progression [27] and HLA-B*35 and HLA-B*53 with acceleration to AIDS [28].

Based on the effects of host genetic variations described on HIV disease progression, our aim was to analyze the association of CCR5/CCL3L1 system and HLA in the presence of clinical symptoms during PHI and disease progression within the first year post-infection.

Materials and Methods

Study population

A group of 70 individuals recruited through 2008–2012 was studied. Inclusion criteria for enrolment in the cohort were: >16 years old at first evaluation, PHI confirmed diagnosis, and first medical and laboratory evaluation (i.e., CD4 T-cell count and plasma HIV RNA) within six months of the probable date of infection. Primary HIV infection is defined as: (1) detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western blot assay [12]; or (2) positive Western blot with a negative test within the previous six months. Hence, it includes both acute and recently infected patients [3].

In this study, PHI was defined as symptomatic if one or more of the following symptoms, associated with acute retroviral syndrome, were present: fever, rash, lymphadenopathy, headache, oral ulcers, dysphagia or pharyngitis. Disease progression was defined by clinical B or C events (according to the Centers for Disease Control and Prevention 1993 classification [29]) and/or CD4 T-cell count <350 cells/mm^3 within the first year of infection [3].

Ethics Statement

International and national ethical guidelines for biomedical research involving human subjects were followed. This research study was reviewed and approved by a local Institutional Review Board (IRB), "*Fundación Huésped*" and was conducted in compliance with all federal regulations governing the protection of human subjects. All potential participants signed an informed consent prior to entering the study.

Study Procedure

Once subjects were identified as PHI, they were included in the cohort. Subjects were evaluated at the time of diagnosis (baseline), at 6 months and at one year. On each visit, HIV plasma VL (branched-DNA, Versant HIV-1 RNA 3.0 assay, Siemmens Healthcare, USA), CD4 T-cell count (flow cytometry double platform, BD FACSCanto, BD Biosciences, USA), and clinical information were updated.

Study samples

Peripheral blood samples were obtained on each visit. Whole blood samples or peripheral blood mononuclear cells (PBMC) were used for DNA extraction using QIAmp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany). Plasma samples from the first visit after HIV diagnosis were used for lipopolysaccharide (LPS) quantification (Limulus Amebocyte Lysate test, LAL assay, QCL-1000, Lonza, DK). HIV tropism was determined by sequencing a region of V3 loop from env gene (HXB2) [30]. Viral DNA was amplified in duplicate by nested PCR and amplicons were sequenced by Big Dye Terminator Kit (Amersham, Sweden). Viral tropism was inferred from Geno2Pheno algorithm (http://coreceptor.bioinf.mpi-inf.mpg.de/index.php) using a false positive rate of 10%.

CCR5 and CCL3L1 characterization

CCR5-Δ32 deletion was identified by differences in PCR products size. CCR2 genotypes and Single Nucleotide Polymorphisms (SNPs) of the CCR5 gene corresponding to positions 29, 208, 627, 630, 676 and 927 (Genbank accession number: AF031236 and AF031237) [31] were determined with Site Directed Mutagenesis-PCR-Restriction Fragment Length Polymorphism (SDM-PCR-RFLP) assay. Primers used in each determination, PCR cycling condition and RFLP assay were

reported previously [15,21,32–33]. Haplotype classification (HHA, HHB, HHC, HHD, HHE, HHF*1, HHF*2, HHG*1 and HHG*2) was determined as reported previously [15,20–21]. CCL3L1 Copy Number (CN) was determined by Taqman real-time PCR [22].

HLA characterization

HLA class I characterization was performed by sequencing-based typing (SBT). HLA-A exons 2 and 3 were amplified together. HLA-B and HLA-C exons 2 and 3 were amplified separately as reported in Table S1 and Figure S1 [34–36]. Amplicons were sequenced using the Big Dye Terminator sequencing kit (Amersham, Sweden) [36]. Sequence interpretation was performed using the NCBI SBT Interpretation software (http://www.ncbi.nlm.nih.gov/gv/mhc/sbt.cgi?cmd=main).

Genetic score

Additive genetic score was used to compile host genetic information [37]. In our model, alleles with a previous reported protective effect were added, and risk alleles were subtracted. For CCR5 polymorphisms, Δ32 and CCR2-64I alleles were considered as protective (1) [21]. Regarding CCR5 genotypes, HHC/HHF*2 and HHC/HHG*2 were considered as protective (1), HHC/HHE, HHE/HHE and HHE/HHG*2 as deleterious (–1), and the others as neutral (0) [21,32]. Two CCL3L1 cpg (mean in the Argentinean population) were considered as neutral (0). Lower CCL3L1 CN than the mean was considered as deleterious (–1) and higher CN as protective (1) [22]. HLA-A*02, HLA-A*32, HLA-A*68, HLA-B*15, HLA-B*13, HLA-B*27, HLA-B*32, HLA-B*39, HLA-B*44, HLA-B*51 and HLA-B*57 were considered as protective (1). HLA-A*11, HLA-A*23, HLA-A*24, HLA-B*08, HLA-B*35, HLA-B*53, HLA-C*04 and HLA-C*07 were considered as deleterious (–1). Other HLA alleles were considered as neutral (0) [11–13,23–24,27–28,37–39]. Heterozygosis for HLA was considered as protective (1) and homozygosis as deleterious (–1) [18].

Statistical analysis

Baseline characteristics were described using mean or medians and standard deviation or interquartile ranges [IQRs] for continuous variables respectively, and counts and percentages for categorical data. Chi-square test or Fisher's exact test were used to compare proportions. Differences among continuous variables were analyzed using Student's t-test or Wilcoxon test. Spearman correlation was calculated for genetic score and HIV viral load and CD4 T-cell count (baseline and follow up). All p values were two-sided; p values$<$0.05 were considered to be statistically significant. Lack of complete data values in table is expressed in numbers. Data analysis was performed using SPSS 15.0, 2007 (Chicago, Illinois).

Results

Characteristics of the study population

We studied 70 HIV-infected adults diagnosed during primary HIV infection (PHI) (49 men and 21 women), 55 were symptomatic and 15 asymptomatic. Sixty of them were also classified according to disease progression within the first year post diagnosis, 18 progressed and 42 did not. Most PHI subjects were recruited during Fiebig stages V and VI [40]. Sexual transmission was reported as the main route: all the women reported heterosexual transmission whereas 82.2% of men reported sexual relationship with other men as the probably route of acquisition of the virus. All subjects were from Buenos Aires City and surrounding areas. The population of this area is mostly descendent from South Europe [41]. Median HIV VL at diagnosis was 61862 RNA copies/ml, whit significantly higher VL in those who presented symptoms and those who progressed (Table 1). The same trend was observed for VL at 6 months. Baseline CD4 T-cell count was 514 cells/mm^3 without statistical differences between symptomatic and asymptomatic subjects. Significantly higher CD4 T-cell counts (baseline, 6 and 12 months) were observed among subjects who did not progress to disease during the first year (Table 1).

Frequency of CCR5 haplotypes/genotypes and CCL3L1

Similar to the results found in Argentinean children exposed perinatally to HIV (including both HIV infected and not infected) [42] and blood donors [43], the most frequent CCR5 haplotypes in the PHI group were HHE (36.4%) and HHC (30.7%). Frequencies of all the other haplotypes were lower than 10% (Figure 1; Table S2). Regarding CCR5 genotypes, HHC/HHE (21.4%) and HHE/HHE (12.9%) were the most commonly found. Other genotypes were present with frequencies lower than 10% (Table 2 and Table S3). The CCL3L1 gene copy number, one of the main ligands of CCR5, was evaluated in 50 PHI subjects with a median of two copies (IQR25-75, 1–4), as reported in persons of European origin [22].

Frequency of HLA variants

Given the essential role of CTL responses during PHI as well as the description of a strong association among certain HLA-I alleles with virus control, HLA-I frequencies were studied in this cohort finding 17 HLA-A, 27 HLA-B and 14 HLA-C different alleles. The HLA-A alleles most frequently found were HLA-A*02 (27.2%) and HLA-A*24 (12.5%). In HLA-B locus, HLA-B*35 (15.6%) and HLA-B*44 (12.9%) were the most frequent. In HLA-C, HLA-C*07 (27.9%), HLA-C*04 (16.2%) and HLA-C*03 (11.8%) were the most frequent. Other HLA-A, B and C alleles showed frequencies lower than 10% (Table S4). HLA class I alleles were found in homozygosis in the following frequencies: 32.4% for HLA-A, 3.0% for HLA-B and 17.6% for HLA-C (Table S5). The most common combinations for HLA-A were A*02-A*02 (11.8%) and A*02-A*68 (8.8%), for HLA-B were B*15-B*35 (4.5%) and B*35-B*44 (4.5%), and for HLA-C, C*04-C*07 (8.8%), C*07-C*07 (8.8%) and C*03-C*07 (7.4%) (data not shown).

Influence of CCR5 haplotypes/genotypes, CCL3L1 copy number, and HLA variants on symptoms present during acute HIV infection

In order to identify individual host genetic determinants of early HIV disease progression, the PHI cohort was stratified according to the presence/absence of symptoms during the seroconversion period. Regarding the CCR5 coreceptor, HHC was overrepresented (40% vs. 28.2%) and HHE (23.3% vs. 40%) was less frequent in asymptomatic as compared to symptomatic subjects, however without statistical significance (Figure 1). Concerning CCR5 genotypes, HHC/HHF*1 was detected in a significantly higher percentage among asymptomatic subjects (26.7% vs. 1.8%, p = 0.006). Even when it was not statistically significant, genotype HHE/HHF*1 was only found among symptomatic subjects (10.9%) (Table 2 and Table S3). No significant differences were found in the CCL3L1 copy number, even when a higher copy number was detected among asymptomatic (median (IQR25-75); 3 (2–3) and 2 (1–4), respectively). No influence of HLA-A, -B and -C alleles was detected in the presence of symptoms during PHI (Table S4). Likewise, no influence of HLA homozygosis was

Table 1. HIV viral load and CD4 T-cell count of the study population diagnosed during primary HIV infection [PHI] (N = 70).

		Symptomatic PHI		p	Progressor at one year		p	All (N = 70)
		Yes (N = 55)	No (N = 15)		Yes (N = 18)	No (N = 42)		
HIV RNA median copies/ml (IQR)	Baseline	77,080	7,024	**0.003**	193,601	41,402	**0.003**	61,862
		(30,449–386,715)	(2,699–76,466)		(80,545–500,000)	(10,409–154,476)		(17,050–257,524)
	6 month	66,002	9,018	**0.004**	166,812	33,508	**0.001**	40,231
		(17.959–178.030)	(3,820–34,624)		(47,167–321,018)	(8,578–73,231)		(117,17–165,238)
CD4 T-cell count median cells/mm^3 (IQR)	Baseline	502	587	0.322	306	602	**<0.001**	514
		(356–649)	(416–876)		(237–346)	(500–741)		(387–671)
	6 month	499	555	0.694	323	602	**<0.001**	503
		(356–665)	(424–665)		(172–386)	(488–690)		(404–65)
	12 month	491	534	0.296	330	534	**0.001**	501
		(389–615)	(436–672)		(289–504)	(435–643)		(400–619)

PHI: primary HIV infection. IQR: interquartile range. Statistically significant p values are in bold.
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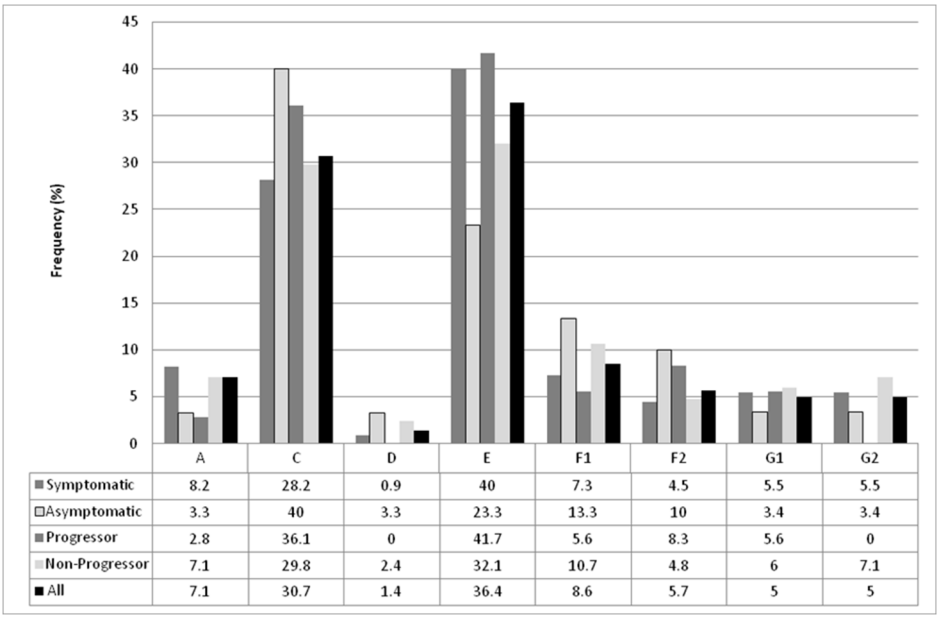

	A	C	D	E	F1	F2	G1	G2
Symptomatic	8.2	28.2	0.9	40	7.3	4.5	5.5	5.5
Asymptomatic	3.3	40	3.3	23.3	13.3	10	3.4	3.4
Progressor	2.8	36.1	0	41.7	5.6	8.3	5.6	0
Non-Progressor	7.1	29.8	2.4	32.1	10.7	4.8	6	7.1
All	7.1	30.7	1.4	36.4	8.6	5.7	5	5

Figure 1. Frequency of CCR5 haplotypes of the study population diagnosed during primary HIV infection [PHI] (N = 70). Full information is available on supplementary material (Table S1).
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observed in the presence of symptoms during seroconversion (Table S5). When HLA pairs were compared, HLA-B*35-B*44 was found in a significantly higher frequency among asymptomatic subjects (21.4% vs. 0%, $p = 0.007$) (data not shown).

Only CCR5 genotypes with a frequency higher than 10% in some of the study groups were included in the table. No significant differences were observed among CCR5 genotypes with frequencies lower than 10%. Full information is on supplementary material (Table S2).

Influence of CCR5 haplotypes/genotypes, CCL3L1 and HLA variants on disease progression within the first year

Additionally, the PHI group was analyzed in order to identify possible genetic factors that might influence the rate of progression within the first year. Several CCR5 haplotypes were most frequently detected in individuals who did not progress (e.g. HHA, HHF*1 and HHG*2) and HHF*2 was most represented in subjects who progressed to disease (Table S2), without statistical differences. Regarding CCR5 genotypes, HHC/HHF*2 was significantly associated with progression ($p = 0.024$) and a higher, but not significant proportion of subject who progress had HHE/HHE also as compared with those who do not progress (22.2% vs. 7.1%) (Table S3). Regarding HLA alleles, a strong association was found between disease progression and higher frequency of HLA-A*11 (16.7% vs. 1.2%, $p = 0.003$) and lower frequency of HLA-C*03 (17.5% vs. 2.8%, $p = 0.035$) (Table S4). No influence of HLA homozygosis was observed in disease progression (Table S5).

Influence of CCR5 haplotypes/genotypes, CCL3L1 and HLA variants on plasma HIV viral load and CD4 T-cell count

As the CD4 T-cell count and HIV plasma VL are good predictors of disease progression [3], the association of these parameters with host genetic factors was also analyzed. Subjects with CCR5 HHE haplotype had higher VL after 6 months (66,001 copies/ml vs. 31,718 copies/ml, $p = 0.039$) and also higher baseline VL (98,684 copies/ml vs. 41,402 copies/ml, $p = 0.082$). On the other hand, HHA was found to be associated with higher baseline CD4 T-cell levels (656 cells/mm^3 vs. 499 cells/mm^3, $p = 0.044$). Regarding CCR5 genotypes, HHC/HHF*1 was associated with lower VL (6,243 copies/ml vs. 53,997 copies/ml, $p = 0.027$) and HHC/HHF*2 with lower CD4 T-cell levels at baseline (379 cells/mm^3 vs. 545 cells/mm^3, $p = 0.046$), at 6 months (355 cells/mm^3 vs. 531 cells/mm^3, $p = 0.024$) and at 12 months (290 cells/mm^3 vs. 510 cells/mm^3, $p = 0.034$).

Concerning the HLA influence on CD4 T-cell count and HIV plasma VL, the presence of several alleles was found to be beneficial for HIV subjects, with an association with higher CD4 T-cell count (HLA-A*01, HLA-A*23, HLA-B*07, HLA-B*39 and HLA-C*07) or lower HIV plasma VL (HLA-A*31 and HLA-B*57). Conversely, some alleles were found to be detrimental for subjects, with an association with higher HIV plasma VL (HLA-A*11, HLA-A*24 and HLA-B*53) or lower CD4 T-cell count (HLA-A*24, HLA-A*33, HLA-B*14, HLA-B*53 and HLA-C*08) (Table 3).

Additive genetic score

Additive genetic score was calculated for each subject and average values were calculated considering symptoms during PHI (2.6 for asymptomatic and 1.4 for symptomatic subjects) and disease progression within the first year (1.8 for those who did not progress and 0.6 for those who progressed). Subjects were grouped according to both characteristics: Group 1: Asymptomatic/Non-progressors, Group 2: Asymptomatic/Progressors and Symptomatic/Non-progressors, and Group 3: Symptomatic/Progressors. Mean genetic score was: 2.8, 1.6 and 0.5 for groups 1, 2 and 3, respectively. Correlation analyses revealed a significant negative correlation between genetic score and HIV viral load at baseline ($p = 0.008$) (Figure 2). No significant association was observed between genetic score and CD4 T-cells count.

Table 2. Frequency of CCR5 human genotypes of the study population diagnosed during primary HIV infection [PHI] (N = 70).

Genotype	Symptomatic PHI		p	Progressor at one year		p	All (N = 70)
	Yes (N = 55)*	No (N = 15)*		Yes (N = 18)*	No (N = 42)*		
HHC/HHC	2 (3.6)	1 (6.7)	0.521	2 (11.1)	1 (2.4)	0.212	3 (4.3)
HHC/HHE	12 (21.8)	3 (20)	1.000	4 (22.2)	9 (21.4)	1.000	15 (21.4)
HHC/HHF*1	**1 (1.8)**	**4 (26.7)**	**0.006**	1 (5.6)	4 (9.5)	1.000	5 (7.1)
HHC/HHF*2	3 (5.5)	1 (6.7)	1.000	**3 (16.7)**	**0**	**0.024**	4 (5.7)
HHC/HHG*1	5 (9.1)	1 (6.7)	1.000	1 (5.6)	5 (11.9)	0.658	6 (8.6)
HHE/HHE	8 (14.5)	1 (6.7)	0.672	4 (22.2)	3 (7.1)	0.220	9 (12.9)
HHE/HHF*1	6 (10.9)	0	0.329	1 (5.6)	4 (9.5)	1.000	6 (8.6)

*Data are no. (%) of CCR5 haplotypes.
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Complementary studies

HIV infection has been associated with disruption of mucosal barrier and CD4 T-cell depletion in the gastrointestinal tract. This damage is caused, at least in part, by increased translocation of microbial products, mainly lipopolysaccharides (LPS), a major component of gram-negative bacterial cell walls [44–46]. Since immune activation is a good predictor of disease progression, plasma LPS levels were determined in the baseline sample of 65 individuals finding a median of 39.0 pg/ml (IQR25-75, 26.7–56.8) with significantly higher levels in the symptomatic than the asymptomatic group (43.5 pg/ml vs. 29.0 pg/ml, $p = 0.040$). No association was found among LPS levels, disease progression, CD4 T-cell count, HIV VL or host genetic factors. HIV tropism was determined given that the presence of X4 tropic viruses was associated with a more rapid disease progression (data not shown). Fourteen out of 59 (23.7%) PHI subjects presented X4 tropic HIV variants. Even when no statistically significant differences were observed, X4 tropic HIV variants were overrepresented among symptomatic subjects (26.1% vs. 15.4%, $p = 0.713$). No differences were observed among HIV tropism, disease progression, CD4 T-cell count or HIV VL.

Discussion

Other countries reported associations between human genes and HIV susceptibility. However, local studies are needed considering differences in genetic background [14,17,19]. In line with this, for the first time in Argentina, this study reports several human genes associated with early HIV disease progression among adults.

Buenos Aires population is mainly descendant of Southern Europe. The frequency of CCR5 haplotypes reported here correlates with reports in Hispanic and other Argentinean groups [21,43], with HHE and HHC being the most common haplotypes. Regarding CCR5 genotypes, the most common were HHC/HHE and HHE/HHE, with other genotypes having frequencies lower than 10%. In comparison with blood donors, PHI individuals were found to have a higher but not significant frequency of HHE/HHE genotype (5.9% vs. 12.9% respectively). This result is consistent with previous reports evidencing an association between presence of HHE/HHE genotype and enhancement of HIV infection [21,42]. Even when the HHE haplotype and the HHE/HHE genotype were overrepresented among symptomatic subjects and those who progressed, no significant associations were observed, maybe due to sample size. Data on HIV VL also supports the same trend with significantly higher VL at 6 months among subjects carrying HHE. This trend is in line with previous studies that associated disease progression with HHE [21,42]. However, this disease-modified effect was not observed among other ethnic groups (i.e., Africans) where the frequency of HHE haplotype was much lower (≈18%) [21]. As HHE is the most frequent CCR5 haplotype in our cohort, the potential adverse effect of this haplotype deserves special attention.

HHC/HHF*1 genotype was associated with asymptomatic PHI and HHC/HHF*2 with disease progression. In line with these results, we found that the HHC/HHF*1 genotype was associated with lower levels of VL and HHC/HHF*2, with lower CD4 T-cell levels at baseline and during one-year follow-up. Only few studies support these findings, maybe due to the fact that these genotypes were found in low frequency in most cohorts [21,42]. One of the most important studies in the subject found a disease accelerating effect for HHC/HHF*1 among African Americans [21]. However, this study also reports that the effect of HHC haplotypes on HIV disease differed among ethnic groups. While the HHC

Table 3. HIV viral load and CD4 T-cell count of the study population diagnosed during primary HIV infection [PHI] according to HLA alleles (N = 70).

Alleles		CD4 T-cell count			HIV RNA	
		median cells/mm^3			median copies/ml	
		Baseline	6 months	12 months	Baseline	6 months
HLA-A*01	Yes	902	810	716	5160	4298
	No	500	499	491	64045	40083
	p	**0.022**	**0.019**	0.112	0.241	0.317
HLA-A*11	Yes	347	344	475	477708	166930
	No	525	517	492	52352	38270
	p	0.070	0.071	0.447	**0.020**	0.059
HLA-A*23	Yes	736	637	534	36101	24322
	No	499	497	475	61862	40232
	p	**0.038**	0.072	0.195	0.374	0.290
HLA-A*24	Yes	393	403	483	500000	89517
	No	576	545	500	41402	30591
	p	**0.049**	**0.048**	0.371	**0.001**	**0.004**
HLA-A*31	Yes	602	563	612	24654	19603
	No	502	502	491	67397	56594
	p	0.494	0.616	0.883	**0.032**	**0.038**
HLA-A*33	Yes	387	387	347	67660	67660
	No	535	528	515	55276	39484
	p	**0.046**	0.100	**0.021**	0.818	1.00
HLA-B*07	Yes	535	818	679	378025	133268
	No	525	499	474	52352	38720
	p	0.972	**0.015**	**0.005**	0.177	0.280
HLA-B*14	Yes	466	485	410	213099	117061
	No	575	542	534	52352	37506
	p	0.167	0.135	**0.002**	0.229	0.260
HLA-B*39	Yes	644	780	573	4383	18062
	No	509	497	483	62679	42753
	p	0.098	**0.027**	0.175	0.073	0.085
HLA-B*53	Yes	288	248	286	500000	349244
	No	545	531	509	54286	39033
	p	**0.046**	**0.036**	0.117	0.058	**0.028**
HLA-B*57	Yes	525	495	654	16926	12971
	No	529	528	492	66821	47077
	p	0.819	0.865	0.272	**0.046**	0.058
HLA-C*07	Yes	525	527	534	66821	60546
	No	497	491	449	62679	40083
	p	0.738	0.527	**0.038**	0.584	0.563
HLA-C*08	Yes	437	499	409	184000	163664
	No	519	499	533	61045	40083
	p	0.290	0.200	**0.001**	0.443	0.286

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haplotype in African Americans was associated with disease acceleration, in Caucasians and Hispanics it was associated with disease retardation. Regarding the HHF*2 haplotype, a previous report found similar results in individuals carrying the allele with lower CD4 T-cell counts during follow-up [47]. However, these results disagree with previous studies that observed a protective effect against disease progression among subjects carrying the CCR2-64I allele [33]. HHC/HHF*2 genotype was also associated with disease retardation among Argentinean children [42]. Even when no statistically significant association was established, the

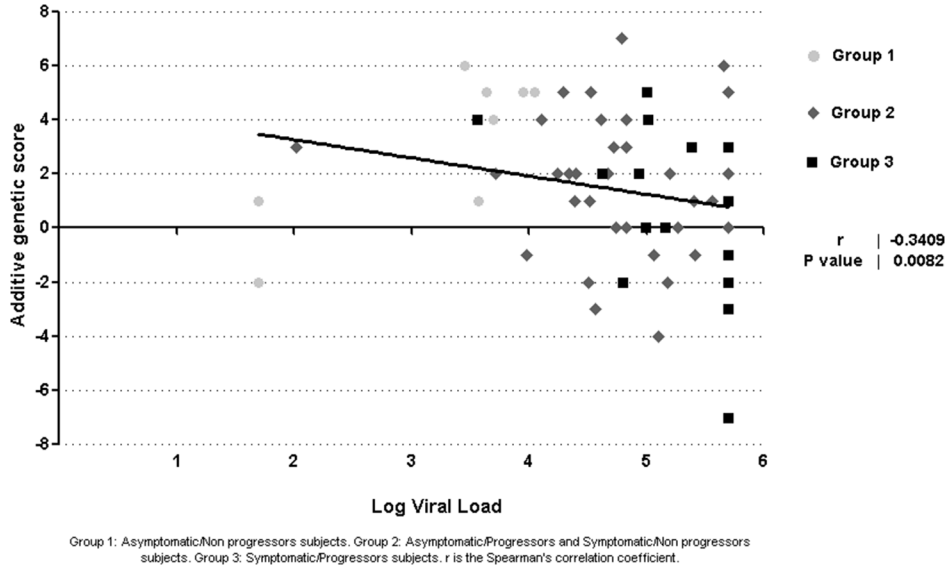

Figure 2. Correlation between baseline HIV viral load and additive genetic score on the study population diagnosed during primary HIV infection [PHI] (N = 70).
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CCR5 genotype HHE/HHF*1 was only detected among symptomatic subjects in more than 10% of the group. CCL3L1 copy number distribution in PHI population was similar to that observed in the European population [22] with a median of two copies. Even when no significant differences were observed, asymptomatic individuals had a higher copy number, maybe suggesting that CCL3L1 would have an impact since the HIV infection onset.

Identifying HLA alleles associations with HIV disease progression is complex due to the extreme variability of the loci. In fact, this study identifies 17, 27 and 14 HLA-A, B and C alleles, respectively. Coincident with previous reports, including our blood donors group, the alleles most frequently reported here were HLA-A*02 and HLA-*24, HLA-B*35 and HLA-B*44, and HLA-C*07, HLA-C*04 and HLA-C*03 [41]. Even when it was proposed that heterozygosis on HLA confers advantages on disease progression revealing a greater variety of the immune response [18,48–49], no significant differences in disease progression were detected between heterozygotes and homozygotes at any individual HLA locus or homozygosis at one, two, or all three class I loci.

Several HLA alleles identified in our study were associated with disease progression. Our results adds more evidence to the protective effect of HLA-B*57 allele on disease progression [23], with significantly lower VL at baseline and also lower, but not significant, VL at 6 months. Moreover, the allele was only found among those who did not progress. Even when HLA-B*57 was previously associated with the absence of symptoms during seroconversion, our study failed to confirm these findings [50]. Regarding HLA-B*27, reported as a protective allele [50], we did not observe this trend or evidence, likely due to the low frequency found (1.5% among HIV positive and 2.0% among blood donors). Another HLA allele, several times associated with disease progression is HLA-B*35 [18,23]. However, our study did not find any statistical association or trend even when the frequency of the allele was around 15% in the overall group.

HLA-A*11 was associated with disease progression during the first year and with higher VL at baseline. We also found a trend in the presence of the allele and higher HIV VL at 6 months and lower CD4 T-cell counts at baseline and during follow-up. These results agree with a previous study that found a higher frequency of HLA-A*1101 among subjects with AIDS compared with other HIV subjects who did not progress [51]. Even when this study performed high resolution HLA-typing, in contrast to our low resolution data, it is important to mention that typing studies reported that most of the typed HLA-A*11 are HLA-A*1101 [41,52].

HLA-B*53 was associated with lower CD4 T-cell counts and higher HIV VL levels, even when only two subjects carried that allele. Elevated VL levels among subjects with HLA-B*53 were previously observed among African seroconverters [53]. Although only few subjects carried the HLA-B*53, the potential impact of this allele on disease progression may deserve more investigation. Another interesting allele is HLA-A*24, associated with lower CD4 T-cell counts and higher VL levels at baseline and during follow-up. This allele frequency was also higher (but not significant) among subjects who presented symptoms during seroconversion as compared with those without them. Previous studies also found a deleterious effect of this allele, enhancing HIV infection [54], showing rapid decline in CD4 T-cells [27] and favouring disease progression [55]. HLA-B*39 confers a beneficial effect on disease evolution yielding high CD4 T-cell counts and low VL levels [16,55]. We also observed a trend in higher frequency of HLA-B*39 among asymptomatic vs. symptomatic (10.7% vs. 2.9%) subjects. Controversial results were found in other alleles. While our study suggests that subjects with HLA-B*14 (with significantly lower CD4 T-cell counts at 12 months and a trend of lower levels of CD4 T-cells at baseline and at 6 months and higher VL) progressed faster to disease, others found significant associations between allele and low disease progression [56] and that the allele had enhanced HIV infection [57].

Previous studies showed that plasma LPS levels among subjects with acute HIV infection were similar to non-infected subjects [58]. In fact, our study found similar levels in the PHI group (39.0 pg/ml) and a group of HIV-negative subjects (37.4 pg/ml, data not shown). However, we found that higher plasma LPS levels are significantly associated with presence of symptoms during PHI.

These results suggest higher immune activation in symptomatic subjects since the establishment of infection.

An important limitation of the current research was the low frequency of asymptomatic subjects included due to the difficulty in identifying them during the seroconversion period. The lack of progression data in a group of patients also influenced the possibility of finding significant associations. It is also important to note the difficulty in finding associations when genetic variants are in low frequency. Given these limitations, a score was constructed in order to combine some of the most important human genetic factors previously associated with HIV/AIDS and to look for associations with presence of symptoms, disease progression and other progression markers like HIV viral load and CD4 T-cell count. Results reveal a higher score in asymptomatic and those who did not progress, revealing the presence of more protective genetic factors in these groups. Even more, when data were analysed considering both variables (symptoms and progression) a higher score was observed for those who did not present symptoms during PHI and did not progress at one year. As described by other authors, the genetic score was a useful tool to evaluate the additive influence of human genetic factors with high variability on small groups [37].

Conclusions

This study reveals that some host genetic variants identified previously as disease-modificating factors influence disease progression from the very beginning of the HIV infection. However, here we also described some associations for the first time. Variability of host genetic factors as well as their association with HIV infection and/or disease progression relies strongly on the ethnic population background. Therefore, the population ethnicities are growing it is becoming increasingly difficult to extrapolate results from one study to other populations. In this context, it is important to highlight the need to perform studies at a in this setting not only these genetic differences in the population but also the environmental variance and the circulating virus.

Supporting Information

Figure S1 PCR Cycle conditions for HLA class I characterization.
(DOC)

Table S1 Sequences of primers used for HLA class I characterization.
(DOC)

Table S2 Frequency of CCR5 haplotypes of the study population diagnosed during primary HIV infection [PHI] (N = 70).
(DOC)

Table S3 Frequency of CCR5 human genotypes among the study population diagnosed during primary HIV infection [PHI] (N = 70).
(DOC)

Table S4 Frequency of HLA class I alleles among the study population diagnosed during primary HIV infection [PHI].
(DOC)

Table S5 Frequency of HLA class I alleles homozygosis among the study population diagnosed during primary HIV infection [PHI].
(DOC)

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Author Contributions

Conceived and designed the experiments: RSC AM HS MAP. Performed the experiments: RSC DD YG GT AR. Analyzed the data: MAP RSC. Contributed to the writing of the manuscript: MAP AM RSC. Participants' recruitment: NL MES MIF OS PC.

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Early Skewed Distribution of Total and HIV-Specific CD8$^+$ T-Cell Memory Phenotypes during Primary HIV Infection Is Related to Reduced Antiviral Activity and Faster Disease Progression

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Abstract

The important role of the CD8$^+$ T-cells on HIV control is well established. However, correlates of immune protection remain elusive. Although the importance of CD8$^+$ T-cell specificity and functionality in virus control has been underscored, further unraveling the link between CD8$^+$ T-cell differentiation and viral control is needed. Here, an immunophenotypic analysis (in terms of memory markers and Programmed cell death 1 (PD-1) expression) of the CD8$^+$ T-cell subset found in primary HIV infection (PHI) was performed. The aim was to seek for associations with functional properties of the CD8$^+$ T-cell subsets, viral control and subsequent disease progression. Also, results were compared with samples from Chronics and Elite Controllers. It was found that normal maturation of total and HIV-specific CD8$^+$ T-cells into memory subsets is skewed in PHI, but not at the dramatic level observed in Chronics. Within the HIV-specific compartment, this alteration was evidenced by an accumulation of effector memory CD8$^+$ T (T_{EM}) cells over fully differentiated terminal effector CD8$^+$ T (T_{TE}) cells. Furthermore, higher proportions of total and HIV-specific CD8$^+$ T_{EM} cells and higher HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio correlated with markers of faster progression. Analysis of PD-1 expression on total and HIV-specific CD8$^+$ T-cells from PHI subjects revealed not only an association with disease progression but also with skewed memory CD8$^+$ T-cell differentiation. Most notably, significant direct correlations were obtained between the functional capacity of CD8$^+$ T-cells to inhibit viral replication *in vitro* with higher proportions of fully-differentiated HIV-specific CD8$^+$ T_{TE} cells, both at baseline and at 12 months post-infection. Thus, a relationship between preservation of CD8$^+$ T-cell differentiation pathway and cell functionality was established. This report presents evidence concerning the link among CD8$^+$ T-cell function, phenotype and virus control, hence supporting the instauration of early interventions to prevent irreversible immune damage.

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Introduction

Human Immunodeficiency Virus (HIV) infection causes an irreversible deterioration of the immune system ultimately leading to the development of AIDS in the vast majority of infected persons. Following virus transmission, acute/early phase of infection is characterized by high levels of peak viremia, rapid loss of CD4$^+$ T-cells in both peripheral blood and mucosal lymphoid tissues, and in some cases clinical symptoms [1]. Emergence of HIV-specific CD8$^+$ T-cell response is associated with the drop of plasma viremia to a stable level; known as the viral set point [2] [3]. Given the central role that HIV-specific CD8$^+$ T-cells play in the control of viral replication [4,5], special emphasis has been focused on this cell population. In order to help design an effective HIV vaccine, different parameters such as the magnitude, specificity and functionality of the CD8 response were extensively studied in different settings. Many of these works asserted that the quality of the response, rather than the quantity, might play an important role [6–10][11–15]. Also, the phenotype of the CD8$^+$ T-cell response is an important component of effective anti-viral immunity. Moreover, phenotype and function are two attributes of the response essentially linked and many research lines are currently being directed at understanding which populations along the CD8$^+$ T-cell differentiation pathway are

most effective in inhibiting viral replication. Recent works shed light on the complex differentiation profiles of the total and HIV-specific CD8+ memory T-cells and their association with antiviral function and disease progression [16–19].

There exists a large amount of publications reporting the characterization of HIV-specific CD8+ T-cells in chronic infection, however works performed during the acute infection are more limited [14,15]. Moreover, in both cases most reports were based on subtype B or C infected cohorts rather than non-subtype B/C cohorts [16]. Our group has previously studied multiple aspects of the HIV-specific CD8+ T-cell subsets during acute/early HIV infection. Our findings were the first to report the immunological aspects and CD8 profile of an Argentinean cohort during the acute/early infection [20,21]. Our last report showed that the early relative immunodominance of Gag-specific cells was associated with delayed disease progression. Also, these Gag-specific CD8+ T-cells had a higher capacity to degranulate, secrete IFN-γ and mediate viral inhibition activity *in vitro* (VIA). The main contribution of this study relied on the correlation between HIV-specific CD8+ T-cell functional properties during acute/early infection and clinical outcomes over the first year post-infection. Here, we present novel results from our ongoing work on an Argentinean cohort of recently infected subjects. As an extension of our preceding works, we aimed at performing an immunophenotypic analysis (in terms of memory markers and Programmed cell death 1 (PD-1) expression) of the CD8+ T-cell differentiation profile found in primary infection with viral control and subsequent disease progression. Additionally, associations between HIV-specific CD8+ T cell phenotype and functional properties (more specifically antiviral capacity) were studied. Evidence supporting these notions is provided.

Materials and Methods

Study population

A total of 63 subjects participated in this study: 10 healthy HIV-seronegative donors (HD) and 53 HIV-infected patients: 32 were enrolled during primary HIV infection (PHI), 10 were chronically infected (Chronics) and 11 were Elite Controllers (EC) (Table 1 and Table S1). PHI subjects were enrolled by the *Grupo Argentino de Seroconversión* Study Group [22] under the following inclusion criteria: (1) detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western Blot assay; or (2) positive Western Blot with a negative test within the previous six months. Chronically infected patients were defined as subjects with established HIV infection for over 3 years, detectable viral load (VL, >50 HIV RNA copies/ml plasma) and HAART (Highly Active Anti-Retroviral Therapy) naïve, while EC were defined as subjects infected for more than 5 years with undetectable VL (<50 HIV RNA copies/ml plasma), CD4+ T-cell counts >450 cells/μl blood, HAART naïve and no record of opportunistic infections and/or AIDS-related diseases. HD samples were obtained from voluntary blood donors at the *Sanatorio Dr Julio Mendez* blood bank (Buenos Aires, Argentina). All donors were >18 years; completed and passed a survey on blood donation; and were screened for serological markers before being accepted as donors.

Human samples

Blood samples were collected from study participants at enrollment, centrifuged to separate plasma, which was stored at −80°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Little Chalfont/Buckinghamshire, UK) and cryopreserved for subsequent phenotypic and functional assays. PBMC cryopreservation and thawing were performed following protocols developed to obtain an optimal detection of antigen specific T-cell responses [23–25]. For PHI subjects, samples were obtained at enrollment (baseline) and at 3, 6, 9, and 12 months post-infection. Samples used for immunophenotypic and functional analyses were obtained from subjects that remained HAART-naïve at the moment of sampling. In the case PHI subject needed to start HAART early, baseline samples were obtained before treatment instauration. Plasma VL (branched-DNA, Versant HIV-1 RNA 3.0 assay; Siemens Healthcare, Sudbury/Suffolk, UK) and CD4+ T-cell count (flow cytometry double platform, BD FACSCanto; BD Biosciences, San Diego/California, USA) were assessed in HIV-infected subjects.

Human Subject Research Ethic Statement

Blood samples from HIV-infected individuals and healthy donors were obtained for this study. Prior to enrollment, the study was reviewed and approved by two institutional review boards (IRB): *Comité de Ética Humana, Facultad de Medicina, Universidad de Buenos Aires* and *Comité de Bioética, Fundación Huésped* (Buenos Aires, Argentina). Both HIV-infected participants and healthy donors provided written informed consents accepting to participate in this study.

Peptides

Potential T-cell epitope (PTE) peptide panels corresponding to Nef, Gag and Env proteins and the CEF (cytomegalovirus -CMV- , Epstein-Barr virus, and influenza virus) peptide pool were obtained from the NIH AIDS Reagent Program (NIH, Bethesda/Maryland, USA) [26,27]. Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis/Missouri, USA) at 40 μg/μl and stored at −20°C.

PTE peptides are 15 amino acids (a.a.) in length and contain naturally-occurring 9 a.a. sequences that are potential T-cell determinants embedded in the sequences of circulating HIV-1 strains worldwide. Here, PTE peptides were grouped in 9 pools: 1xNef (N = 127 peptides), 3xGag (corresponding to p17 (N = 97), p24 (N = 128) and p2p7p1p6 (denoted as RG, N = 95), and 5xEnv (Gp120A1 -N = 73, spans HXB2 Env a.a. positions 1–154-, Gp120A2 -N = 73, 157–284-, Gp120B -N = 105, 287–511-, Gp41A -N = 114, 513–689-, Gp41B -N = 115, 689–842-).

Phenotypic and functional analysis of CD8+ T-cells by flow cytometry

T-cell phenotypic and functional markers were measured to identify total and HIV-specific CD8+ T-cell memory populations following the protocol published before [20,21] with the following modifications: Cryopreserved PBMCs were thawed and rested overnight in RPMI medium (Sigma-Aldrich, St Louis/Missouri, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island/NY, USA), 2 mM L-glutamine (Sigma-Aldrich, St Louis/Missouri, USA), 100 U/ml penicillin (Sigma-Aldrich, St Louis/Missouri, USA), 100 mg/ml streptomycin (Sigma-Aldrich, St Louis/Missouri, USA), and 10 mM HEPES (Gibco, Grand Island/NY, USA). Cell viability was checked both immediately after thawing and after overnight rest by trypan blue exclusion. Only samples with good cell recovery (>70%) and good viability (>95%) both after thawing and resting, were used in the assays. PBMCs were dispensed in U-bottom 96-well plates (5×10^5 cells/well) in duplicate wells. Costimulatory antibodies (anti-CD28 and anti-CD49d antibodies (1 μg/ml; BD Biosciences, San Jose/California, USA), monensin (Golgistop, 0.7 μl/ml; BD Bioscienc-

Table 1. Summary of clinical data corresponding to HIV+ subjects enrolled per study group.

		Viral load[a,c]				
Group (No. of subjects)	**Median Days post-infection (IQ)**	**Median RNA copies/ml (IQ)**	**Mean log_{10} ± SD**	**Viral set point[d] (mean log_{10} ± SD)**	**CD4+ T-cell count[b,c], median No. of cells/μl (IQ)**	**CD4 set point[d], median No. of cells/μl (IQ)**
PHI						
All (n = 32)	60 (30–90)	34,800 (8,843–252,588)	4.6±0.9	4.5±0.7	503 (320–682)	517 (404–629)
PHI >350 (n = 20)	60 (49–113)	18,471 (6,010–98,650)	4.3±0.9	4.3±0.6	618 (510–771)	573 (454–652)
PHI <350 (n = 12)	55 (30–90)	151,026 (34,510–500,000)	5.1±0.8	5.1±0.7	281 (230–338)	256 (158–319)
Chronic (n = 10)	-	28,435 (9,449–197,984)	4.5±0.7		141 (11–563)	
EC (n = 11)	-	<50	<1.7		602 (562–888)	

[a]Versant HIV-1 RNA 3.0 assay, Siemens. Lower and upper detection limits are 50 and 500,000 RNA copies/ml, respectively (1.7 and 5.7log10).
[b]Flow cytometry double platform, FACSCanto, BD Biosciences.
[c]For PHI subjects, data correspond to baseline samples. For chronic and elite controller subjects, data correspond to samples obtained at enrollment.
[d]Set points were not calculated for subjects that initiated HAART during the first year post-infection.
IQ: Interquartile range.
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es, San Jose/California, USA), brefeldin A (10 μg/ml; BD Biosciences, San Jose/California, USA), and the corresponding peptide pool (2 μg/ml) were added. Also, a mixture of anti-CD107A-fluorescein isothiocyanate (FITC) and anti-CD107B-FITC antibodies (BD Biosciences, San Jose/California, USA) was added in each well. An unstimulated (peptide-free medium plus 0.5% DMSO and costimulatory antibodies) and two positive controls (2 μg/ml CEF peptide pool and phorbol myristate acetate (PMA)-ionomycin (Sigma-Aldrich, St Louis/Missouri, USA)) were included in each assay. Cells were incubated for 5 hours at 37°C, washed, and stained for 30 min at 37°C with anti-CCR7-phycoerythrin (PE) (BD Biosciences, San Jose/California, USA), followed by staining for 30 min at 4°C, with LIVE/DEAD Fixable NEAR-IR (Invitrogen, Life Technologies, Carlsbad/California, USA), in order to exclude dead cells, and anti-CD3, -CD8 and -CD45RO antibodies conjugated to -PECy7, -APC and -PeCy5.5 (BD Biosciences, San Jose/California, USA) respectively. For PD-1 analysis, stimulated PBMCs were surface stained with a separate panel that included anti-PD-1-PE (BD Biosciences, San Jose/California, USA), anti-CD3-PECy7, anti-CD8-APC and LIVE/DEAD Fixable NEAR-IR. Then, cells were permeabilized and fixed using the Cytofix/Cytoperm kit (BD Biosciences, San Jose/California, USA), following the manufacturer's protocol. After the permeabilization/fixation step, cells were stained intracellularly with anti-IL-2, anti-TNF-α, and anti-IFN-γ antibodies, all of them conjugated to FITC (BD Biosciences, San Jose/California, USA). Cells were then washed, resuspended in 0.5% paraformaldehyde (PFA, Merck & Co, Whitehouse station/New Jersey, USA) and stored until data acquisition in a 2-laser, 6-color BD FACSCanto flow cytometer. Data acquisition and analysis were performed using the BD FACSDiva v 6.1.3 software (BD Biosciences, San Jose/California, USA). FlowJo Vx.0.7 free trial software (FlowJo Enterprise, Treestar Inc., Ashland/Oregon, USA) was used to generate Figure 1A and Figure S1 only for illustration purposes. Instrument settings and fluorescence compensation were performed on each testing day using unstained and single-stained samples. Isotype controls, consisting of stimulated cells stained with anti-CD3 and anti-CD8 conjugated antibodies and isotype controls corresponding to phenotype (CCR7, CD45RO and PD-1) and intracellular markers, were performed for each patient in order to accurately set negative populations. First, a plot of forward scatter area (FSC-A) versus height (FSC-H) was constructed to remove doublets. Then, gating was performed on small lymphocytes in a FSC versus side scatter (SSC) plot. At least 80,000 events were acquired in the lymphocyte gate. Dead cells were then excluded on the bases of LIVE/DEAD fluorescence. Subsequently, $CD3^+$ $CD8^+$ cells were gated in a CD3-versus-CD8 dot plot. HIV-specific $CD8^+$ T-cells were identified in a CD8 vs. cytokines (FITC) density plot (the gating strategy is provided in Figure S1). A positive cytokine response was defined as at least twice background, >0.05% after subtraction of background, and at least 50 events. This criterion was established to minimize the possibility of error due to a low number of events when further subdividing these cells into memory subsets. For phenotypic analysis, CD45RO vs. CCR7 density plot or PD-1 histogram were performed on gated $CD8^+$ cells. Distribution of different phenotype subsets were analyzed both in total and HIV-specific $CD8^+$ T-cell compartments.

Immune Activation

$CD4^+$ and $CD8^+$ lymphocyte activation was analyzed on thawed and over-night rested PBMCs by flow cytometry. Cells were stained for 30 min at 4°C with LIVE/DEAD Fixable NEAR-IR in order to exclude dead cells, and with the following fluorochrome-conjugated antibodies (all of them obtained from BD Biosciences, San Jose/California, USA): anti-HLA-DR-FITC, anti-CD4-PerCP, anti-CD38-APC, anti-CD3-PeCy7 and anti-CD8-PE.

Data acquisition and analysis was performed using the BD FACSDiva software. Initial gating was performed on living lymphocytes followed by gating on $CD3^+CD4^+$ or $CD3^+CD8^+$ events. Isotype-matched FITC- and APC-conjugated non-specific antibodies were used in each sample in order to accurately set HLA-DR and CD38 negative populations.

$CD8^+$ T-cell Virus Inhibitory Activity (VIA) and $CD8^+$ T-cell polyfuntionality

The *ex vivo* ability of $CD8^+$ T-cell to inhibit viral replication in primary autologous $CD4^+$ T-cells (VIA) and the capacity of HIV-specific $CD8^+$ T-cells to produce cytokines (IL-2, IFN-γ and TNF-α) and degranulate (evidenced by CD107A/B mobilization) upon stimulation as well as its polyfunctionality were evaluated exactly as published elsewhere [20].

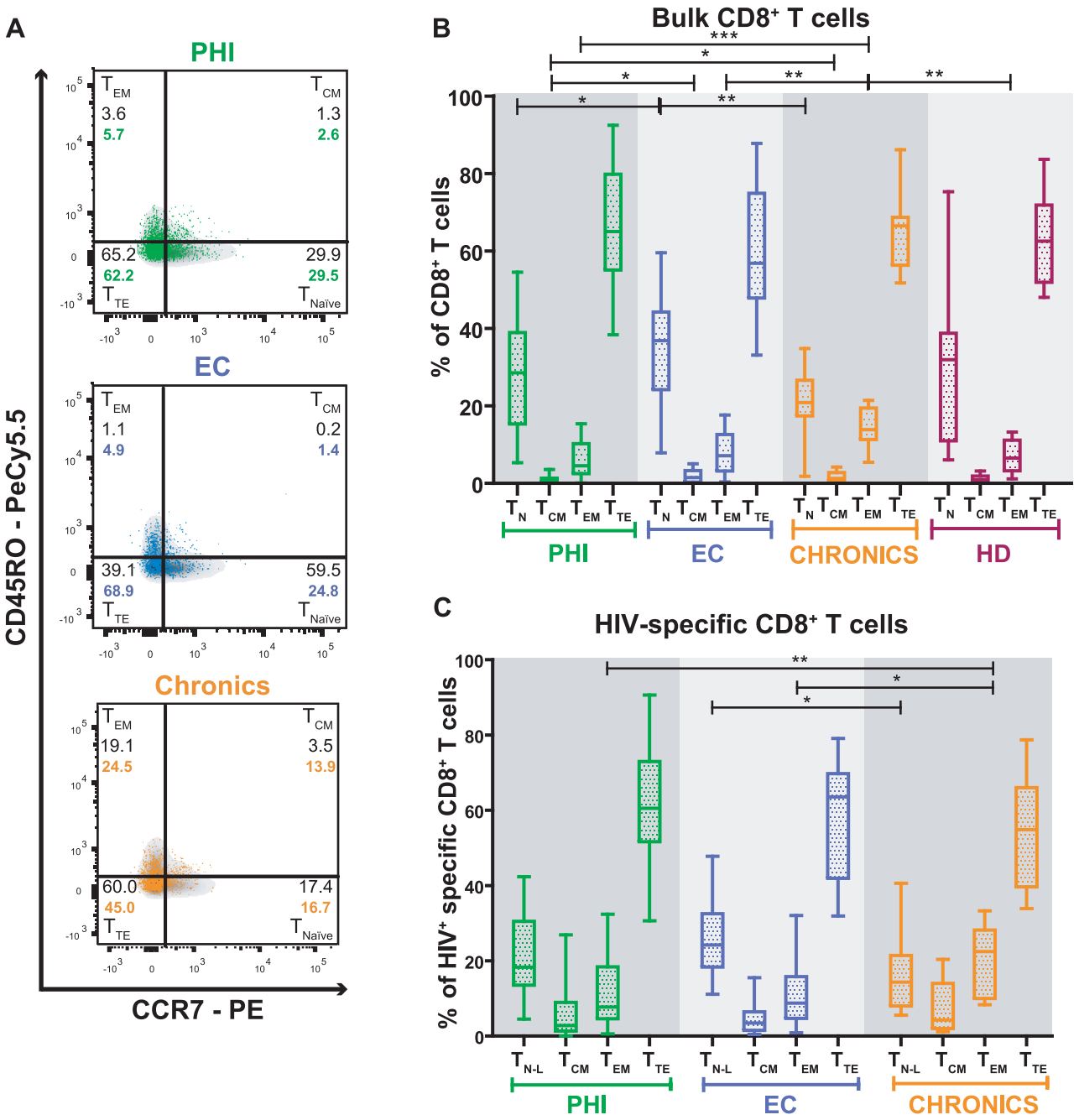

Figure 1. Distribution of memory sub-populations within bulk and HIV-specific CD8+ T-cells. (A) Density and overlay dot plots of memory subsets in total (density) and HIV-specific CD8+ T-cells (colored dots) of one representative subject per study group. The four defined sub-populations are identified within each quadrant: T_N and T_{N-L} = Naïve and Naïve-like T-cells, respectively ($CCR7^+/CD45RO^-$); T_{CM} = central memory T-cells ($CCR7^+/CD45RO^+$); T_{EM} = effector memory T-cells ($CCR7^-/CD45RO^+$); T_{TE} = terminal effectors T-cells ($CCR7^-/CD45RO^-$). Proportions of each memory subset of total (in black) and HIV-specific (in color) $CD8^+$ T-cells are also shown. Panels B and C: Percentage of bulk (B) and HIV-specific (C) $CD8^+$ T-cells subsets of subjects enrolled per study group. Primary HIV infection (PHI) N = 24 subjects (31 specific responses); Elite Controllers (EC) N = 11 subjects (16 specific responses); Chronics N = 10 subjects (13 specific responses); Healthy Donors (HD) N = 10. Horizontal lines stand for median values. P values were calculated using Mann-Whitney test. Asterisks denote different P values: * $P<0.05$; ** $P<0.005$; *** $P<0.001$.
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Quantification of soluble plasma factors

Simultaneous determination of the following 39 cytokines and chemokines was performed in plasma samples from a subset of 18 PHI subjects (at baseline time-point only) using Luminex technology (MILLIPLEX MAP Human Cytokine/Chemokine, Merck Millipore, Billerica/Massachusetts, USA): EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, sCD40L, sIL-2Rα, TGF-α, TNF-α, TNF-β, VEGF. Samples were processed and analyzed as described by Giavedoni et al [28].

Data analysis

For PHI subjects, presumed date of infection was calculated as described in [22]. Viral and $CD4^+$ T-cell set-points were calculated as the geometric mean of the determinations obtained between 6 and 12 months post-presumed date of infection. Set-points were not calculated for those subjects who started HAART during the first 12 months of infection or if no stable set-point was reached during that period. Starting from pilot sample data, pre-study estimations of final sample sizes were determined using Harris, Horvitz, Mood method in order to provide 80% power, at the 5% level of significance. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla/California, USA). All data, except $\log_{10}$ VL, were analyzed using nonparametric statistics. Two-tailed Wilcoxon and Mann-Whitney tests were used to compare intra- and inter-group variables, respectively. Correlations were determined using Spearman's rank test. All tests were considered significant if the p value obtained was less than 0.05.

Results

Study subjects description

In order to accomplish the aims of this study, three groups of HIV-infected subjects were enrolled: 32 subjects were recruited during HIV seroconversion and/or within 6 months since the presumed date of infection (PHI group), 10 chronically-infected subjects (Chronics), and 11 subjects defined as Elite Controllers (EC) according to the criteria defined in Materials and Methods. Detailed description of the HIV infected participants is shown in Table 1, Figure S2, and Table S1. Additionally, samples from 10 HIV-negative healthy donors (HD) were obtained. Baseline sample for PHI subjects were obtained at a median of 60 days post-presumed date of infection and most corresponded to Fiebig stages V and VI [29]. As regards chronically infected subjects, the individuals enrolled in this study include subjects with preserved immune status as well as subjects with advanced immune deterioration (as observed in Table S1 and Figure S2), providing evidence of the natural heterogeneity of such HIV-positive population. Additionally, immune activation was evaluated in all groups of HIV-infected subjects as it is known to be a major predictor of disease progression [30]. The percentages of $CD4^+$ and $CD8^+$ T-cells that expressed the immune activation markers CD38 and HLA-DR, either alone or in combination, were determined by flow cytometry. Results are shown in Figure S2C and Table 2. As expected, highest levels of immune activation were observed in baseline samples from PHIs, followed by Chronics and ECs.

For certain analyses, PHI subjects were further divided into two subgroups whether their $CD4^+$ T-cell count dropped below 350 cells/μl at any time during the first year post-infection or not (PHI<350 and PHI>350, respectively, Table S1). By doing so, we aimed to differentiate subjects with more rapid or aggressive progression of infection (PHI<350 group) and to investigate the association of this pattern with alterations in the phenotypic distribution of CD8 subsets. The 350 cells/μl-endpoint was chosen based on the national and international recommendations for HAART initiation by the year 2010, when most of these individuals were already enrolled [22]. PHI<350 showed significantly higher VLs and lower $CD4^+$ T-cell counts, both at baseline ($p = 0.0321$ and $p<0.0001$ respectively) and set-point ($p = 0.0466$ and $p = 0.0008$, respectively), compared to the PHI>350 group (Table 1).

Distribution of memory T-cell phenotypes in total and HIV-specific $CD8^+$ T-cells identified during primary HIV infection as well as viremic and aviremic chronic infections

In a previous study, we showed that $CD8^+$ T-cell specificity and function were related to control of early disease progression [20]. Based on that analysis, now we aimed to investigate the distribution of memory phenotypes in both total and HIV-specific $CD8^+$ T-cell compartments during primary HIV infection (using samples from 24 PHI subjects obtained at baseline, Table S1) and its association with subsequent disease progression. Also, these parameters were screened in 10 HDs, 10 Chronics and 11 ECs for comparison purposes. For this, we performed a phenotypic analysis of $CD8^+$ T-cells by flow cytometry, which allowed us to define four $CD8^+$ T-cell sub-populations (Figure 1A): naïve ($T_{Naïve}$, $CCR7^+CD45RO^-$), central memory (T_{CM}, $CCR7^+CD45RO^+$), effector memory (T_{EM}, $CCR7^-CD45RO^+$) and terminal effector (T_{TE}, $CCR7^-CD45RO^-$) cells. The distribution of these subsets was analyzed both in bulk and HIV-specific $CD8^+$ T-cells. The latter was identified as cells able to degranulate (mobilize CD107A/B) and/or express cytokines (IFN-γ, IL-2 and/or TNF-α) upon stimulation with peptide pools corresponding to Nef, p24, p17 or p2p7p1p6 proteins. All these molecules (CD107A/B, IFN-γ, IL-2 and TNF-α) were identified using antibodies stained with the same fluorochrome (FITC) in order to detect all cells responsive to stimulation, regardless of their specific functionality. Figure S1 illustrates the gating strategy used.

i. Distribution of memory subsets within bulk $CD8^+$ T-cell is abnormal in viremic Chronics but does not significantly distinguish PHI subjects, ECs or HDs. The distribution of memory phenotypes within the total $CD8^+$ T-cell compartment showed the following hierarchy in all groups of subjects analyzed: $T_{TE}>T_{Naïve}>T_{EM}>T_{CM}$ (Figure 1B). Although this hierarchy was conserved in all groups, differences in the proportion of the various sub-populations among groups were observed. The highest median proportion of total $CD8^+$ $T_{Naïve}$ cells (as defined for the purposes of this work) was found in ECs (36.9%; IQ25–75: 24.2–44.2), followed by HDs (31.9%; IQ25–75: 11.0–38.8), PHI subjects (29.0%; IQ25–75: 15.4–38.9) and Chronics (20.9%; IQ25–75: 17.4–26.6). The difference was not statistically significant between ECs and HDs, it barely reached statistical significance between ECs and PHI subjects ($p = 0.0493$) and differed significantly in ECs versus Chronics ($p = 0.0018$). This is in line with a previous report that indicates that naïve T-cells still comprise a large proportion of the T-cell compartment at early times post-infection [19]. On the other hand, the highest proportion of total $CD8^+$ T_{TE} cells was observed in Chronics (66.5%; IQ25–75: 56.4–68.7), followed by PHI subjects (64.2%; IQ25–75: 55.3–79.5), HDs (62.6%; IQ25–75: 51.9–71.8) and ECs (56.8%; IQ25–75: 47.9–74.9) (Figure 1B). The median proportion of total $CD8^+$ T_{CM} cells was statistically lower in PHI subjects (0.6%; IQ25–75: 0.2–1.3) compared both to Chronics (1.3%; IQ25–75: 0.6–2.8; $p = 0.0127$) and ECs (1.5%; IQ25–75: 0.3–3.3; $p = 0.0405$). Finally, it was observed that Chronics had a statistically higher proportion (≈2-fold) of total $CD8^+$ T_{EM} cells (13.9%; IQ25–75: 11.3–19.5), compared to ECs (7.1%; IQ25–75: 3.2–13.0; $p = 0.0048$), HDs (6.5%; IQ25–75: 3.2–11.2; $p = 0.0076$) and PHI subjects (4.5%; IQ25–75: 2.5–10.1; $p<0.0001$). When a similar analysis was performed within PHI subgroups (PHI>350 versus PHI<350) no statistically significant difference was observed. Overall, these results indicate that the distribution of memory phenotypes within the total $CD8^+$ T-cell compartment evaluated at early time-points post-infection is similar to that of

Table 2. Immune Activation Panel corresponding to HIV+ subjects enrolled per study group[a,b].

	Median % (IQ)					
Group (No. of subjects)	**CD4/CD38**	**CD4/HLA-DR**	**CD4/CD38/HLA-DR**	**CD8/CD38**	**CD8/HLA-DR**	**CD8/CD38/HLA-DR**
PHI						
All (n = 32)	21.7 (13.8–35.1)	5.0 (1.5.–8.4)	1.4 (0.4–2.2)	44.3 (22.5–55.7)	32.4 (15.5–44.8)	15.8 (7.4–34.7)
PHI >350 (n = 20)	23.1 (13.8–33.4)	6.7 (1.5–6.4)	1.2 (0.4–2.1)	33.7 (21.3–48.5)	24.9 (11.8–39.7)	9.7 (6.0–27.8)
PHI <350 (n = 12)	18.3 (11.9–36.3)	8.2 (1.4–12.6)	1.5 (0.5–5.6)	48.2 (39.0–64.1)	40.2 (27.9–57.8)	33.4 (14.8–43.6)
Chronic (n = 10)	41.3 (28.1–46.1)	12.0 (5.2–18.5)	2.7 (1.1–18.5)	35.9 (24.4–91.7)	19.0 (18.2–49.8)	11.2 (5.6–24.4)
EC (n = 11)	22.6 (12.9–32.9)	4.7 (2.3–9.1)	0.6 (0.4–1.7)	33.5 (28.2–44.5)	17.2 (12.3–31.9)	8.2 (3.2–12.8)

[a]Flow cytometry double platform, FACSCanto, BD Biosciences.
[b]For PHI subjects, data correspond to baseline samples. For chronic and elite controller subjects, data correspond to samples obtained at enrollment.
IQ: Interquartile range.
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HDs and ECs. In contrast, a higher proportion of the most differentiated T_{EM} and T_{TE} cells was found in viremic Chronics while a higher proportion of the less differentiated $T_{Naïve}$ and T_{CM} cells (similar to the scenario observed in HDs) was found in aviremic chronically-infected subjects (i.e. ECs) (Figure 1B).

ii. HIV-specific CD8+ T-cells from recently infected subjects preserve a maturation hierarchy similar to that of ECs, whereas it is skewed in chronically viremic infected subjects. The distribution of memory phenotypes within the HIV-specific CD8+ T-cell compartment mostly mirrored that of the total compartment. However, important differences were also observed. It is worth noting here that, due to technical constraints, our definition of naïve T-cells is not strict enough to completely avoid inclusion of very early differentiated memory cells such as the so called stem-cell memory T-cells. To completely avoid this problem, additional surface markers should be included in the panel [31]. Hence, when referring to HIV-specific $CCR7^+CD45RO^-$ CD8+ T-cells, we used the term "naïve-like" ($T_{naïve\text{-}like}$).

The highest proportion of HIV-specific CD8+ $T_{naïve\text{-}like}$ cells was observed in ECs (24.2%; IQ25–75: 18.4–32.5) followed by PHI subjects (18.5%; IQ25–75: 14.0–33.3) and Chronics (14.34%; IQ25–75: 8.0–21.4; p = 0.0164) (Figure 1C). Chronics had a significantly higher proportion of HIV-specific CD8+ T_{EM} cells (22.5%; IQ25–75: 10.0–28.2), compared to PHI subjects (7.4%; IQ25–75: 4.6–18.3; p = 0.002) and ECs (8.8%; IQ25–75: 4.7–15.8; p = 0.0111) and tended to have a lower proportion of HIV-specific CD8+ T_{TE} cells (54.9%; IQ25–75: 39.7–66.0) than PHI subjects (58.9%; IQ25–75: 51.3–72.9) and ECs (63.5%; IQ25–75: 41.9–69.8). This is in line with previous studies which indicate that, during chronic progressive HIV infection, there is a maturation arrest of HIV-specific CD8+ T-cells from T_{EM} to T_{TE} [6,32]. To provide further insights into this notion, the HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio was analyzed in all groups in order to picture the proportion of T_{EM} cells out of the total of the most differentiated subsets from our panel (T_{EM} plus T_{TE}). In consonance with the hypothesis mentioned above, the $T_{EM}/(T_{EM}+T_{TE})$ ratio was significantly higher in Chronics compared to ECs (p = 0.0144) and PHI (p = 0.0042) (Figure 2A). No statistically significant difference was observed between ECs and PHIs or within PHI subgroups (PHI>350 versus PHI<350). Moreover, the percentages of HIV-specific CD8+ T_{EM} and T_{TE} cells negatively correlated in PHI subjects (r = −0.458; p = 0.0095; Figure 2B) and ECs (r = −0.546; p = 0.0351; Figure 2C) whereas no correlation at all was observed in Chronics (r = 0.258; p>0.05; Figure 2D). These results support the notion that differentiation of T_{TE} cells from T_{EM} cells is altered in viremic chronic HIV-1 infection. However, it might not be the case in ECs or, more importantly, in recently infected subjects (PHI group) where these results indicate that there exists an early preservation of the CD8+ T-cell compartment before going into the chronic stage of infection.

Finally, based on previous results indicating that Gag-specific CD8+ T-cells improved antiviral functions [20], it was hypothesized that the distribution of memory phenotypes within the HIV-specific CD8+ compartment would vary according to the antigens used as stimulus; i.e. Nef versus Gag (p24, p17 or RG) peptide pools. However, no clear association was found between antigen specificity and CD8+ T-cell memory/effector phenotype in either group analyzed (data not shown).

Baseline higher proportions of total and HIV-specific CD8+ T_{EM} cells as well as higher HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio correlated with markers of faster progression while higher proportions of T_{naive} and $T_{naive\text{-}like}$ CD8+ T-cells associated with markers of slower disease progression

According to the data described above on the distribution of memory phenotypes within both total and HIV-specific CD8+ T-cell compartment in groups with differential disease outcome, together with data collected from the bibliography, it was hypothesized that within the PHI group, the relative frequency of the different memory subsets evaluated at baseline would be associated with disease progression. Thus, the relative frequency of a given subset measured at early time-points post-infection could be postulated as an indicator of subsequent disease progression rate. To test this, correlation analyses were performed between the percentages of the different memory subsets and the clinical data of the subjects enrolled, obtained at baseline and during the first year post-infection.

As regards the total CD8+ T-cell compartment, and always within the PHI group, the proportion of T_{EM} cells correlated inversely with baseline CD4+ T-cell count (r = −0.363; p = 0.0274; Figure 3A) and directly with baseline activation levels within the

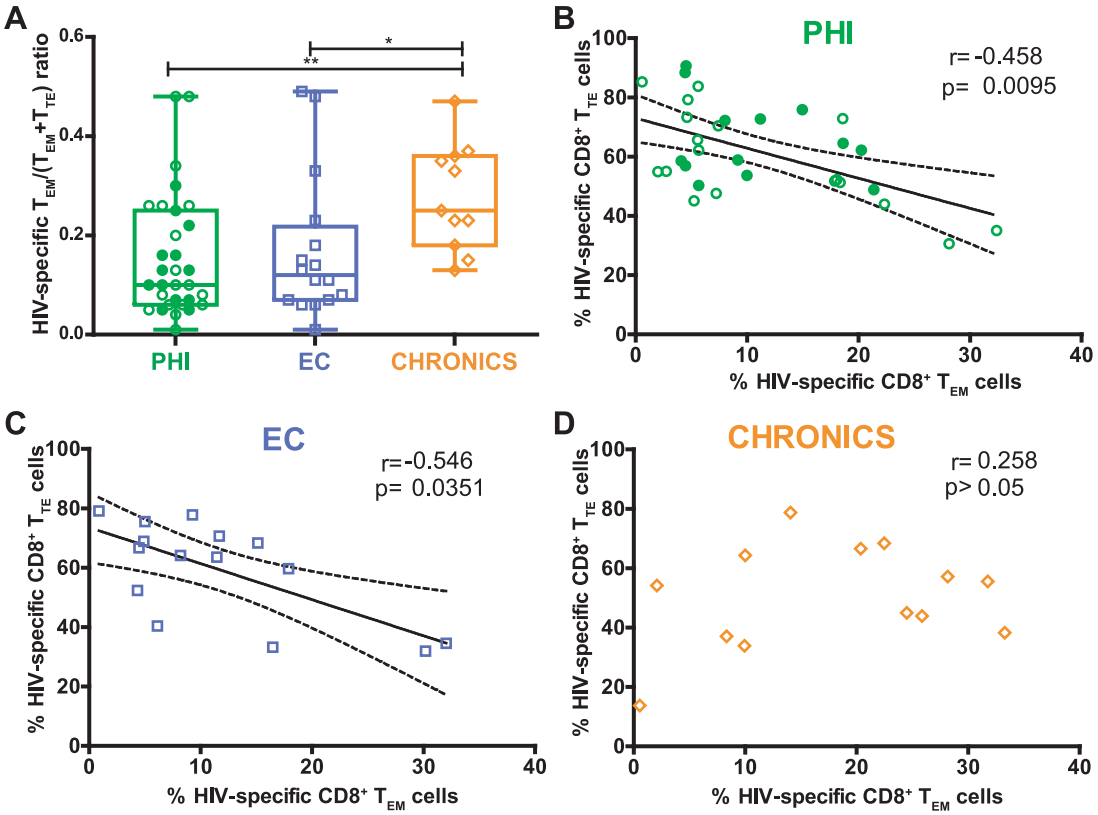

Figure 2. Arrest of HIV-specific CD8+ T-cells from effector memory (T_{EM}) to terminal effector (T_{TE}) of subjects enrolled. HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio (A) of subjects enrolled per study group. Correlation among HIV-specific CD8+ T_{TE} and the T_{EM} cells in primary HIV infection (PHI) (B), elite controllers (EC) (C) and Chronic (D) groups. PHI group N = 24 subjects (31 specific responses); EC N = 11 subjects (16 specific responses); Chronics N = 10 subjects (13 specific responses). Panels A and B, open and filled green dots denote PHI>350 and PHI<350 subjects, respectively. Panel A, P values were calculated using Mann-Whitney test. Asterisks denote different P values: * P<0.05; ** P<0.005. Panels B–D, r and P values correspond to Spearman's test.
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CD8+ T-cell compartment (r = 0.349; p = 0.0368; Figure 3C). Moreover, the proportion of T_{EM} cells also correlated negatively with the CD4+ T-cell count set-point (r = −0.443; p = 0.0142; Figure 3B) indicating that the early proportion of this subset is associated with the subsequent level of immune set-point. When analyzing the HIV-specific CD8+ T-cells compartment it was found that the proportion of HIV-specific T_{EM} cells inversely correlated with baseline CD4+ T-cells (r = −0.448; p = 0.0167, Figure 3D) and the CD4+ T-cell count set-point (r = −0.599; p = 0.0086, Figure 3E). The same significant trends were observed when the HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio was measured (r = −0.472; p = 0.0129, Figure 3G; r = −0.629; p = 0.0091, Figure 3H, respectively). Additionally, the proportion of HIV-specific T_{EM} cells directly correlated with viral set-point (r = 0.571; p = 0.0208, see Figure S3F). Then, the CD8+ T-cell activation was analyzed. Direct correlations both with the percentage of HIV-specific T_{EM} cells (r = 0.489; p = 0.0132, Figure 3F), and with the HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio (r = 0.482; p = 0.0146, Figure 3I) were found. Furthermore, the HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio was also found to directly correlate with baseline plasma levels of cytokines associated with disease progression such as IP-10 (r = 0.499; p = 0.0251), IL-1a (r = 0.509; p = 0.0220), and IL-15 (r = 0.538; p = 0.0144, not shown). On the other hand, the proportion of bulk CD8+ $T_{Naïve}$ cells correlated directly with baseline CD4+ T-cell count (r = 0.330; p = 0.0432, Figure S3A) and inversely with baseline CD4+ T-cell activation (r = −0.542; p = 0.0008; Figure S3B). Similarly, the proportion of $T_{naïve\text{-}like}$ cells directly correlated with the percentage of baseline CD4+ T-cells (r = 0.383; p = 0.0368, Figure S3C) while it inversely correlated with baseline VL (r = −0.460; p = 0.0093, Figure S3D) and viral set-point (r = −0.630; p = 0.0022, Figure S3E).

Overall, in line with the inter-group analysis performed above, these results indicate that a higher relative proportion of both total and HIV-specific CD8+ $T_{Naïve}$ (or $T_{naïve\text{-}like}$) cells during early time-points post-infection correlated with better immune status in terms of CD4+ T-cell count, at baseline and set-point. Conversely, higher proportions of the more differentiated T_{EM} and its accumulation relative to terminally differentiated cells within the HIV-specific compartment (evaluated as the HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio) correlated with markers of faster disease progression: lower baseline and set-point CD4+ T-cell counts, higher viral set-point (only for the HIV-specific subset), and higher baseline levels of cellular and soluble markers of immune activation.

PD-1 expression on CD8+ T-cells during PHI is related to disease progression and also with memory CD8+ T-cell differentiation

In order to provide more insights into the phenotype of CD8+ T-cells found in HIV infection, the expression of Programmed cell death 1 (PD-1), a molecule commonly linked to immune exhaustion, was evaluated on bulk and HIV-specific CD8+ T-

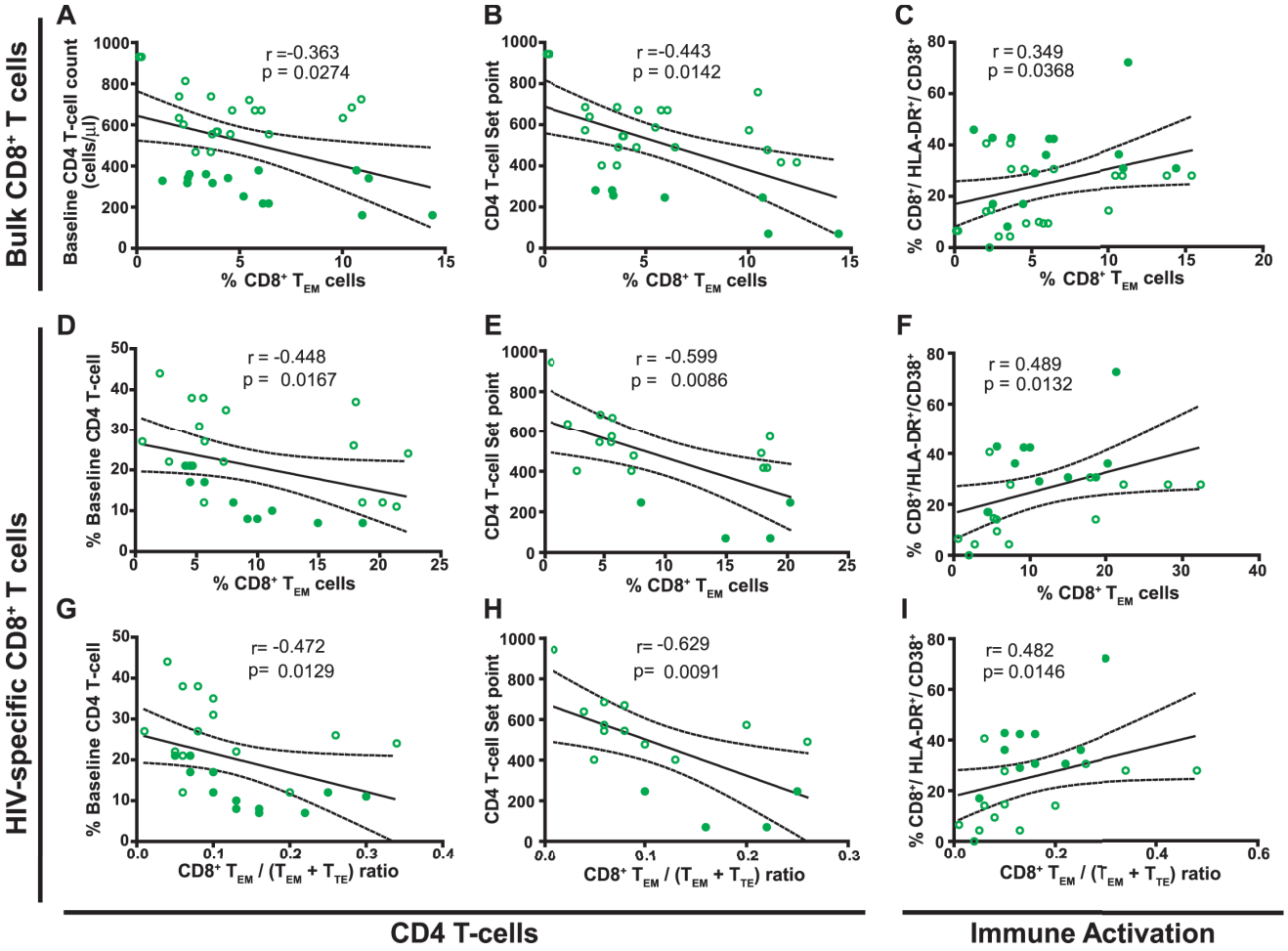

Figure 3. Correlations between the proportion of CD8$^+$ T_{EM} cells within bulk (A–C) and HIV-specific (D–F) compartments as well as the HIV-specific CD8$^+$ $T_{EM}/(T_{EM}+T_{TE})$ ratio (G–I) with clinical parameters measured in baseline samples from primary HIV infected (PHI) subjects. Baseline CD4 T-cell counts (A), immune set point (B) and baseline immune activation (C) versus proportion of bulk CD8$^+$ effector memory (T_{EM}) cells. Percentage of baseline CD4 T-cell (D), immune set point (E) and baseline immune activation (F) versus proportion of HIV-specific CD8$^+$ T_{EM} cells. Percentage of baseline CD4 T-cell (G), immune set point (H) and baseline immune activation (I) versus HIV-specific CD8$^+$ $T_{EM}/(T_{EM}+T_{TE})$ ratio. PHI group N = 24 subjects (39 responses analyzed for bulk compartment and 31 responses for the specific compartment). For set point correlations N = 15 subjects. In all panels, open and filled green dots denote PHI>350 and PHI<350 subjects, respectively. All r and P values correspond to Spearman's test.
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cells from a subset of 19 PHI subjects using samples obtained at 8±1 months post-infection (due to cell sample availability). The relationship between PD-1 expression and markers of disease progression as well as with the distribution of memory subsets were studied in this group. First, it was observed that PHI<350 subjects displayed higher proportions of both total and HIV-specific PD-1$^+$ CD8$^+$ T-cells, compared to PHI>350 (45.6% versus 28.8%, p = 0.0709 and 40% versus 25%, p = 0.0109, respectively; Figure 4A). Despite not reaching statistical significance, a clear tendency was observed. This trend was also recorded when PD-1$^+$ events were subdivided into PD-1^{low} (Figure 4B) and PD-1^{high} (Figure 4C) phenotypes according to the intensity (on the basis of mean fluorescence intensity) of PD-1 expression. Interestingly, bulk and HIV-specific CD8$^+$ T-cells only differed significantly regarding the proportion of PD-1^{high} cells (p = 0.0294, Figure 4C). In this sense, strong positive correlations were observed between bulk and HIV-specific compartments regarding the proportion of total PD-1$^+$ cells (r = 0.7008, p<0.0001) and PD-1^{low} cells (r = 0.7674, p<0.0001, not shown). However, no significant correlation was found in PD-1^{high} cells (r = 0.3308, p = 0.07, not shown). This indicates that PD-1 is preferentially up-regulated in HIV-specific cells as described elsewhere [33–35]. Also, regarding specific cells, no PD-1 expression difference was observed in cells with different specificities (i.e. when Nef, Gag of CEF pools were used as stimuli). According to the difference observed between PHI>350 and PHI<350 subjects, negative correlations were found between both bulk and HIV-specific PD-1$^+$ CD8$^+$ T-cells and CD4$^+$ T-cell percentages (r = −0.510, p = 0.0109 and r =

−0.457, p = 0.0216, respectively, Figures 4D and 4E). Otherwise, no other association between PD-1 expression (measured as %PD-1 or PD-1 MFI) on CD8+ T-cells (either total or HIV-specific) and markers of disease progression (viral load, viral set-point, soluble or cellular immune activation markers) was found. Alternatively, when analyzing the proportion of PD-1^{low} and PD-1^{high} phenotypes, a direct correlation between PD-1^{high} CD8+ T-cells and viral load was found (r = 0.447, p = 0.0287, Figure 4F).

Then, we sought to investigate the relationship between the pattern of PD-1 expression and the distribution of memory subsets during primary HIV infection. It was found that the percentages of PD-1^{high} CD8+ T-cells, both within bulk and HIV-specific compartments, negatively correlated with the proportion of bulk and HIV-specific CD8+ T_{EM} cells (r = −0.501, p = 0.0341 and r = −0.668, p = 0.0047, respectively; Figures 4G and 4I). Conversely, positive correlations were observed with the proportion of bulk and HIV-specific CD8+ T_{TE} cells (r = −0.510, p = 0.0308 and r = −0.564, p = 0.0228, respectively; Figures 4H and 4J). Additionally, HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio also correlated inversely with the proportion of PD-1^{high} CD8+ T-cells (r = −0.674, p = 0.0042; Figure 4K). This is in consonance with the notion that PD-1 is not only a marker of immune cell exhaustion but also its expression is related to CD8+ T-cell differentiation stage and activation status [36].

CD8+ T-cell antiviral activity is related to CD8+ T-cell memory differentiation but not to PD-1 expression during primary HIV infection

We have previously shown that higher HIV-specific CD8+ T-cell antiviral capacity during primary HIV infection was associated with higher CD4+ T-cell counts at set-point [20]. Thus, we sought to investigate the relationship between CD8+ T-cell phenotype and functionality in a subset of 11 PHI subjects from our cohort. This subset of 11 PHI subjects remained HAART-naïve during the study period. In the first place, no association was found between the memory phenotype and the capacity of specific CD8+ T-cell to exert a particular function (degranulate or secrete cytokines or chemokines) or with the proportion of polyfunctional CD8+ T-cells. Additionally, no association was found among the expression of PD-1 by CD8+ T-cells and its functionality. Contrarily, significant inverse correlations were observed between CD8+ T-cell antiviral activity (VIA) evaluated at baseline with the concurrent proportion of HIV-specific CD8+ T_{EM} cells (r = −0.593, p = 0.0096; Figure 5A) and the HIV-specific CD8+ $T_{EM}/(T_{EM}+T_{TE})$ ratio (r = −0.613, p = 0.0069; Figure 5C). Conversely, a positive correlation was obtained with the proportion of HIV-specific CD8+ T_{TE} cells (r = 0.718, p = 0.0008; Figure 5B). This result indicates that the magnitude of CD8+ T-cell antiviral activity during recent infection is related to a higher proportion of HIV-specific cells with a fully differentiated phenotype, rapidly able to exert effector functions. Concomitantly, a higher level of CD8+ T-cell differentiation arrest (evidenced by higher $T_{EM}/(T_{EM}+T_{TE})$ ratios) translates into lower CD8+ T-cell antiviral activity. Moreover, identical correlations were obtained when analyzing CD8+ T-cell phenotype at baseline and antiviral activity at 12 months post-infection (Figure 5D to 5F) indicating that the early CD8+ T-cell differentiation hierarchy is also associated with CD8+ T-cell antiviral function beyond the establishment of the set-point.

Discussion

Data accumulated over the last years have established that the HIV-specific CD8+ T-cell response plays a critical role in viral control (reviewed in [4,5,37]). For this reason, efforts have been made to understand the properties of CD8+ T-cells (in terms of function and/or phenotype) that best correlate with control of viral replication [5]. Moreover, this information will be instrumental for developing and enhancing immunization strategies focused on eliciting appropriate immune responses as well as for defining currently-lacking immune correlates of protection in order to evaluate the performance of vaccine candidates. In line with this, research studies on primary HIV infection (PHI) are increasing worldwide to better understand the natural history of HIV infection and to identify the early pathogenic events that may set the course for subsequent disease progression. More specifically, cohort studies addressing the association of particular features of CD8+ T-cell responses arising during acute/early HIV infection with potential markers associated with disease progression are fundamental. However, most of these studies were performed in developed countries, and scarce information exists from other settings, such as South America, where local studies are needed to comprehend particular characteristics of the infection. It is worth highlighting that the capacity of cohort studies to provide meaningful contributions relies on the definition of rigorous inclusion criteria, which in turn allows for powerful comparisons of both intra- and inter-studies. In this line, our group studied multiple aspects of the HIV-specific CD8+ T-cell subset (specificity, *ex vivo* viral inhibitory capacity and polyfunctionality) arising early after infection, in a well-defined cohort of acute/early infected subjects from Argentina, in comparison with that found in, also local, viremic Chronics and ECs [20]. As an extension of that preceding work, here we aimed at performing immunophenotypic analyses (in terms of memory markers and PD-1 expression) of the CD8+ T-cells, using the same cohorts of subjects, in order to seek for associations with both CD8+ T-cell functional properties and with viral control and subsequent disease progression. Major findings indicate that i) the distribution of total and HIV-specific CD8+ T-cell memory subsets is severely altered in chronically infected subjects (excluding ECs). Although it is also altered in recently infected subjects (PHI group), the shift is not so profound as in Chronics (Figures 1 and 2); ii) within the PHI group, higher proportions of T_{naive} and $T_{naive\text{-}like}$ CD8+ T-cells associated with markers of slower disease progression while higher proportions of total and HIV-specific CD8+ T_{EM} cells as well as higher HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio correlated with markers of faster progression (Figures 3 and S3); iii) analysis of PD-1 expression on total and HIV-specific CD8+ T-cells from PHI subjects revealed an association not only with disease progression but also with memory CD8+ T-cell differentiation (Figure 4); and iv) hierarchy of memory CD8+ T-cell subsets correlated with CD8+ T-cell activity during primary HIV infection (Figure 5). Of note, we consider that this study represents an important extension beyond the scope of previous studies on CD8+ T-cell phenotype during HIV infection, focused only on acute/early infection, ECs or in comparing two opposite study populations (for instance, ECs versus rapid progressors). Here, we simultaneously studied three groups of HIV-infected subjects at different disease stages (acute versus chronic) and with different clinical outcomes (controlled versus non-controlled). Moreover, clinical follow-up of subjects identified during primary infection allowed us to correlate the CD8+ T-cell phenotype observed at baseline with subsequent disease progression. In addition, we simultaneously studied cell phenotype both in bulk and HIV-specific CD8+ T-cells, contrary to other studies focused on the total compartment only. Finally, a relationship between the distribution of CD8+ T-cell phenotypes and CD8+ T-cell antiviral function could be established, a field in which only a few reports exist (see below).

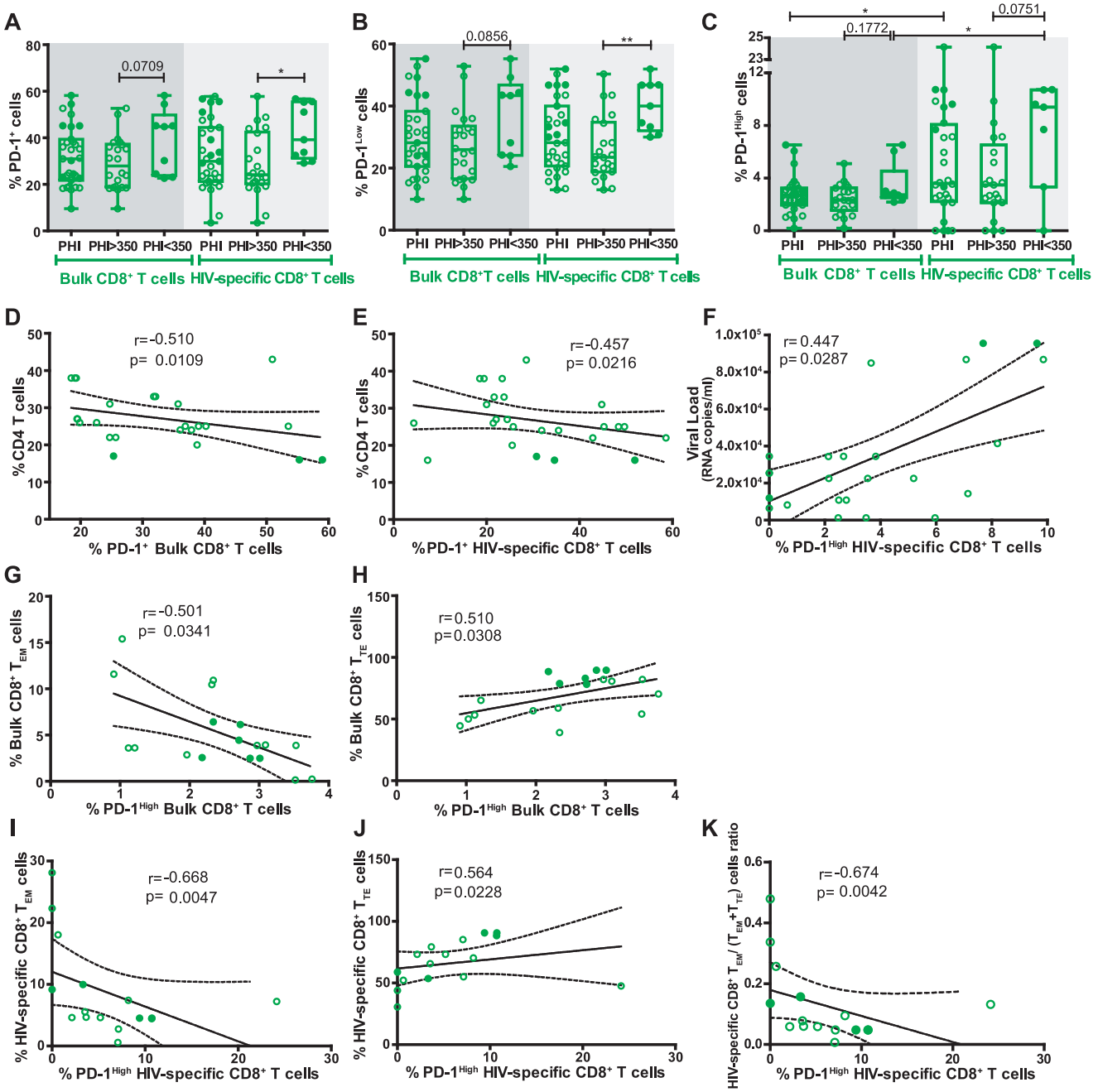

Figure 4. The relationship between PD-1 expression and markers of disease progression as well as with the distribution of memory subsets were studied in primary HIV infection (PHI) group. Percentage of PD-1 (A), PD-1^{Low} (B), and PD-1^{High} (C) cells out of bulk and HIV-specific CD8$^+$ T-cells, in samples from PHI subjects (N = 19; 30 specific responses) obtained at 8 months post-infection. (D to F) Correlations between clinical parameters and percentage of PD-1 expression in the CD8$^+$ T-cell subset, within the PHI group: Percentage of CD4 T-cells versus percentage of bulk PD-1$^+$ CD8$^+$ T-cells (D) or PD-1$^+$ HIV-specific CD8$^+$ T-cells (E). Viral load versus percentage of PD-1^{High} HIV-specific CD8$^+$ T-cells (F). (G to K) Correlation between baseline CD8$^+$ T-cell memory subsets and percentage of PD-1 expression at 8 months post-infection (N = 11 subjects; 18 specific responses): percentage of bulk CD8$^+$ effector memory (T_{EM}) (G) or terminal effector (T_{TE}) (H) cells versus percentage of bulk PD-1^{High} CD8$^+$ T-cells. Percentage of HIV-specific CD8$^+$ T_{EM} (I), CD8$^+$ T_{TE} (J) cells, or $T_{EM}/(T_{EM}+T_{TE})$ ratio (K) versus percentage of PD-1^{High} HIV-specific CD8$^+$ T-cells. Panels A–C: horizontal lines stand for median values. P values were calculated using Mann-Whitney test. Asterisks denote different P values: * P<0.05; ** P<0.005; *** P<0.001. Panels D–K: r and P values correspond to Spearman's test. In all panels, open and filled green dots denote PHI>350 and PHI<350 subjects, respectively.

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So far, several groups have studied the distribution of memory CD8$^+$ subsets in HIV infection. The use of different markers and the different numbers of such markers included in each study make it a difficult task to compare and interpret inter-study results. With some discrepancies, the general picture indicates that the hallmark of HIV infection in terms of memory CD8$^+$ T-cell subsets consists in an accumulation of not fully differentiated cells. This phenomenon was first described as a block toward terminal

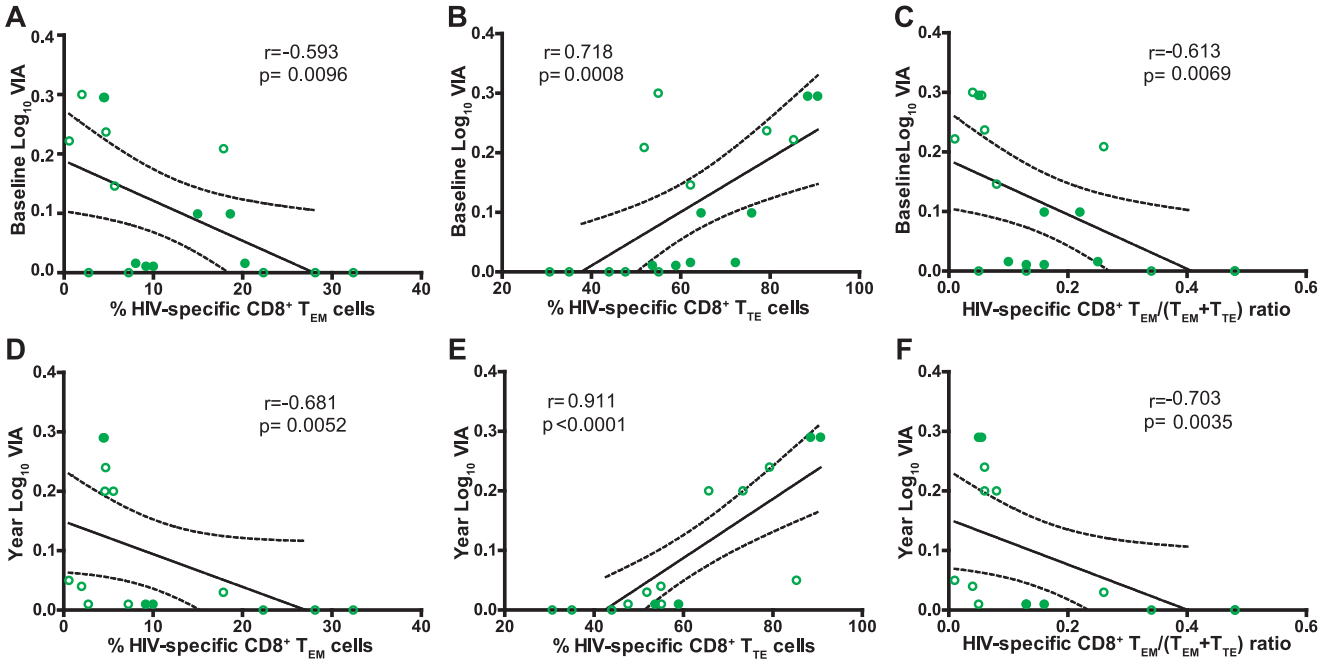

Figure 5. Correlation between CD8$^+$ T-cell capacity to suppress HIV replication *ex vivo* (VIA) and percentage of baseline HIV-specific CD8$^+$ T-cell subsets: CD8$^+$ T-cell antiviral capacity measured at baseline versus percentage of HIV-specific CD8$^+$ effector memory (T_{EM}) cells (A), CD8$^+$ terminal effector (T_{TE}) cells (B), or CD8$^+$ $T_{EM}/(T_{EM}+T_{TE})$ ratio (C). Antiviral CD8$^+$ T-cell capacity measured at 12-months post-infection versus percentage of baseline HIV-specific CD8$^+$ T_{EM} cells (D), CD8$^+$ T_{TE} cells (E), or CD8$^+$ $T_{EM}/(T_{EM}+T_{TE})$ ratio (F). N = 11 subjects (18 specific responses). In all panels, open and filled green dots denote PHI>350 and PHI<350 subjects, respectively. All r and P values correspond to Spearman's test. VIA: Viral inhibitory activity.
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differentiation within the HIV-specific compartment in progressive chronic HIV infection [38] and subsequently confirmed even in other settings such as acute infection [6,34,39]. Interestingly enough, many studies have established a link between HIV-specific memory CD8$^+$ T-cell differentiation and disease progression [16,40,41]. Additionally, differentiation of total CD8$^+$ T-cells is also skewed in HIV infection and related to progression [6,16,19,42,43]. In this context, our results reinforce the level of knowledge into this field providing further support into these notions. In the first place, and as reported elsewhere [16,19], our results indicate that the distribution of memory subsets within bulk CD8$^+$ T-cell is abnormal in viremic Chronics but does not significantly distinguish PHI subjects, ECs or HDs. Similarly, the HIV-specific CD8$^+$ T-cell compartment was only severely compromised in Chronics but not in the PHI group. In this scenario, early HAART initiation has been shown to provide not only virological but also immunological benefits to HIV-infected subjects. For instance, it was shown that the normal hierarchy in CD8$^+$ T-cell subset differentiation is not restored after HAART-driven viral suppression [43–45] and that very early HAART initiation limits the seeding of the HIV reservoir, particularly in long-lived T_{CM} cells [46,47]. Altogether, these data argue in favor of early initiation of HAART, in order to prevent irreversible deterioration of the mechanisms involved in immune homeostasis. Further studies involving the relationship between immune preservation (in terms of function and phenotype), size and features of viral reservoir together with disease progression in a PHI cohort are guaranteed.

Intriguingly, it was observed that the memory differentiation hierarchy between the total and HIV-specific compartments did not differ significantly in either group analyzed. This might reflect that the force driving CD8$^+$ T-cell differentiation in HIV infection equally affects both compartments. Also, the memory differentiation pattern did not differ between Gag-specific versus Nef-specific responses, as previously reported [41,48]. Contrary to this, Meyer-Olson *et al.* [39] described that epitope-specific CD8$^+$ T-cell maturation into memory/effector phenotypes is a TCR-dependent process. The absence of difference in the specificities observed in our study may be associated with the use of peptide pools as stimuli which may mask such single-epitope differences.

As stated above, HIV infection is characterized by an accumulation of preterminally differentiated (CD45RO$^+$/CCR7$^-$ or T_{EM} as defined for the purposes of this work) HIV-specific CD8$^+$ T-cells and relative diminished frequency of fully differentiated effector cells (CD45RO$^-$/CCR7$^-$ or T_{TE}). Our inter-group analysis supports this notion since Chronics had a significantly higher proportion of HIV-specific CD8$^+$ T_{EM} cells than PHI subjects and ECs ($p = 0.002$ and $p = 0.0111$, respectevily) and a trend to a lower proportion of HIV-specific CD8$^+$ T_{TE}. Most important, Chronics had significantly higher HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratios reflecting the accumulation of T_{EM} over T_{TE} in Chronics but not in ECs (Chronics vs ECs $p = 0.0144$) or PHI (Chronics vs PHI $p = 0.0042$). The same could be observed in the correlation analysis of proportions of T_{EM} versus T_{TE} in all groups of subjects: significant inverse correlations were obtained for PHI ($p = 0.0095$) (as in [40]) and ECs ($p = 0.0351$) but not for Chronics. Even more notably, the correlation analysis performed within the PHI group indicated that the HIV-specific $T_{EM}/(T_{EM}+T_{TE})$ ratio evaluated at baseline inversely correlated with baseline and set-point CD4$^+$ T-cell levels and directly with cellular and soluble markers of immune activation. This result, which adds support to previously reported findings in other cohorts [16,40], clearly indicate that the early deterioration of CD8$^+$ T-cell differentiation pathway associates with disease progression even at

very early times post-infection. In this sense, our results, among many other reports [16,44,49], have suggested a direct link between maturation misbalance of the $CD8^+$ T-cell memory compartment and the generalized and persistent immune activation found in HIV^+ subjects. However, which is the cause and which the consequence is still not fully understood. A recent study focused on ECs [50] suggested that, in these particular subjects, maintenance of a less mature memory $CD4^+$ T-cell population provides the necessary T-cell help for optimal maturation of effective $CD8^+$ T-cell responses. In other words, the skewed memory $CD8^+$ T-cell phenotype might be the result of improper $CD4^+$ T-cell help. Alternatively, it was proposed that higher proportions of $T_{Naïve}$ cells in EC may reflect increased thymic output in these subjects (compared to Chronics) which would contribute to the replenishing of such compartment [51]. Similar investigations in primary HIV infection are guaranteed to elucidate the impact of memory $CD4^+$ T-cell differentiation as well as thymic function on the $CD8^+$ T-cell subset in the context of progressive HIV infection.

Expression of PD-1 on virus-specific $CD8^+$ T-cells has been consistently associated with a state of cellular exhaustion in the context of persistent viral infections, with HIV infection not being an exception [33,52–54]. However, data on acute HIV infection is much more scarce [16,34,43,55]. Here, the expression of PD-1 was evaluated in bulk and HIV-specific $CD8^+$ T-cells at 8 months post-infection and related to clinical outcome as well as memory $CD8^+$ T-cell differentiation and functionality, aiming to provide a broader picture of $CD8^+$ T-cell phenotype hallmark in primary HIV infection. In the first place, it was found that PD-1^+ $CD8^+$ T-cells (both total and HIV-specific) were augmented in the PHI$<$ 350 group, which had significantly lower $CD4^+$ T-cell counts compared to the PHI$>$350 group ($p = 0.0341$ and $p = 0.0308$, respectively), suggesting a relationship of PD-1 expression with faster disease progression. In line with this observation, inverse correlations were obtained for PD-1^+ $CD8^+$ T-cells and $CD4^+$ T-cell counts. Similarly, other reports on primary HIV infection [16,34] (and contrary to that described for chronic infection [33,53]), found no associations between total PD-1 expression and viral load. However, a relationship between the magnitude of PD-1 expression and viral replication became evident when total PD-1^+ cells were split into PD-1^{Low} and PD-1^{High} cells: higher proportions of PD-1^{High} cells directly correlated with higher viral load. This is consistent with the notion that PD-1 is up-regulated on $CD8^+$ T-cells due to T-cell activation in the presence of high viral loads [36]. In this line, there is an increasing body of evidence suggesting that PD-1 might be linked to T-cell exhaustion and also, it would be a marker of cell activation [16,36] and a key regulator of memory cell differentiation [35,43,56] and survival [57]. Our results indicate that, at early times post-primary infection (8 months post infection), higher proportions of PD-1^{high} $CD8^+$ T-cells correlated with lower $CD8^+$ T_{EM} and higher $CD8^+$ T_{TE} proportions. Likewise, other authors indicated that PD-1 is expressed in all memory subsets, and that PD-1 up-regulation is associated with cellular activation, with a reduction in proliferative potential and with a higher sensitivity to cell death [16,36,43,53,56,57]. Moreover, these results provide further support to the revisited idea that, during acute infections, PD-1 is a cellular activation marker rather than an exhaustion marker as it is in chronic infections [43,58]. Also in this context, several reports have demonstrated that PD-1^+ $CD8^+$ T-cells can be fully functional [34,58–60]. This is consistent with our failure in identifying associations between PD-1 expression and HIV-specific $CD8^+$ T-cell functionality during primary HIV infection, as Petrovas *et al*. [57] reported for chronic infection.

Contrary to what was obtained for PD-1 expression, significant associations were found between memory $CD8^+$ T-cell phenotype and functionality. More precisely, lower and higher baseline proportions of HIV-specific T_{EM} and T_{TE} $CD8^+$ T-cells, respectively, correlated with higher *ex vivo* $CD8^+$ T-cell antiviral activity (VIA), both at baseline ($p = 0.0096$ and $p = 0.0008$, respectively) and at 12 months post-infection ($p = 0.0052$ and $p < 0.0001$, respectively). Antiviral activity as evaluated in this work encompasses both lytic and non-lytic antiviral mechanisms, evidencing the overall capacity of HIV-specific cells to mediate virus control. Previous reports have demonstrated, by isolating pure populations of $CD8^+$ T-cells (based on their memory differentiation) from chronically HIV-infected subjects and vaccinees, that cells from all subsets could mediate VIA [15,61,62]. In these reports, Elite Controller status [62] and control of breakthrough infections in vaccinated monkeys [61] were associated with improved antiviral activity of the $CD8^+$ T_{EM} compartment. In this line, here we were able to find a relationship between the hierarchy of memory $CD8^+$ T-cell differentiation and $CD8^+$ T-cell antiviral function in primary infection. Even when *a priori* these results seem to be in contradiction, it must be noted that the experimental approach and, most important, the setting are completely different: we evaluated $CD8^+$ T-cell antiviral function in the context of high viral load and immune activation such as acute/early infection meanwhile $CD8^+$ T-cells from ECs and vaccines are not subjected to such a hostile environment affecting its functionality. This raises concerns over whether signatures of $CD8^+$ T-cell function or phenotype found in individuals such as ECs are the cause or the consequence of virus control. Many reports argue in favor of the latter hypothesis so caution should be taken when interpreting such data. Conversely to VIA, no association was found between memory $CD8^+$ T-cell differentiation and polyfunctionality. This is in contradiction with a report by Riou *et al*. [41] showing that HIV-specific $CD8^+$ T-cells show decreasing polyfunctionality coinciding with an increase in differentiation from early to terminally differentiated memory subsets during HIV acute/early infection. Due to technical constraints, these differences may be masked by our experimental design.

Overall, here we report that normal maturation of total and HIV-specific $CD8^+$ T-cells into memory subsets is skewed in PHI but not at the dramatic level observed in chronic infection. Furthermore, the magnitude of this alteration in maturation translates into a decrease in $CD8^+$ T-cell antiviral capacity which is directly correlated with early disease progression. Unscrambling relationships among T-cell differentiation, T-cell functionality, immune activation, viral control, and disease progression in multiple settings (primary infection, viremic Chronics and ECs), such as those observed in this work, are increasingly important to advance our understanding of HIV pathogenesis. As well, this information will be instrumental for therapeutic and sterilizing vaccine design in order to boost our ability to elicit beneficial responses.

Supporting Information

Figure S1 Gating strategy used for the identification of different CD8 sub-populations, based on their phenotype, on bulk and HIV-specific T-cells. Illustration data were derived from one representative subject, stimulated with an HIV peptide pool. Initial gating was performed on a plot of

forward scatter area (FSC-A) versus height (FSC-H) to remove doublets. Then, gating was performed on small lymphocytes in a plot of forward scatter (FSC) versus side scatter (SSC). Dead cells were then excluded on the basis of LIVE/DEAD fluorescence. Subsequently, $CD3^+$ $CD8^+$ cells were gated in a CD3-versus-CD8 dot plot. Following identification of these cells, HIV-specific $CD8^+$ T-cells were identified in a CD8 versus. cytokines (FITC) density plot. Then, the distribution of different phenotype subsets were analyzed both in total and HIV-specific $CD8^+$ T-cell compartments. For this, CD45RO versus CCR7 density plots or PD-1 histograms (also PD-1^{Low} or PD-1^{High}) were performed on gated $CD3^+CD8^+$ cells (bulk) or $CD8^+$ $Cytokines^+$ cells (HIV-specific). Simultaneous use of CD45RO and CCR7 markers allowed us to define four $CD8^+$ T-cell sub-populations: naïve ($T_{Naïve}$, $CCR7^+CD45RO^-$), central memory (T_{CM}, $CCR7^+CD45RO^+$), effector memory (T_{EM}, $CCR7^-CD45RO^+$) and terminal effector (T_{TE}, $CCR7^-CD45RO^-$) cells.
(EPS)

Figure S2 Three groups of HIV infected subjects were enrolled for this study: 32 subjects were recruited during HIV seroconversion and/or within 6 months since the presumed date of infection (PHI group), 10 chronically infected subjects (Chronics), and 11 subjects defined as Elite Controllers (EC) according to the criteria defined in materials and methods. Viral load (A) $CD4^+$ T-cell count (B) and Immune Activation (C) were determined. Panels A and B, values corresponding to both baseline and set point samples are shown for Primary HIV infected (PHI) subjects. Viral and $CD4^+$ T-cell set-points were calculated as the geometric mean of determinations obtained between 6 and 12 months post-presumed date of infection. Also, subjects included in either PHI>350 and PHI<350 subgroups (defined in materials and methods) are indicated by open and filled green dots, respectively. Horizontal lines stand for median values. P values were calculated using Mann-Whitney test. Asterisks denote different P values: * $P<0.05$; ** $P<0.005$; *** $P<0.001$. Within the PHI group, median baseline VL and CD4+ T-cell counts were 34,800 RNA copies/ml (interquartile range (IQ)25–75: 8,843–252,588 copies/ml) and 503 cells/μl (IQ25–75: 320–682 cells/μl), respectively. As regards chronically infected subjects, median VL was 28,435 RNA copies/ml (IQ25–75: 9,449–197,984) and median CD4+ T-cell count was 141 cells/μl (IQ25–75: 11–563) which was significantly lower than the other groups ($p = 0.016$ and $p = 0.0028$ compared to PHI and ECs, respectively). On the other hand, all ECs had undetectable plasma VL (<50 RNA copies/ml) and the median $CD4^+$ T-cell count was 602 cells/μl (IQ25–75: 562–888). PHI<350 showed, both at baseline and set-point, significantly higher VLs ($p = 0.0321$ and $p<0.0001$, respectively) and lower $CD4^+$ T-cell counts ($p = 0.0466$ and $p = 0.0008$, respectively), compared to the PHI>350 group and (see also Table 1).
(EPS)

Figure S3 Correlations between the proportion of the different $CD8^+$ T-cell subsets within bulk (A and B) and the HIV-specific compartment (C to F) and clinical parameters measured in baseline samples from primary HIV infected (PHI) subjects: Baseline $CD4^+$ T-cell counts (A) and baseline CD4 immune activation (B) versus percentage of $CD8^+$ T_{Naive} cells. Percentage of HIV-specific $CD8^+$ $T_{Naive\text{-}like}$ cells versus percentage of baseline $CD4^+$ T-cell (C), baseline viral load (D) and viral set-point (E). (F) Percentage of HIV-specific $CD8^+$ T_{EM} cells versus viral set-point. PHI group N = 24 subjects (39 responses analyzed for bulk compartment and 31 responses for the specific compartment). For set point correlations N = 15 subjects. In all panels, open and filled green dots denote PHI>350 and PHI<350 subjects, respectively. All r and P values correspond to Spearman's test.
(EPS)

Table S1 Characteristics of HIV^+ subjects enrolled per study group.
(DOCX)

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Conceived and designed the experiments: YG MMG GT. Performed the experiments: YG JF MJR. Analyzed the data: YG MMG HS GT. Contributed reagents/materials/analysis tools: NL MES PC LG OS. Contributed to the writing of the manuscript: YG MMG HS GT.

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Early Gag Immunodominance of the HIV-Specific T-Cell Response during Acute/Early Infection Is Associated with Higher CD8$^+$ T-Cell Antiviral Activity and Correlates with Preservation of the CD4$^+$ T-Cell Compartment

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The important role of the CD8$^+$ T-cell response on HIV control is well established. Moreover, the acute phase of infection represents a proper scenario to delineate the antiviral cellular functions that best correlate with control. Here, multiple functional aspects (specificity, *ex vivo* viral inhibitory activity [VIA] and polyfunctionality) of the HIV-specific CD8$^+$ T-cell subset arising early after infection, and their association with disease progression markers, were examined. Blood samples from 44 subjects recruited within 6 months from infection (primary HIV infection [PHI] group), 16 chronically infected subjects, 11 elite controllers (EC), and 10 healthy donors were obtained. Results indicated that, although Nef dominated the anti-HIV response during acute/early infection, a higher proportion of early anti-Gag T cells correlated with delayed progression. Polyfunctional HIV-specific CD8$^+$ T cells were detected at early time points but did not associate with virus control. Conversely, higher CD4$^+$ T-cell set points were observed in PHI subjects with higher HIV-specific CD8$^+$ T-cell VIA at baseline. Importantly, VIA levels correlated with the magnitude of the anti-Gag cellular response. The advantage of Gag-specific cells may result from their enhanced ability to mediate lysis of infected cells (evidenced by a higher capacity to degranulate and to mediate VIA) and to simultaneously produce IFN-γ. Finally, Gag immunodominance was associated with elevated plasma levels of interleukin 2 (IL-2) and macrophage inflammatory protein 1β (MIP-1β). All together, this study underscores the importance of CD8$^+$ T-cell specificity in the improved control of disease progression, which was related to the capacity of Gag-specific cells to mediate both lytic and nonlytic antiviral mechanisms at early time points postinfection.

Human immunodeficiency virus (HIV) still represents a major public health concern. Although the instauration of highly active antiretroviral treatment (HAART) had a tremendous impact on the epidemic dynamics, the development of an effective prophylactic vaccine is still a main objective in the HIV-related research field. As HIV is highly diverse among different isolates, evolves continuously under selective pressure, infects immune cells, and encodes proteins with the capacity to modulate immune cell functions, it imposes definite challenges that should be overcome in the race of getting a successful vaccine. However, the description of (i) infected subjects able to control HIV replication over long periods of time to very low levels without therapy (known as long-term nonprogressors [LTNP] and elite controllers [EC]); (ii) uninfected subjects who, despite being highly exposed to the virus, remain seronegative (exposed seronegatives [ESN]); and (iii) the results from the Thai vaccine trial RV-144, which showed 30% efficacy (1), suggests that the objective is reachable. In this line, much of the research work conducted over the past few years was aimed to define the immune correlates of protection, i.e., desirable characteristics that the vaccine-elicited immune response should have in order to contain viral challenge. Within this field, special emphasis has been focused on the HIV-specific CD8$^+$ cytotoxic T lymphocytes (CTLs), which are thought to play a key role in reducing viral replication (2, 3).

The first evidence that specific CD8$^+$ T cells were involved in the control of viral replication was reported in studies conducted in humans and nonhuman primates during the acute phase of infection. After infection, emergence of specific CD8$^+$ T cells correlates with the decline of peak viremia toward set point establishment, which varies from person to person and is a strong predictor of disease progression (4). Also, CTL escape mutants have been described (5, 6), and superior viral control has been attributed to specific human leukocyte antigen (HLA) class I alleles (7, 8). Moreover, recent proof-of-concept vaccine studies in nonhuman primates indicate that vaccine-elicited CD8$^+$ T-cell responses are associated with partial protection from infection and with en-

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TABLE 1 Summary of clinical data corresponding to enrolled HIV$^+$ subjects per study group

Group (no. of subjects)	Viral load[a,c]		Viral set point[d] (mean log10 ± SD)	CD4$^+$ T-cell count,[b,c] median no. of cells/μl (IQ)	CD4 set point,[d] median no. of cells/μl (IQ)
	Median RNA copies/ml (IQ)	Mean log10 ± SD			
PHI					
All ($n = 44$)	98,684 (13,161–477,708)	4.6 ± 1.2	4.5 ± 0.8	525 (320–678)	526 (357–656)
PHI > 350 ($n = 23$)	32,473 (9,532–116,129)	4.2 ± 1.2	4.3 ± 0.6	633 (587–786)	586 (491–680)
PHI < 350 ($n = 18$)	269,532 (101,394–500,000)	5.2 ± 1.0	5.0 ± 0.8	292 (244–375)	281 (234–327)
Chronic ($n = 16$)	16,682 (2,136–41,677)	4.1 ± 0.7		455 (164–577)	
Chronic excluding viremic controllers ($n = 12$)	22,267 (13,296–44,758)	4.4 ± 0.5		287 (140–544)	
EC ($n = 11$)	<50	<1,7		595 (562–817)	

[a] Versant HIV-1 RNA 3.0 assay, Siemens. Lower and upper detection limits are 50 and 500,000 RNA copies/ml, respectively (1.7 and 5.7log10).
[b] Flow cytometry double platform, FACSCanto, BD Biosciences.
[c] For PHI subjects, data correspond to baseline samples. For chronic and elite controller subjects, data correspond to samples obtained at enrollment.
[d] Set points were not calculated for subjects that initiated HAART during the first year postinfection.

hanced control of breakthrough infections (9, 10), reinforcing the notion that specific CD8$^+$ T cells exert a pivotal role in viral control. In-depth analyses of this cellular population, performed in different cohorts and models, suggest that specificity, quality, and phenotype are all determinants of CD8$^+$ T-cell ability to mediate control: specificity in terms of viral targets (11–15); quality in terms of avidity and capacity to mediate viral suppression, proliferate, and secrete a broad spectrum of chemokines and cytokines (16–20); and phenotype in terms of memory sub-subsets and expression of exhaustion markers (21–23).

Cell samples obtained during the acute/early HIV infection constitute invaluable tools to understand the functional features of the HIV-specific CD8$^+$ T cells that best correlate with the lower-set-point/protection-from-progression axis and future control. For sure, these approaches will help dissect the correlates of protection needed to develop an effective prophylactic vaccine. Besides, vaccine-elicited highly suppressive specific CD8$^+$ T cells would help constrain viral replication to very low levels in breakthrough infections occurring in vaccinees, which in turn would contribute to a slower progression of the newly infected person as well as lower transmission risk (24).

We have previously worked with acute phase samples in order to evaluate Nef-specific cross-clade T-cell reactivity in samples from subtype B- and BF-infected subjects (25). In that study, differences in the CD8$^+$ T-cell population functional profile were observed among subjects, finding an association between CD8$^+$ T-cell polyfunctionality and viral control. In the manuscript, we sought to analyze multiple aspects of the HIV-specific CD8$^+$ T-cell compartment (specificity, *ex vivo* viral inhibitory capacity, and polyfunctionality) arising early after infection in a larger cohort, in comparison with the response found in viremic chronics and elite controllers, with the aim to delineate CD8$^+$ T-cell features that best associate with disease control and that would contribute to rational vaccine design. Briefly, it was found that a higher relative proportion of Gag- than Nef-specific cells, even very early after infection, strongly associated with delayed progression during the first year postinfection, in consonance with Gag immunodominance in EC and chronically infected "viremic controllers." The advantage of Gag-specific cells may result from their enhanced ability to mediate lysis of infected cells (evidenced by higher capacity to degranulate and to mediate viral inhibition activity *in vitro*) and simultaneously produce IFN-γ. Also, a direct association between the level of Gag immunodominance and plasma levels of interleukin 2 (IL-2) and macrophage inflammatory protein 1β (MIP-1β) was observed. These data underscore the importance of considering both cell specificity and quality in the design and evaluation of HIV vaccine candidates.

MATERIALS AND METHODS

Study subjects. A total of 81 subjects participated in this study: 10 healthy HIV-seronegative donors (HD) and 71 HIV-infected patients, of whom 44 were enrolled during acute/early primary HIV infection (PHI), 16 were chronically infected patients (chronics), and 11 were elite controllers (EC) (Table 1; see also Table S1 in the supplemental material). PHI subjects were enrolled by the Grupo Argentino de Seroconversión Study Group under the following inclusion criteria (26): (i) detection of HIV RNA or p24 antigen with a simultaneous negative or indeterminate Western blot assay or (ii) positive Western blot assay with a negative test within the previous 6 months. Chronically infected patients were defined as subjects with established HIV infection older than 3 years, with detectable viral load (VL; >50 HIV RNA copies/ml plasma), and who are HAART naive, while EC were defined as subjects infected for more than 5 years with undetectable VL (<50 HIV RNA copies/ml plasma) who are HAART naïve and have no record of opportunistic infections and/or AIDS-related diseases. The study was reviewed and approved by two institutional review boards (IRB): Comité de Ética Humana, Facultad de Medicina, Universidad de Buenos Aires, and Comité de Bioética, Fundación Huésped (Buenos Aires, Argentina). Both HIV-infected participants and healthy donors provided written informed consent accepting to participate in this study.

Samples. Blood samples were collected from study participants at enrollment and centrifuged to separate plasma, which was stored at −80°C until use. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation (Amersham, Sweden) and cryopreserved for subsequent functional assays. For PHI subjects, additional samples were obtained at 3, 6, 9, and 12 months postinfection. In the case of chronic, EC, and PHI subjects, plasma VL (branched-DNA, Versant HIV-1 RNA 3.0 assay; Siemens Healthcare) and CD4$^+$ T-cell count (flow cytometry double platform, BD FACSCanto; BD Biosciences) were determined.

HLA typing. Human leukocyte antigen (HLA) class I typing was performed using an in-house protocol consisting of PCR amplification, nucleotide sequencing with nested primers, and Web-based sequence interpretation (R. S. Coloccini and D. C. Monaco, unpublished data). HLA-A

exons 2 and 3 were amplified in one amplicon. HLA-B exons 2 and 3 were amplified separately. The amplification of exon 3 was performed by heminested PCR, using 2 different reverse primers. Similarly, HLA-C exons 2 and 3 were amplified separately using a heminested PCR strategy (2 different forward primers) in the case of exon 2. Amplicons were directly sequenced using the BigDye Terminator sequencing kit (Amersham, Sweden) on an automatic sequencer (Applied Biosystems DNA Sequencer 3100). Nucleotide sequences were analyzed and manually adjusted using Sequencher 4.10.1 software (Gene Codes Co.). Sequence interpretation was performed using the NCBI SBT Interpretation software, available online (http://www.ncbi.nlm.nih.gov/gv/mhc/sbt.cgi?cmd=main).

Peptides. Potential T-cell epitope (PTE) peptide panels corresponding to Nef, Gag, and Env proteins and the cytomegalovirus (CMV), Epstein-Barr virus, and influenza virus (CEF) peptide pool were obtained from the NIH AIDS Reagent Program (27, 28). Lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO) at 40 μg/μl and stored at −20°C.

The PTE peptides are 15 amino acids (aa) in length and contain naturally occurring 9-aa sequences that are potential T-cell determinants embedded in the sequences of circulating HIV-1 strains worldwide. Here, the PTE peptides were grouped in 9 pools: 1× Nef ($n = 127$ peptides), 3× Gag (corresponding to p17 [$n = 97$], p24 [$n = 128$], and p2p7p1p6 [denoted as RG; $n = 95$]), and 5× Env (Gp120A1 [$n = 73$, spans HXB2 Env aa positions 1 to 154], Gp120A2 [$n = 73$, aa 157 to 284], Gp120B [$n = 105$, aa 287 to 511], Gp41A [$n = 114$, aa 513 to 689], Gp41B [$n = 115$, aa 689 to 842]).

ELISPOT assay. Gamma interferon (IFN-γ)-secreting cells were evaluated using enzyme-linked immunospot (ELISPOT) assays as described previously (25). Briefly, cryopreserved PBMCs were thawed in complete RPMI medium (RPMIc; RPMI 1640 [Gibco BRL], 10% fetal bovine serum [FBS; Gibco BRL], 2 mM L-glutamine [Gibco BRL], 100 U/ml penicillin [Gibco BRL], 100 μg/ml streptomycin [Gibco BRL], 10 mM HEPES [Gibco BRL]) supplemented with 50 U/ml DNase I (Benzonase nuclease; Sigma-Aldrich) and then rested overnight in DNase-free medium at a density of 10^6 cells/ml. Cell viability was checked by trypan blue exclusion after thawing and overnight rest. Rested PBMCs with >95% viability were plated on sterile 96-well plates (MultiScreen IP plates; Millipore), previously coated with mouse anti-human IFN-γ monoclonal antibody (BD Biosciences) at 10^5 cells/well, and peptide pools were added at a final concentration of 2 μg/ml of each peptide. Final DMSO concentration was always lower than 0.7%. Negative (peptide-free medium plus 0.5% DMSO), CEF peptide pool (2 μg/ml of each peptide), and phorbol myristate acetate (PMA)-ionomycin (5 ng/ml PMA plus 500 ng/ml ionomycin; Sigma-Aldrich) controls were assayed for each patient. Plates were developed using biotinylated anti-human IFN-γ monoclonal antibody, streptavidin-peroxidase complex, and the 3-amino-9-ethylcarbazole (AEC) substrate reagent set (BD Biosciences). Plates were scanned on an ImmunoSpot reader (Cellular Technology Ltd.). Specific spot count and spot size were calculated using the ImmunoSpot software. Results were expressed as spot-forming units (SFU)/10^6 PBMCs after subtraction of the negative-control values. Thresholds for positive responses for the test wells were defined as at least 50 SFU/10^6 PBMCs or as mean SFU greater than three times the mean SFU of the negative-control wells, whichever was higher.

Virus inhibitory activity. *Ex vivo* $CD8^+$ T-cell capacity to inhibit viral replication in primary autologous $CD4^+$ T cells was assayed following the protocol published by Sáez-Cirión et al. (29) with minor modifications: bulk $CD4^+$ T cells were isolated from thawed and overnight rested PBMCs by positive selection with anti-CD4 antibody-coated magnetic beads (BD Biosciences) and kept for 3 days in RPMIc supplemented with 1 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich). $CD4^+$ T-cell-depleted PBMCs were then subjected to $CD8^+$ T-cell-positive selection with anti-CD8 antibody-coated magnetic beads (BD Biosciences). Sorted $CD8^+$ cells were cultured for 3 days in RPMIc without PHA.

At day three postisolation, $CD4^+$ and $CD8^+$ cells were washed, counted, and plated in U-bottom 96-well plates at a 1:1 ratio. Cells were then infected with X4-tropic (HIV_{LAI}) or R5-tropic (HIV_{BAL}) HIV-1 laboratory strains at a multiplicity of infection (MOI) of 0.001. In order to improve infection efficiency, plates were first centrifuged at 1,200 × *g* for 1 h at 22°C (spinoculation), and then adsorption was let to proceed for an extra hour at 37°C in a humidified CO_2 incubator. After infection, cells were washed twice and resuspended in RPMIc medium containing IL-2 at a final concentration of 10 U/ml. At day 3 postinfection, the cocultures were fed by replacing one-half of the culture supernatant with fresh medium. At day 7 postinfection, supernatants were removed and p24 antigen was quantified by enzyme-linked immunosorbent assay (ELISA; Vironostika HIV-1 antigen kit; bioMérieux, France). Uninfected cocultures were included as negative controls, and infected $CD4^+$ T cells without added effectors ($CD8^+$ T cells) served as 100% infectivity controls. All conditions were assayed in triplicate. $CD8^+$ T-cell anti-HIV suppressive capacity was calculated as the $\log_{10}$ of the percentage of p24 antigen lost when $CD8^+$ T cells were present in the culture.

Intracellular cytokine staining (ICS). Production of cytokines and degranulation of HIV-specific cells were measured upon stimulation by flow cytometry following the protocol published before (25) with the following modification: thawed and overnight rested PBMCs were dispensed in U-bottom 96-well plates (5×10^5 cells/well) in duplicate wells. Cell viability was checked before and after overnight rest by trypan blue exclusion (only samples with >95% viability after the overnight rest were used for the assays). Costimulatory antibodies (anti-CD28 and anti-CD49d antibodies, 1 μg/ml; BD Biosciences), monensin (Golgistop, 0.7 μl/ml; BD Biosciences), brefeldin A (10 μg/ml; BD Biosciences), and the corresponding peptide pool (2 μg/ml) were added. An unstimulated (peptide-free medium plus 0.5% DMSO and costimulatory antibodies) and two positive controls (2 μg/ml CEF peptide pool and PMA-ionomycin) were included in each assay. A mixture of anti-CD107A-fluorescein isothiocyanate (FITC) and anti-CD107B-FITC antibodies (BD Biosciences) was added to one of the replicates. Cells were incubated for 6 h at 37°C, washed, and stained for 30 min at 4°C with LIVE/DEAD Fixable NEAR-IR (Invitrogen), in order to exclude dead cells, and with anti-CD3-PerCP and anti-CD8-APC surface antibodies (BD Biosciences). Then, cells were permeabilized and fixed using the Cytofix/Cytoperm kit (BD Biosciences). After the permeabilization/fixation step, one of the replicates was stained using anti-IL-2–phycoerythrin (PE), anti-tumor necrosis factor alpha (TNF-α)-FITC, and anti-IFN-γ–PECy7 antibodies (BD Biosciences), while replicates already containing the anti-CD107A/B mix were stained with anti-IL-2–PE and anti-IFN-γ–PECy7. Cells were then washed and stored until data acquisition in a 2-laser, 6-color BD FACSCanto flow cytometer. Data acquisition and analysis were performed using the BD FACSDiva software. Instrument settings and fluorescence compensation were performed for each day of testing using unstained and single-stained samples. Isotype controls (consisting of stimulated cells stained with conjugated antibodies to surface molecules and isotype controls corresponding to intracellular markers) were performed for each patient in order to accurately set negative populations. First, a plot of forward scatter area (FSC-A) versus height (FSC-H) was constructed to remove doublets. Then, gating was performed on small lymphocytes in a plot of FSC versus side scatter (SSC). At least 80,000 events were acquired in the lymphocyte gate. Dead cells were then excluded on the bases of LIVE/DEAD fluorescence. Subsequently, $CD3^+$ $CD8^+$ cells were gated in a CD3-versus-CD8 dot plot. Following identification of these cells, a gate was made for each function studied (see Fig. 5A). To study 2-function and 3-function positive populations, "derived gate tools" available in the FACSDiva software was used. For this purpose, intersections of two and three gates were created, respectively. Once the percentages of events were determined for each derived gate, the value of triple-positive events was subtracted from those of double-positive events and, in turn, double- and triple-positive events were subtracted from the total events positive for a given function to calculate monofunctional cells. Samples with a nonspecific background higher than 0.05% for any function were retested using a new vial of frozen cells. Data presented corre-

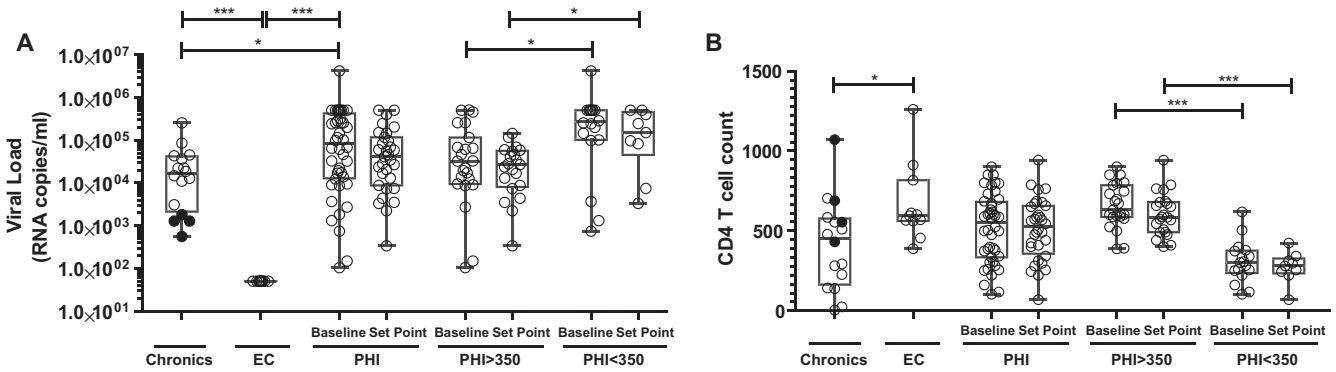


FIG 1 Viral load (A) and CD4$^+$ T-cell count (B) of enrolled HIV$^+$ subjects per study group. For PHI subjects, values corresponding to both baseline and set point are shown. Also, PHI subjects are shown as a whole group (PHI) and split into PHI $>$ 350 and PHI $<$ 350 subgroups whether their CD4$^+$ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not. Within the chronic group, black dots correspond to viremic controllers (see Table S1 in the supplemental material and the text). Horizontal lines stand for median values. *P* values were calculated using Mann-Whitney test. VEGF, vascular endothelial growth factor; MDC, macrophage-derived chemokine; GRO, growth-related protein. Asterisks denote different *P* values: *, $P < 0.05$; ***, $P < 0.005$.

spond to background-subtracted results using the CD28/CD49d-only stimulation. This was performed on a cytokine-subset-by-cytokine-subset basis, i.e., subtracting the result from the CD28/CD49d-only condition for a given cytokine subset to the same subset of a peptide-stimulated condition. Two standard deviations (SDs) above background was set as the threshold for determining positive responses. Values below this threshold were set at 0.

For certain cell functions, relative mean fluorescence intensity (rMFI) was calculated as the ratio between MFI corresponding to specific versus total CD8$^+$ T cells for a given channel.

Quantification of plasma soluble factors. Simultaneous determination of the following 39 cytokines and chemokines was performed in plasma samples from a subset of 28 PHI subjects (at baseline time point only) using Luminex technology (MILLIPLEX MAP Human Cytokine/Chemokine; Millipore): epidermal growth factor (EGF), eotaxin, FGF-2, Flt-3 ligand, fractalkine, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1rα, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, monocyte chemoattractant protein 1 (MCP-1), MCP-3, MDC (CCL22), MIP-1α, MIP-1β, sCD40L, sIL-2Rα, transforming growth factor alpha (TGF-α), TNF-α, TNF-β, VEGF. Samples were processed and analyzed as described by Giavedoni (30).

Data analysis. For PHI subjects, viral and CD4 set points were calculated as the geometric mean of the determinations obtained between 6 and 12 months after the presumed date of infection. Set points were not calculated for those subjects who started HAART during the first 12 months from infection or if no stable set point was reached during that period.

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software). All data except $\log_{10}$ VL and breadth of response were analyzed using nonparametric statistics. Two-tailed Wilcoxon and Mann-Whitney tests were used to compare intra- and intergroup variables, respectively. Correlations were determined using Spearman's rank test. All tests were considered significant if the *P* value obtained was less than 0.05. For correlations between plasma cytokine levels and immune parameters, *P* values were adjusted for multiple comparisons using a false discovery rate (FDR) procedure, according to the Benjamini & Hochberg method, with R Project software version 2.10.0. Adjusted *P* values were considered significant when less than 0.1.

RESULTS

Study subject descriptions. Three groups of HIV-infected subjects were enrolled as follows in order to attempt to dissect immune mechanisms associated with control over disease progression during acute/early HIV infection: 44 subjects recruited during HIV seroconversion and/or within 6 months from presumed date of infection (PHI group), 16 chronically infected subjects (chronics), and 11 subjects defined as elite controllers (EC) according to the criteria defined in Materials and Methods. Detailed descriptions of the HIV-infected participants are shown in Table S1 in the supplemental material. Baseline samples for most PHI subjects were obtained during Fiebig stages V and VI (31). Within this group, median baseline VL was 98,684 RNA copies/ml (25 to 75% interquartile range [IQ], 13,161 to 477,708 copies/ml). As expected, it was statistically higher than chronics' VL (median, 16,682 RNA copies/ml [IQ, 2,136 to 41,677], $P = 0.012$]) and, of course, EC's VL (Table 1 and Fig. 1). Median baseline CD4$^+$ T-cell count for PHI (525 cells/μl [IQ, 320 to 678]) did not differ significantly from the median CD4$^+$ T-cell count of chronics (455 cells/μl [IQ, 164 to 577]) or EC (595 cells/μl [IQ, 562 to 817]), but a statistically significant difference was found between chronics and EC ($P = 0.018$) (Fig. 1). For those PHI subjects who remained HAART naive during the first year postinfection, viral and CD4$^+$ T-cell set points were calculated (Table 1 and Fig. 1; see Table S1 in the supplemental material). These parameters did not differ significantly from chronics' VL and CD4$^+$ T-cell count. Of note, the group of chronically infected subjects enrolled for this work is very heterogeneous and includes four subjects that could be classified as what some authors refer to as viremic controllers, i.e., individuals able to spontaneously control viral load below 2,000 RNA copies/ml (32) (C03, C05, C07, C08; Fig. 1; see also Table S1 in the supplemental material). After removing these subjects from the analysis, median VL for the chronic group was 22,267 RNA copies/ml (IQ, 13,296 to 44,758), and median CD4$^+$ T-cell count was 287 cells/μl (IQ, 140 to 544) (Table 1).

PHI subjects were further divided into two subgroups whether their CD4$^+$ T-cell count dropped below 350 cells/μl at any time during the first year postinfection or not (PHI $<$ 350 and PHI $>$ 350). By doing this, we were aimed to differentiate subjects with more rapid or aggressive progression of early infection (PHI $<$ 350 group) and to investigate the association of this pattern with the immune parameters analyzed in this work. The 350-cells/μl endpoint was chosen based on the national and international recommendations for initiation of HAART current by the year 2010,

when most of these individuals were already enrolled (26). The PHI < 350 and PHI > 350 groups differed significantly in both baseline and set point VLs and CD4 T-cell counts (Table 1, Fig. 1).

Distribution of HLA alleles in enrolled subjects as a whole reflected the frequency distribution described for Argentina's urban population (www.allelefrequencies.net). To exclude the possibility of enrichment of particular HLA alleles associated with faster (A01, A68, B35) or slower (B27, B51, B57) HIV disease course, the frequency of these alleles was studied per study group. No significant differences in the frequency of A01, A68, or B35 alleles were found among groups, even for the EC group, where these alleles were found in 4 out of 11 subjects (36% versus 41% of chronics and 47% of PHI subjects) (see Table S1 in the supplemental material). On the other hand, "protective alleles" B27, B51, or B57 were present in one subject from the PHI < 350 group, three subjects from the PHI > 350 group, one PHI subject with undetermined progression status, three chronically infected subject, and only three EC. So, the different disease progression rates among groups cannot be explained merely by differences in the genetic background, at least based on the HLA-I locus level.

Screening of HIV-specific T-cell responses by ELISPOT revealed differences between progressive and nonprogressive infection regarding magnitude, preferred targets of specific response, and quality. To evaluate how the central targets, magnitude, and quality (in terms of breadth and capacity to respond upon stimulation) of the HIV-specific cellular immune response during the acute/early phase of infection impact early disease progression, HIV-specific IFN-γ-secreting cells were screened by ELISPOT in PHI subjects using samples obtained at baseline (defined as the first sample obtained after enrollment according to the criteria defined in Materials and Methods). PTE peptide pools spanning the viral proteins Nef (1 pool), Gag (3 pools), and Env (5 pools) were used as stimuli. Also, these HIV-specific immune responses were screened in chronics and EC for comparison purposes.

(i) Magnitude and targets of HIV-specific cellular immune response in PHI, chronics, and EC. The median magnitude of the specific immune response (expressed as the number of SFU/10^6 PBMCs) was higher in EC for all three antigens evaluated than in chronic and PHI subjects. This difference was particularly important in terms of Gag antigen: EC showed a >5-fold-higher median Gag-specific magnitude than chronic and PHI subjects (median Gag-specific magnitude = 3,570, 692, and 145 SFU/10^6 PBMCs for EC, chronics, and PHI subjects, respectively [EC versus chronics, $P = 0.025$; EC versus PHI subjects, $P = 0.0003$]; Fig. 2A). Intragroup analysis showed that anti-Gag magnitude was significantly higher (≈6-fold) in EC than Nef (median, 656 SFU/10^6 PBMCs; $P = 0.005$) and Env (median, 590 SFU/10^6 PBMCs; $P = 0.007$), while the anti-Nef magnitude in the PHI group (median, 308 SFU/10^6 PBMCs) tended to be higher than anti-Gag magnitude (median, 145 SFU/10^6 PBMCs; $P = 0.06$). Also in the PHI group, both anti-Nef and anti-Gag magnitude were significantly higher than anti-Env magnitude (median, 35 SFU/10^6 PBMCs; $P = 0.015$ and $P = 0.014$, respectively) (Fig. 2A).

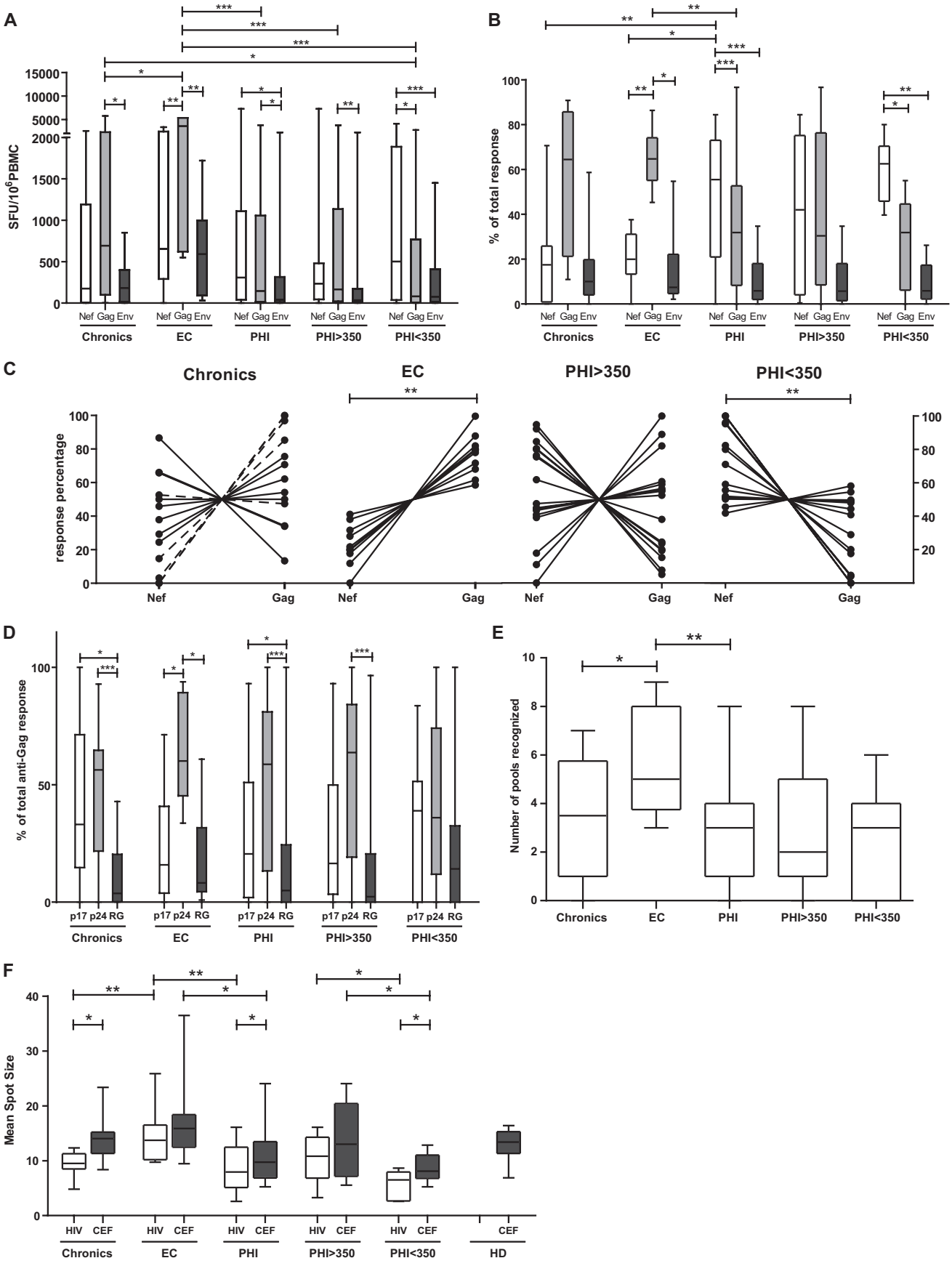
The relative contribution of each antigen to the total response, i.e., the proportion of cells specific for each protein relative to the total anti-HIV response, is an important factor to be taken into account when evaluating the association of HIV-specific immune response with viral control (11). Thus, the proportion of specific cells against each HIV antigen in relation to the total response (sum of the T-cell responses against the individual antigens) was analyzed (Fig. 2B). Again, the contribution of the different targets differed among the different study groups: PHI subjects showed a statistically higher proportion of Nef-specific cells (median, 55%) than chronics (median, 17%; $P = 0.005$) and EC (median, 20%; $P = 0.007$) and lower proportion of anti-Gag-specific cells (PHI, 32% versus EC, 64% [$P = 0.004$]; PHI versus chronics, 64% [$P > 0.05$]). In the same line, intragroup analysis revealed that anti-Gag response (median, 64%) clearly predominated over Nef (20%)- and Env (7.4%)-specific cells in EC (Gag versus Nef, $P = 0.005$; Gag versus Env, $P = 0.007$), whereas anti-Nef cells predominated in PHI (median, 55%) over Gag (32%, $P = 0.001$) and over Env (6%, $P < 0.001$). The chronic group showed the same immunodominance pattern (Gag > Nef > Env) than EC, although the response dispersion among chronics was much higher, which reflects the heterogeneity of this group. When analyzing separately the chronic subjects referred to as viremic controllers, it could be observed that the median percentage of Gag immunodominance in this chronic subgroup was 86%, considerably higher than that of regular chronics (43%).

When a similar analysis was performed within PHI subgroups, it could be observed that the scenario is clearly different between PHI > 350 and PHI < 350 groups: while no statistically significant difference could be observed between anti-Nef and anti-Gag magnitude or proportion of total response in the PHI > 350 group, anti-Nef cells clearly dominated the anti-HIV response in the PHI < 350 group (median magnitude, 500 SFU/10^6 PBMCs; median proportion of total response, 62%) over Gag and Env both in terms of magnitude (80 SFU/10^6 PBMCs [$P = 0.0125$] and 77 SFU/10^6 PBMCs [$P = 0.001$], respectively) and proportion (32% [$P = 0.01$] and 7% [$P = 0.003$], respectively) (Fig. 2A and B).

Given the significant role that anti-Nef and anti-Gag responses seem to play in defining the different rates of progression among groups, the contribution of anti-Nef and anti-Gag T-cell responses (relative to the sum of the magnitude obtained for both antigens) was analyzed per individuals included in the different groups (Fig. 2C). Anti-Gag responses clearly dominated over Nef in all EC ($P = 0.002$). On the opposite sense, anti-Nef responses dominated the anti-HIV response in the group of rapid progressors, PHI < 350 ($P = 0.008$). In chronics and the PHI > 350 group, an intermediate situation was observed: Gag dominated over Nef only in a subset of individuals, while the rest showed either Nef immunodominance or a balanced situation between both antigens. Among the chronic group, those with clear Gag immunodominance included those previously referred to as "viremic controllers" (C03, C05, C07, and C08).

It must be noted that the immunodominance hierarchy obtained with baseline samples was maintained when using samples obtained 1 year postinfection (data not shown), thus similar tendencies were observed when analysis was performed with the 12-month samples.

Then, a more detailed analysis of the Gag polyprotein subunits targeted by cells from the recruited individuals was performed. In all groups (chronic, EC, and PHI subjects as a whole), the immunodominance pattern (according to medians) was as follows: p24 > p17 > RG (Fig. 2D). Only in EC, the anti-Gag response was focused into p24 protein in a significantly higher proportion over both p17 and RG ($P = 0.047$ and 0.032, respectively). In chronics and PHI subjects, the proportion of anti-p24 response was significantly higher than the anti-RG response ($P = 0.0012$ and $P =$



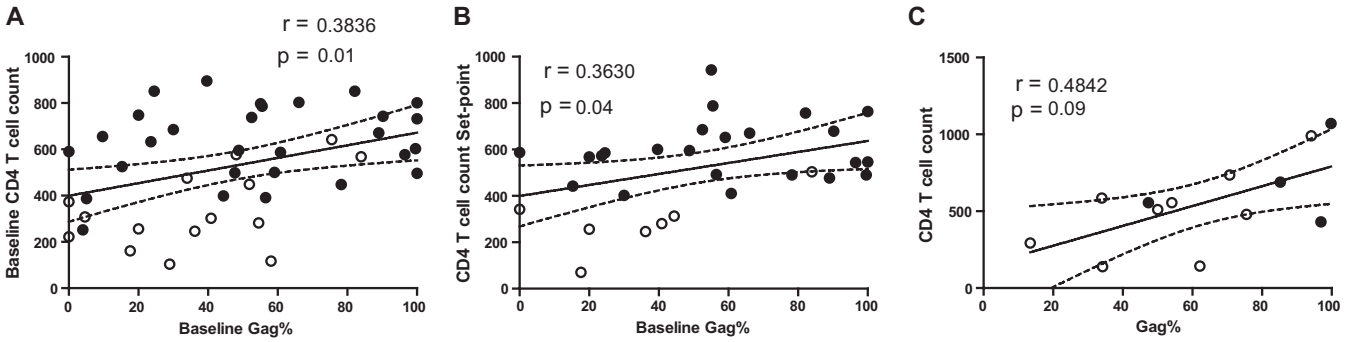


FIG 3 Correlation between the relative baseline immunodominance of anti-Gag response and $CD4^+$ T-cell counts. (A) Baseline $CD4^+$ T-cell count versus baseline percentage of anti-Gag response in PHI subjects. (B) Set point $CD4^+$ T-cell count versus baseline percentage of anti-Gag response in PHI subjects. (C) $CD4^+$ T-cell count versus percentage of anti-Gag response in chronics. In panels A and B, black and white dots denote PHI > 350 and PHI < 350 subjects, respectively. In panel C, black dots correspond to viremic controllers within the chronic group. All *r* and *P* values correspond to Spearman's test.

0.0021, respectively) and also tended to be higher than the anti-p17 response, although the difference was not statistically significant in either group. When PHI subjects were split into PHI > 350 and PHI < 350, it was observed that the PHI > 350 group maintained the immunodominance pattern observed before, whereas a shift to p17 > p24 > RG was observed in the PHI < 350 group.

(ii) Quality (in terms of breadth and intensity of IFN-γ production upon stimulation) of the HIV-specific cell immune response in PHI, chronic, and EC groups. Afterward, the breadth of the response was calculated for each individual as the number of peptide pools recognized (out of the 9 pools used to screen HIV-specific response) (Fig. 2E). This analysis revealed that EC were able to recognize a higher number of pools than chronics and PHI ($P = 0.05$ and 0.002, respectively). Even more, EC were able to recognize more Gag-specific pools ($n = 3$ pools) than chronics and the PHI group (not shown). No statistically significant difference was observed between the PHI < 350 and PHI > 350 groups.

Finally, the mean spot size obtained for both CEF and HIV peptide pools was recorded as the measure of the amount of IFN-γ produced by the individual specific T cells (which in turn correlated with the T-cell functional avidity) (33) (Fig. 2F). No intrasubject difference was observed among the different HIV-specific pools (Nef, Gag, and Env) (data not shown). Hence, for the purpose of this analysis, the mean spot size for all HIV antigens was averaged, taking into account only those pools for which a positive response was obtained in the ELISPOT assay on a subject-by-subject basis. Regarding the control CEF-specific spots, PHI subjects showed the smallest size being statistically different from EC (EC CEF-specific spots were 1.5 times larger than in the PHI group, $P = 0.029$), which in turn showed the largest CEF-specific spots, even slightly larger than healthy donors ($P > 0.05$). This difference in the PHI group was driven by PHI < 350 subjects who even differed substantially from their countergroup, PHI > 350 (spots from the PHI > 350 group doubled, on average, those of the PHI < 350 group). A similar scenario was observed for HIV-specific spots but, in this case, differences among groups were stronger. EC showed significantly larger spots than the chronic and PHI groups (1.5× [$P = 0.004$] and 1.7 × [$P = 0.002$], respectively). Within the PHI group, a significant difference could be observed between the PHI > 350 and PHI < 350 groups, showing the former group had larger HIV-specific spots (1.87×, $P = 0.022$). Interestingly, when CEF- versus HIV-specific spot size was analyzed intragroup, the HIV spots were smaller than CEF spots. In fact, the spot size difference between the two antigens reached statistical significance in chronics ($P = 0.036$), PHI as a whole ($P = 0.011$), and the PHI < 350 group ($P = 0.018$). Although the trend was also observed in EC and the PHI > 350 group, the breach between both antigens was less important, especially in EC.

(iii) In acute/early infected subjects (PHI group), baseline relative immunodominance of anti-Gag cellular response correlates with both baseline and set point $CD4^+$ T-cell counts. In Fig. 2C, it can be observed that the EC and PHI < 350 group (opposed groups in terms of disease progression) showed exactly opposite immunodominance patterns regarding Nef and Gag proteins. Based on this observation, it was hypothesized that in the much heterogenous chronic and PHI > 350 groups, Gag immunodominance correlates with slower disease progression. To test this hypothesis, correlation analyses were performed between the percentages of anti-Gag responses and clinical data of enrolled subjects. It was found that baseline relative Gag immunodomi-

FIG 2 ELISPOT screening of HIV-specific T-cell response magnitude, immunodominant targets, breadth, and functionality in primary HIV infection (PHI, baseline samples), chronic, and elite controller (EC) groups. (A) Magnitude of total anti-Nef, anti-Gag, and anti-Env T-cell responses, expressed as SFU/10^6 PBMC. (B) Contribution of each antigen relative to the total HIV response expressed as the percentage out of the total sum of the specific response (sum of the magnitude obtained for all Nef, Gag, and Env antigens). (C) Contribution of anti-Nef and anti-Gag responses (relative to the sum of the magnitude obtained for both antigens) on a subject-by-subject basis (each represented by a line) in chronic, EC, and PHI groups. "Viremic chronics" are denoted in the corresponding panel with dashed lines. (D) Gag subunits targeted per study group, expressed as percentage out of the total anti-Gag response. RG = p2p7p1p6. (E) Breadth of the response expressed as the number of peptide pools recognized out of the 9 HIV pools assayed. (F) Mean spot size obtained for both CEF and HIV peptide pools. HIV mean spot sizes represent the average mean spot sizes out of all Nef, Gag, and Env pools for which a positive response was obtained in the ELISPOT assay on a subject-by-subject basis. All data presented in the figure for PHI subjects correspond to baseline samples. PHI > 350 and PHI < 350 stand for subgroups within the PHI group in which subjects were segregated according to whether their $CD4^+$ T-cell count dropped below 350 cells/μl at any time during the first year of infection or not. Horizontal lines within boxes represent the median values. Intra- and intergroup differences were analyzed using Wilcoxon and Mann-Whitney tests, respectively. Asterisks denote different *P* values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

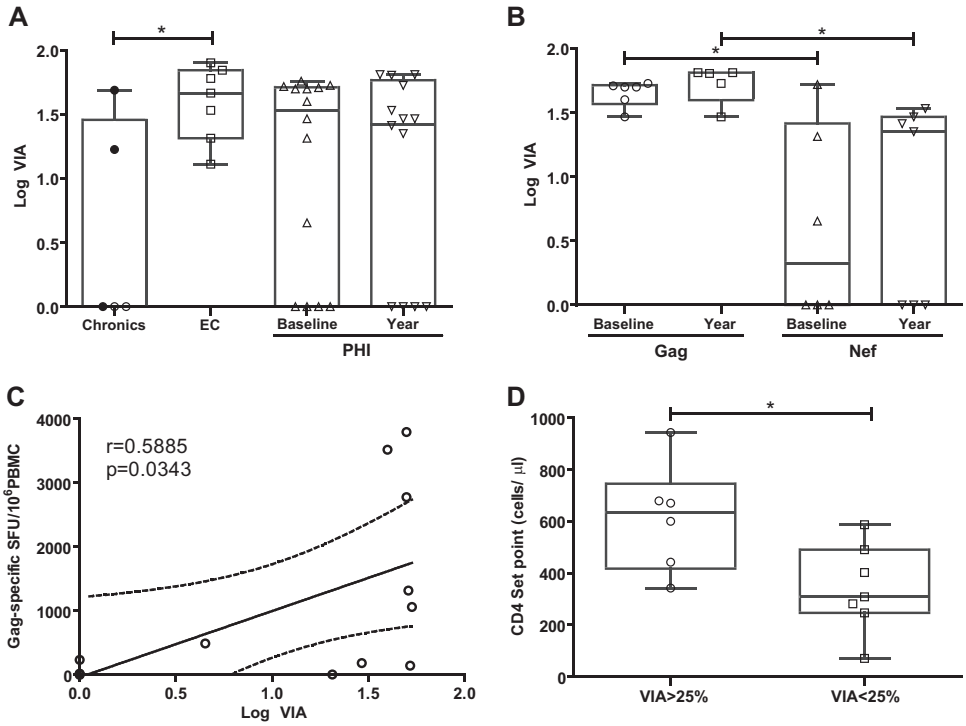


FIG 4 *In vitro* viral inhibitory activity (VIA) mediated by HIV-specific $CD8^+$ T cells. VIA is expressed as the $\log_{10}$ of the proportion of p24 antigen lost when $CD8^+$ T cells were present in the culture, compared to $CD4^+$ cells alone. (A) Viral inhibitory activity found in chronic, EC, and PHI subjects, against lab-adapted R5 HIV strains; (B) viral inhibitory activity in PHI individuals, reassorted into subjects with Gag immunodominance or Nef immunodominance according to preferred (>50% of total response) viral protein targeted by the immune response at baseline sample in the ELISPOT assay; (C) correlation between the magnitude of Gag-specific response in PHI subjects at baseline sample and VIA; *r* and *P* values correspond to Spearman's test; (D) PHI subjects with substantial capacity to mediate VIA (>25%) also showed higher $CD4^+$ T-cell set points. In panels A, B, and D, symbols show the values for each individual subject. Within the chronic group (A), viremic controllers are denoted with black dots. Horizontal lines within boxes represent the median values. Intergroup and intragroup (PHI baseline versus year) differences were analyzed using Mann-Whitney and Wilcoxon tests, respectively; *, $P < 0.05$.

nance in PHI subjects (baseline Gag%) significantly correlated with baseline $CD4^+$ T-cell counts (Spearman's $r = 0.3836$, $P = 0.01$; Fig. 3A). More interestingly, baseline Gag% also significantly correlated with set point $CD4^+$ T-cell count (Spearman's $r = 0.3630$, $P = 0.04$; Fig. 3B), indicating that Gag immunodominance is an important factor shaping disease progression in terms of $CD4^+$ T-cell loss. A similar association between these parameters was found within the chronic group, although the trend did not reach statistical significance, likely due to the smaller sample size (Spearman's $r = 0.4842$, $P = 0.09$; Fig. 3C).

All together, data collected so far demonstrate that although Nef dominates the anti-HIV response in acute/early infection, a higher proportion of early T-cell responses targeting preferentially Gag (in particular p24) and larger spot size (indicative of improved capacity to secrete IFN-γ and higher avidity) were associated with delayed progression during the first year of infection. Also, these same factors were observed in chronically infected elite controllers (EC), indicating that these T-cell features may play an important role in protection from progression. In particular, early Gag immunodominance correlated with higher $CD4^+$ T-cell counts, both at baseline and set point, further denoting it represents a key immune factor involved in delayed progression.

HIV-specific $CD8^+$ T cells elicited during PHI were capable of inhibiting heterologous viral replication *in vitro*, and the magnitude of this activity was related to Gag immunodominance. We next aimed to investigate whether HIV-specific $CD8^+$ T cells arising during acute/early HIV infection could suppress the replication of lab-adapted X4 and R5 HIV strains and to which extent, in comparison with chronic infection. It was also our aim to establish a possible relationship between this viral inhibitory activity (VIA) and the viral proteins targeted by the immune response during the early stage of infection. To evaluate this, $CD4^+$ and $CD8^+$ cells were isolated from PBMCs obtained from seven chronic subjects (including viremic controllers C03, C07, and C08), seven EC, and 19 PHI subjects (10 from the PHI > 350 group and 9 from the PHI < 350 group). In PHI subjects, VIA was evaluated at baseline samples as well as samples collected at 1 year postinfection. $CD4^+$ T cells were isolated, activated, infected, and then cultured either alone or in combination with autologous $CD8^+$ T cells at a 1:1 ratio. Viral replication was assayed at day seven postinfection by p24 ELISA. As previously described (16, 19, 20, 34, 35), $CD8^+$ T cells from EC mediated stronger VIA than cells from chronics against both R5 (Fig. 4A) and X4 (data not shown) viruses. Of note, EC equally inhibited both R5 and X4 viruses, indicating that these individuals had a broad VIA. It is worth noting here that the use of heterologous lab-adapted viral strains might lead to a VIA underestimation, and stronger differences among groups may be masked due to this reason (16). As for the PHI group, most subjects (70%) demonstrated to have $CD8^+$ T cells capable of mediating VIA (median, 32%, in p24 reduction, which corresponds to $1.5\log_{10}$ VIA) against the R5 virus at baseline, indicating that HIV-specific $CD8^+$ T cells able to mediate

VIA arise early during infection. Longitudinal analysis using samples obtained at 12 months postinfection indicated that this activity persists over time even beyond set point establishment (Fig. 4A).

When the PHI group was split into the PHI > 350 and PHI < 350 groups, no significant differences were observed in VIA either at baseline or 12-month samples. Given our observation obtained from the ELISPOT analysis, where Gag immunodominance during PHI was associated with preservation of the $CD4^+$ T-cell count over the first year postinfection, we decided to reassort PHI individuals into Gag responders or Nef responders according to preferred (>50% of total response) viral protein target. This analysis revealed that $CD8^+$ cells from PHI individuals in which Gag-specific cells dominated the early HIV-specific T-cell response had a stronger capacity to mediate VIA against the R5 virus than Nef responders, both at baseline ($P = 0.043$) and at the 12-month samples ($P = 0.011$) (Fig. 4B). This result agrees with the fact that EC consistently show the strongest VIA compared to other HIV^+ groups (in this and other studies) and concomitantly show a robust anti-Gag immunodominance of the HIV-specific cellular immune response. In the same line, a statistically significant correlation was found within the PHI group between VIA and the magnitude of Gag-specific SFU (Spearman's $r = 0.5885$, $P = 0.0343$; Fig. 4C). Overall, these results suggest that the ability of HIV-specific $CD8^+$ T cells arising early during infection to suppress viral replication in autologous $CD4^+$ T cells might be related, among other factors, to the relative Gag immunodominance out of the total HIV-specific $CD8^+$ T-cell response as well as the absolute number of Gag-specific $CD8^+$ T cells. Also, it was observed that EC and PHI Gag responders more frequently showed VIA against both R5 and X4 viruses, while chronic and PHI Nef responders recognized one, other, or no viruses, suggesting that the former groups have broader activity (data not shown).

Given the consistent association of higher VIA with EC status observed in this and other studies (16, 19, 20, 34, 35), we decided to seek for any association of stronger VIA with clinical parameters related to acute/early infection. No association was observed between VIA magnitude and baseline viral load, viral set point, or baseline $CD4^+$ T-cell count. However, it could be observed that those subjects showing substantial capacity to mediate VIA (>25%) had higher $CD4^+$ T-cell set points ($P = 0.02$, Fig. 4D), suggesting that the association of Gag immunodominance with delayed progression in terms of $CD4^+$ T-cell count preservation described earlier in the manuscript (Fig. 3) would be related to a higher capacity of Gag-specific cells to mediate VIA.

Low frequency of polyfunctional HIV-specific $CD8^+$ T cells was detected during PHI; it slightly increased over time during the first year postinfection but did not associate with viral or $CD4^+$ T-cell set points. In order to further characterize HIV-specific $CD8^+$ T cells arising early during infection, the functionality of these cells, in terms of their ability to degranulate (evidenced by CD107A/B mobilization) and to produce IFN-γ, TNF-α, and/or IL-2 upon peptide stimulation, was studied by flow cytometry. For these assays, data obtained in the ELISPOT screening was used as a starting point to define which peptide pools were used as stimuli. So for each subject, only those peptide pools for which positive responses were found by ELISPOT were used as stimuli in the ICS assay. The gating strategy used is illustrated in Fig. 5A. The proportion of cells expressing each of these functions alone or in combination was analyzed, not only in PHI but also in chronic and EC subjects, in order to help distinguish any association with disease progression.

As previously reported by our group (25), HIV-specific $CD8^+$ T cells expressing all the functions analyzed (either alone or in combination) could be measured very early during infection. Among PHI baseline samples, mean ± SD percentages of HIV-specific $CD3^+$ $CD8^+$ cells for each single function were 0.16 ± 0.28 for IFN-γ, 1.13 ± 5.23 for TNF-α, 0.06 ± 0.14 for IL-2, and 0.21 ± 0.4 for CD107. Figure 5B depicts the relative contribution to the total specific $CD8^+$ response made by each function or function combination. No particular function or function combination could be distinguished that differed substantially among PHI, EC, or chronic subjects, except for bifunctional $CD107A/B^+$ $IFN\text{-}\gamma^+$ $CD8^+$ T cells. All EC individuals had detectable bifunctional $CD107A/B^+$ $IFN\text{-}\gamma^+$ $CD8^+$ T cells (mean, 30% ± 11% out of the specific $CD8^+$ T cells), differing, in this sense, significantly from chronic (13% ± 23%; $P = 0.007$) and PHI individuals (9% ± 14%; $P = 0.0004$), indicating that this sub-subset of specific $CD8^+$ T cells could be relevant to virus control. Then, the proportion of cells expressing one, two, or three functions was studied among groups independently of any particular function. As expected, the proportion of polyfunctional cells was greater in EC than in chronic and PHI subjects (Fig. 5C). In particular, EC had a significantly higher proportion of trifunctional cells (mean, 8.1% ± 7%) and significantly lower proportion of monofunctional cells (mean, 46% ± 19%) than chronic subjects (mean, 2.7% ± 9% [$P = 0.013$] and 68% ± 27% [$P = 0.033$], respectively). As mentioned above, bifunctional and trifunctional cells could be measured very early in PHI, together accounting for 25.5% of the HIV-specific $CD8^+$ pool in these subjects. Moreover, the proportion of these cells seemed to slightly increase over time to the 12-month sample (32.3%), although the difference between the baseline and year samples did not differ significantly. Within the PHI group, no significant differences were observed in any particular function or function combination when individuals were segregated into PHI > 350 and PHI < 350 groups. Similarly, no difference was observed between both groups in the proportion of mono-, bi-, or trifunctional cells. Also, no significant correlation was observed for any function, combination, or proportion either with baseline or set point VL or $CD4^+$ T-cell count (data not shown).

It was then hypothesized that the stronger $CD8^+$ T-cell VIA found in PHI subjects in whom Gag dominated the cellular immune response, as well as the slower progression observed within this subgroup, could be related to higher polyfunctionality of Gag-specific cells. Consequently, the proportion of Gag- and Nef-specific $CD8^+$ T cells expressing one, two, or three functions was compared within the PHI group at baseline and 12-month samples (Fig. 5D). No differences were observed for both antigens, indicating that polyfunctionality was not related to specificity. Moreover, both Gag- and Nef-specific responses showed a nonstatistically significant increase in polyfunctionality over time.

Overall, these results suggest that although a higher proportion of polyfunctional $CD8^+$ T cells is associated to EC status and even when these cells could be measured very early in HIV infection, they would not be associated with better or worse resolution of acute phase. Also, the benefits of Gag-specific cells would not be related to polyfunctionality in the terms analyzed here.

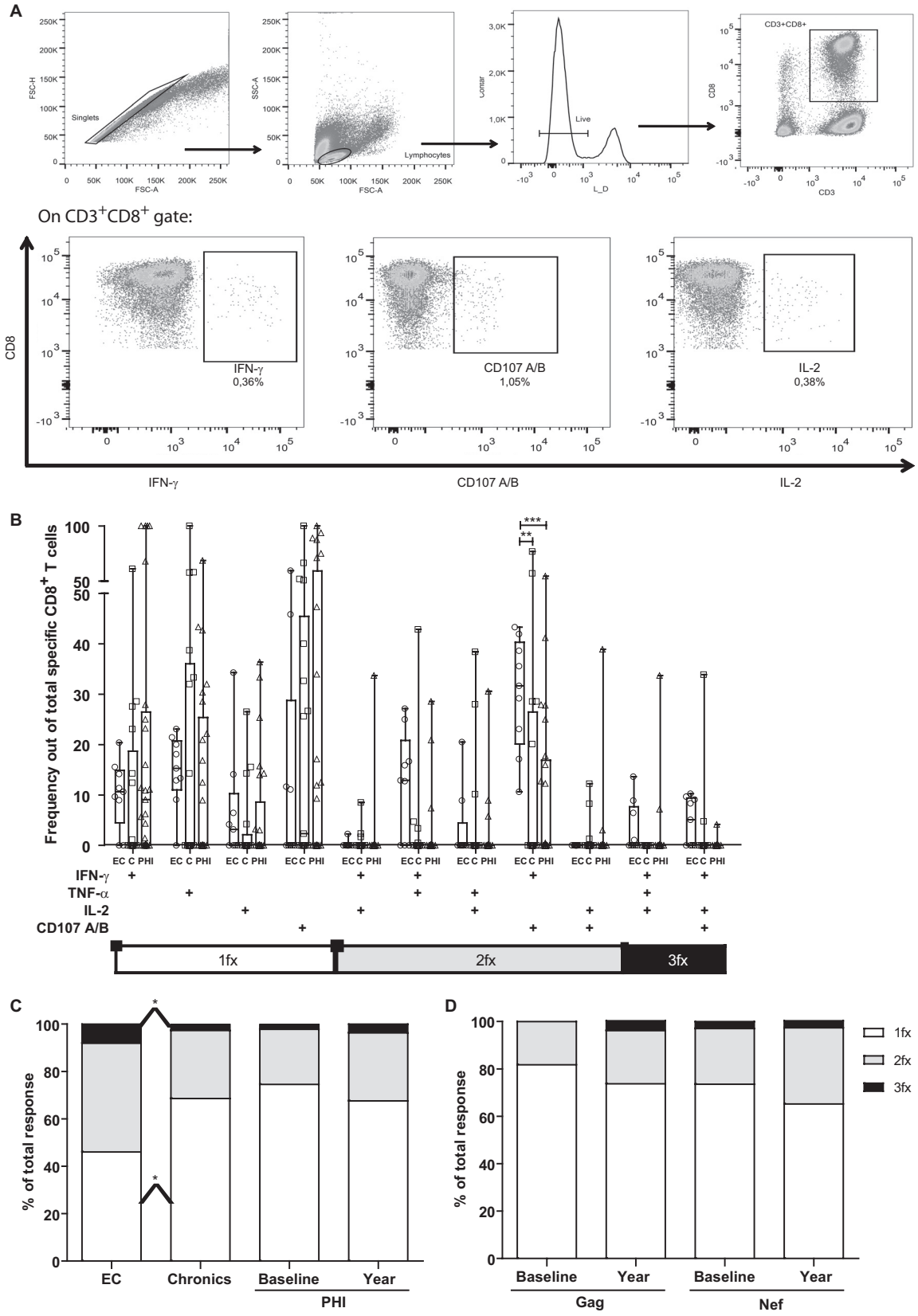


FIG 5 (A) Gating strategy used for the identification of degranulating and cytokine-secreting HIV-specific T cells. Illustration data were derived from one representative subject, stimulated with an HIV peptide pool. Initial gating was performed on a plot of forward scatter area (FSC-A) versus height (FSC-H) to remove doublets. Then, gating was performed on small lymphocytes in a plot of forward scatter (FSC) versus side scatter (SSC). Dead cells were then excluded

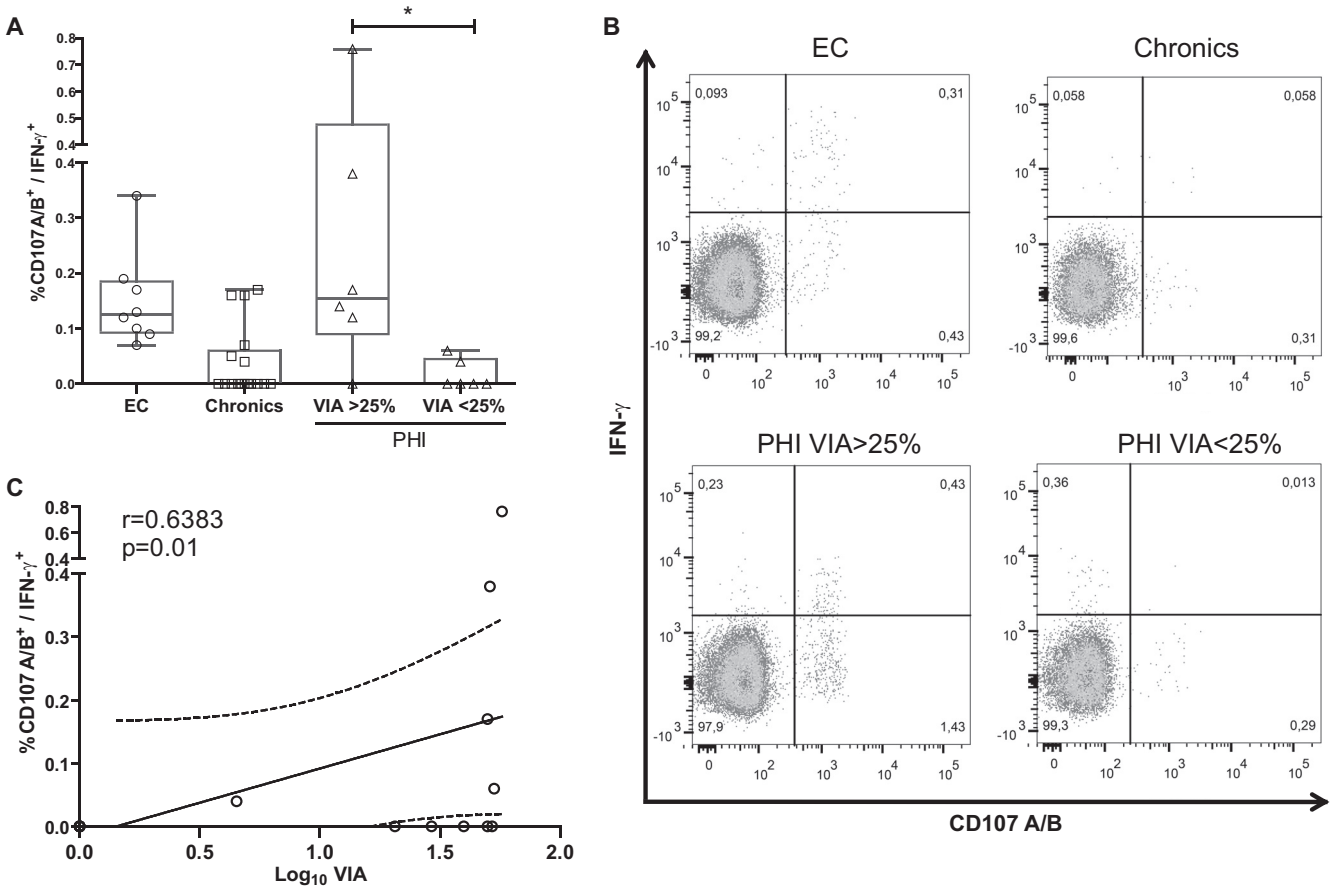


FIG 6 Association between the $CD8^+$ T-cell capacity to suppress HIV replication *in vitro* and the frequency of HIV-specific T cells able to degranulate and secrete IFN-γ^+ upon stimulation. (A) Frequency of HIV-specific CD107A/B^+ IFN-γ^+ $CD8^+$ T cells in EC, chronic, and PHI subjects (segregated according to $CD8^+$ T-cell VIA magnitude). *, $P < 0.05$ Mann-Whitney test. (B) Representative IFN-γ^+ versus CD107A/B^+ dot plots (gated on $CD3^+CD8^+$ events) obtained for subjects of each group. (C) Correlation between the frequency of HIV-specific CD107A/B^+ IFN-γ^+ $CD8^+$ T cells and VIA in PHI subjects at baseline sample. *r* and *P* values correspond to Spearman's test.

A higher proportion of HIV-specific $CD8^+$ T cells able to degranulate and secrete IFN-γ was associated with improved capacity to suppress viral replication during PHI. As mentioned before, out of the multiple $CD8^+$ T-cell effector functions studied by flow cytometry, the combined expression of CD107A/B and IFN-γ was significantly associated with EC status (Fig. 5B). Given this, it was reasoned that the particular combination of these two functions (instead of polyfunctionality as a whole) would be associated with improved resolution of acute infection. Thus, associations among percentages of specific CD107A/B^+ IFN-γ^+ $CD8^+$ T cells and virological and immunological parameters (all corresponding to baseline samples) were analyzed. It was found that, within PHI subjects, those showing substantial $CD8^+$ T-cell VIA (>25%) had higher numbers of CD107A/B^+ IFN-γ^+ $CD8^+$ T cells than those having weak (<25%) VIA ($P = 0.025$). Even more, the percentage of CD107A/B^+ IFN-γ^+ $CD8^+$ T cells in the PHI VIA > 25% group was comparable to that of EC (Fig. 6A and B). Moreover, a significant correlation between the percentage of specific CD107A/B^+ IFN-γ^+ $CD8^+$ T cells and the CD8 T-cell VIA was found for PHI subjects (Spearman's $r = 0.6383$, $P = 0.01$; Fig. 6C).

These observations indicate that the capacity to degranulate and express IFN-γ might play a relevant role in the ability of specific cells to mediate VIA (i.e., to suppress viral replication *in*

on the bases of LIVE/DEAD fluorescence. Subsequently, $CD3^+CD8^+$ cells were gated in a CD3-versus-CD8 dot plot. Following identification of these cells, a gate was made for each function studied: degranulation (evidenced as CD107A/B surface expression) and production of IFN-γ, IL-2, and TNF-α. (B) Relative contribution to the total specific $CD8^+$ response made by each function or function combination in PHI (baseline sample), chronic, and EC subjects. Symbols represent the percentage out of the total specific $CD8^+$ T cells expressing the particular combination of functions indicated on the axis, for each subject. Responses shown correspond to background-subtracted results using the CD28/49d control. Values corresponding to bi- and monofunctional cells were corrected by subtracting the values corresponding to triple-positive events and double- and triple-positive events, respectively. Horizontal lines within boxes represent the median values. (C) Summary of functional profile in chronic, EC, and PHI subjects (baseline and year samples in the case of the PHI group). The distinct cellular subsets shown in panel B were grouped by number of functions, so each section of the bars represent, the mean proportion of specific $CD8^+$ T cells expressing one (white), two (gray), or three (black) functions, independently of any particular function, matching the color code used in panel B. (D) Results corresponding to PHI subjects, using either Gag or Nef peptide pools as stimuli. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

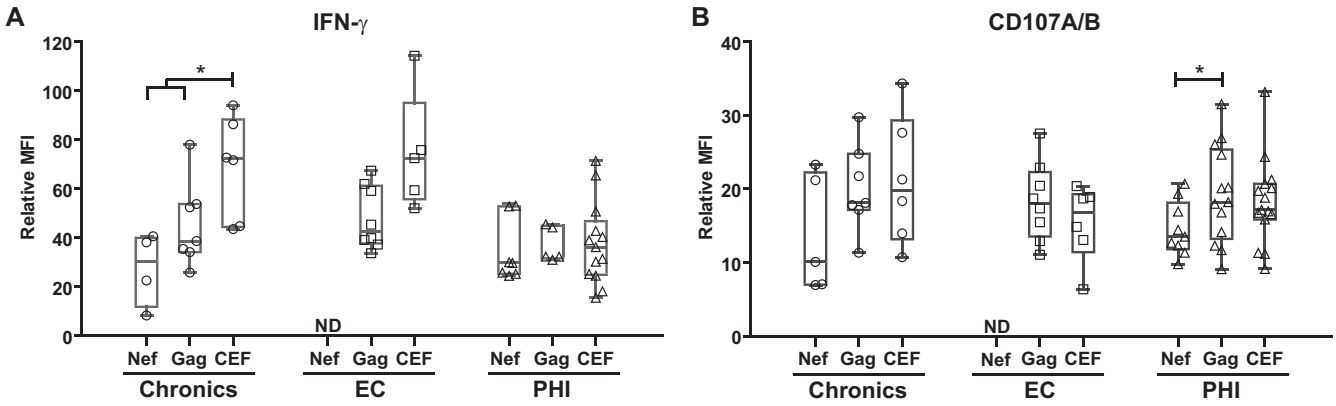


FIG 7 Relative mean fluorescence intensity (rMFI) of IFN-γ (A) and CD107A/B (B) in Nef-, Gag-, and CEF-specific $CD8^+$ T cells, calculated as the ratio between MFI corresponding to specific versus total $CD8^+$ T cells for a given channel, in chronic, EC, and PHI subjects. Symbols show the values for each individual subject. Horizontal lines within boxes represent the median values. *, $P < 0.05$; ND, not determined.

vitro), which in turn associated both with the preservation of the $CD4^+$ T-cell subset during acute/early infection and the status of EC. On these bases, the subpopulation of specific $CD107A/B^+$ $CD8^+$ T cells was subjected to a deeper analysis. This analysis revealed that, out of the total $CD107A/B^+$ HIV-specific $CD8^+$ cells, most of them coexpressed IFN-γ and/or IL-2 in the EC cohort, i.e., most degranulating HIV-specific $CD8^+$ T cells from EC were bi- or trifunctional. Of note, all EC had measurable HIV-specific $CD107A/B^+$ IFN-γ^+ $CD8^+$ T cells, and around 60% of subjects had measurable HIV-specific $CD107A/B^+$ IL-2^+ $CD8^+$ T cells. On the contrary, only a minor portion of chronic and PHI subjects exhibited polyfunctional HIV-specific $CD107A/B^+$ $CD8^+$ T cells. When a similar analysis was performed but considering response specificity, it could be observed that Gag-specific $CD107A/B^+$ $CD8^+$ T cells showed higher levels of polyfunctionality than Nef-specific cells in chronics, even comparable to Gag-specific cells from EC. However, no difference was observed between Nef- and Gag-specific $CD8^+$ T cells in terms of polyfunctionality associated to degranulation capacity in the PHI group, either at baseline or 12-month samples (not shown).

Finally, the relative mean fluorescence intensity (rMFI) of IFN-γ and CD107A/B was studied in specific $CD8^+$ T cells as an indicator of cell function strength (Fig. 7). IFN-γ rMFI analysis (Fig. 7A) produced results that paralleled those obtained after the examination of spot sizes in the ELISPOT assays. CEF-specific cells showed 1.7-fold-higher (1.7×) IFN-γ rMFI than HIV-specific cells in the chronic ($P = 0.03$) and EC ($P > 0.05$) groups but not in the PHI group. As for CD107 expression, no difference was observed among groups regarding CEF-specific cells (Fig. 7B). When CD107A/B rMFI was compared intragroup, it was found that Gag-specific cells showed higher CD107A/B rMFI than Nef-specific cells both in chronic (1.7×, $P > 0.05$) and PHI subjects (1.4×, $P = 0.033$). Even more, CD107A/B rMFI for Gag-specific cells was comparable to that of CEF-specific cells in all three groups of subjects analyzed. When the same analysis was performed but gated on double-positive $CD107A/B^+$ IFN-γ^+ $CD8^+$ T cells, it could be observed that rMFI was higher for each function, as already reported for polyfunctional cells (36), and the same trends shown in Fig. 7 were maintained. Even more, the differences on CD107A/B expression between Nef- versus Gag-specific cells became more pronounced. However, this observation was derived from only a minor subset of patients and a small number of events (especially for chronic and PHI subjects) in order to draw definite statements.

In summary, EC showed a higher number of degranulating HIV-specific $CD8^+$ cells than chronic and PHI subjects. Even more, in these subjects, this activity was more frequently accompanied by the capacity to secrete cytokines (IFN-γ and/or IL-2), while in the chronic and PHI groups, these cells were mostly monofunctional. Within PHI, a direct correlation between the percentage of HIV-specific $CD107A/B^+$ IFN-γ^+ $CD8^+$ T cells and the magnitude of $CD8^+$ T-cell VIA was found. Finally, Gag specificity was associated with a stronger ability to degranulate (evidenced by higher CD107 rMFI), which seems consistent with these cells having a stronger capacity to mediate viral inhibition.

Plasma levels of MIP-1β and IL-2 are associated with Gag immunodominance. Cytokines play a key role in many infectious diseases, both shaping the immune response mounted against pathogens and contributing to pathogenesis. Several reports have established that plasma cytokine levels measured during acute HIV infection may predict subsequent disease progression (37–39). In this scenario, we sought to analyze the relationship between plasma cytokine levels during acute/early HIV infection and the Gag or Nef immunodominance hierarchy obtained also at PHI baseline samples. This was performed with the aim of determining if there existed an association between both soluble and cellular immune signatures associated with protection from disease progression and following the hypothesis that if two immune parameters are truly determinants of protective immunity, then a direct association between them should be observed. It was found that the percentage of Gag-specific cells out of the total HIV-specific cells (baseline Gag%) significantly correlated in a direct fashion with plasma levels of MIP-1β and IL-2, among PHI subjects at baseline samples (Fig. 8A and B). Although the correlation between plasma MIP-1β and baseline Gag% was not significant after the adjusted analysis, the clear trend indicating a direct association between both parameters is still worth noting. Conversely, the proportion of Nef-specific cells inversely correlated with plasma MIP-1β (Fig. 8C). These findings are of particular importance, since these molecules are produced by $CD8^+$ T cells upon stimulation and the percentage of cells expressing IL-2 and MIP-1β either alone or in combination with other functions were previ-

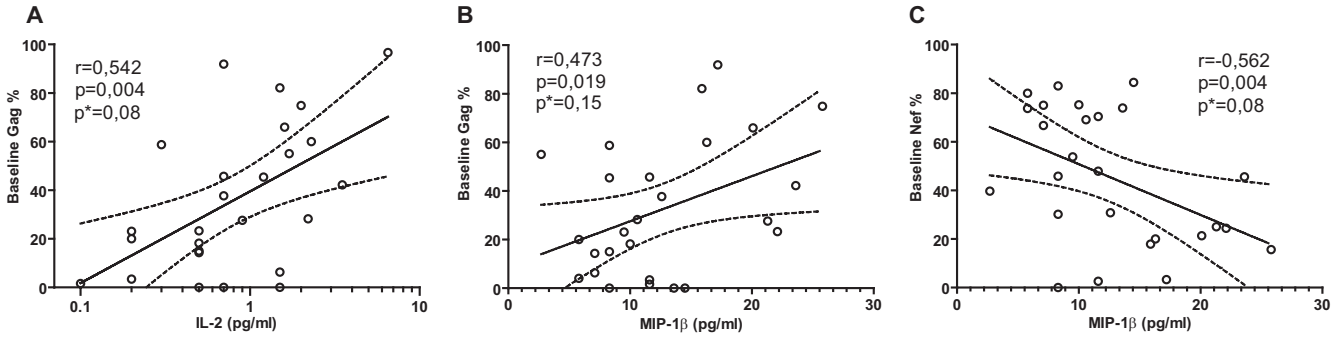


FIG 8 Correlation between anti-Gag and anti-Nef cellular immune response immunodominance hierarchy and plasma cytokine/chemokine levels in PHI subjects at baseline samples. Significant correlations were found between the relative immunodominance of Gag (A and B) and Nef (C) with plasma IL-2 (A) and MIP-1β (B and C) levels. *r* and *P* values correspond to Spearman's test. *, *P* values adjusted by the Benjamini and Hochberg method for false discovery rate (FDR) procedure.

ously associated with virus control (6, 17, 18, 20, 40). Moreover, soluble MIP-1β was also reported to mediate viral inhibition *in vitro* (41). The fact that there exists an association between important soluble antiviral mediators such as IL-2 and MIP-1β and Gag immunodominance is in line with the evidence indicating that early relative Gag immunodominance is somehow related to the generation of a robust, efficient, and multifaceted antiviral immune response, thus reinforcing the notion that it is one key determinant of protective immunity contributing to slower disease progression.

DISCUSSION

Data accumulated over the past years, based on both experimental infections in nonhuman primate models and natural infections in humans, have established that the HIV-specific $CD8^+$ T-cell response plays a critical role on viral control (reviewed in references 2 and 3). This is particularly evident during acute infection, where the up-slope of arising HIV-specific $CD8^+$ T cells is temporarily associated with the decline in the initial peak viremia (4). For this reason, efforts have been made to understand which particular functions and/or phenotypes, out of the global $CD8^+$ T-cell subset, best correlate with control of viral replication (3). Moreover, it is thought that this information will be instrumental for developing and enhancing immunization strategies as well as for defining immune correlates of protection, which are currently lacking, in order to evaluate the performance of vaccine candidates. In line with this, cohort studies addressing the association of particular features of $CD8^+$ T-cell responses arising during acute/early HIV infection with potential markers associated with disease progression are fundamental. To date, most of these studies come from cohorts settled in Europe, North America, or Africa, and scarce information exists on this issue from other settings, such as South America. Here, we report the study of multiple functional aspects of the HIV-specific $CD8^+$ T-cell compartment arising early after infection in a subset of subjects from a well-characterized cohort of Argentinean seroconverters, in comparison to that found in, also local, viremic chronics and elite controllers. Epidemiological, clinical, immunological, and virological characteristics of the patients enrolled in the cohort are described elsewhere (26). Aiming at delineating $CD8^+$ T-cell features that best associate with disease progression in our population, it was found that (i) there exist early differences regarding the immunodominant viral targets of the specific T-cell response between subjects with different rates of progression to disease (in terms of $CD4^+$ T cell loss) during the first year of infection (Fig. 2 and 3), (ii) polyfunctional HIV-specific $CD8^+$ T cells could be detected during PHI but their frequency was not associated with virus control or protection from progression (Fig. 5), (iii) $CD8^+$ T cells capable of mediating viral inhibitory activity (VIA) *in vitro* could also be detected during PHI (Fig. 4), and (iv) the improved capacity to mediate VIA during PHI was associated with a higher $CD4^+$ T-cell set point and was related to Gag immunodominance and a higher proportion of HIV-specific $CD8^+$ T cells able to degranulate and secrete IFN-γ (Fig. 4 and 6). The main contribution of this study relies on the correlation between the HIV-specific $CD8^+$ T-cell functional properties during acute/early infection and the clinical outcomes during the first year postinfection. On the other hand, to our knowledge, this is the first report to perform an immunological characterization of the T-cell responses in a cohort of acute/early HIV-infected subjects from South America.

Clear differences in the viral proteins that are targeted by the HIV-specific cellular response have been described between the acute/early and chronic phases of infection: while Nef-specific cells dominate the early antiviral response (42), this response afterward broadens toward epitopes contained within other viral proteins, such as Gag, Env, and Vpr (11–15). Many of these studies have also established that Gag-specific $CD8^+$ T-cell responses are associated with low viremia in chronic infection. Other reports indicate that Gag is the immunodominant target in elite controllers (43), both in blood and mucosal tissues (44), providing further support to the important role of Gag-specific cells in restricting viral replication. Possible mechanisms to explain this better ability to control viral replication include ability to kill very recently infected cells (even before viral genome integration to host genome) (45, 46), Gag fitness constraints to escape immune pressure (47, 48), and higher capacity to mediate antiviral activity, which will be discussed in the following paragraphs. In line with these findings, we found that Gag-specific cells (particularly p24-specific cells) dominated the HIV-specific response in EC, both in terms of magnitude and hierarchy (Fig. 2A and B). Also, Gag immunodominance was observed in a subgroup of chronically infected subjects referred to as "viremic controllers." On the contrary, Nef-specific cells dominated the response in PHI subjects. However, when this group was split into PHI $>$ 350 and PHI $<$ 350 groups, it was observed that in the group with more rapid

progression (PHI < 350), Nef clearly dominated the anti-HIV response (Fig. 2A to C). More importantly, significant correlations between baseline Gag immunodominance and both baseline and set point $CD4^+$ T-cell counts were observed in the PHI group (Fig. 3). In line with this result, other authors described a direct correlation between the magnitude of the anti-Nef $CD8^+$ T-cell response and viral load in a cohort of HIV subtype C acutely infected subjects (49). Overall, these results argue in favor of that an earlier and higher contribution of Gag-specific cells to the hierarchy of the total anti-HIV response at very early time points postinfection contributes to a slower disease progression.

The ability of $CD8^+$ T cells to kill virus-infected cells may rely in their specificity, phenotype, and/or functionality and could even be mediated by distinct mechanisms (mediated by soluble factors or cell-to-cell contact). The evaluation of the *ex vivo* virus inhibitory activity (VIA) results in an overall measurement of the total $CD8^+$ T-cell antiviral potency. Previous reports have used this assay to evaluate VIA mediated by $CD8^+$ T cells obtained from elite controllers, treated and untreated chronically infected individuals (16, 19, 20, 34, 35), as well as human and simian vaccinees (20, 50). In these studies, it was demonstrated that VIA correlates with the magnitude of the Gag-specific $CD8^+$ T-cell response (16, 35), that the expression of "protective" HLA-I alleles is not a required condition (20, 35), and that it is associated with higher frequencies of degranulating (as measured by CD107A/B expression) specific $CD8^+$ T cells, usually accompanied by other functions, such as secretion of IFN-γ and MIP-1β (20, 35, 50). In agreement with this background, we found that $CD8^+$ T cells from EC mediated stronger and broader VIA than those cells from untreated chronic subjects. Additionally, we also demonstrate in this work that VIA can be measured very early after infection in most acutely infected subjects (although to a lower magnitude than EC) and that this activity persists over time (Fig. 4). To our knowledge, there exists only one very recent report studying VIA in acute HIV infection (41). In that work, Freel and collaborators also demonstrate (i) that VIA magnitude correlates with the percentage of HIV-specific $CD8^+$ T cells expressing CD107A, MIP-1β, and IFN-γ and with the secretion of MIP-1α, MIP-1β, IFN-γ, IP-10, and IL-1α after cell stimulation with peptide antigens and (ii) that early escape from $CD8^+$ T-cell-mediated VIA occurs during PHI. In our work, further insights are provided into the role of $CD8^+$ T-cell-mediated VIA during PHI. Aiming at delineating the specificity and functionality of VIA-mediating cells during PHI, it could be established that $CD8^+$ cells from PHI individuals in whom Gag-specific cells dominated the early HIV-specific T-cell response had stronger capacity to mediate VIA than Nef responders, and a statistically significant direct correlation was found within the PHI group between VIA and the magnitude of Gag-specific cellular immune response (Fig. 4). This is in line with results obtained for EC and chronic subjects with broad anti-Gag responses (16, 35) and could provide an additional mechanistic explanation for the pivotal role of Gag-specific cells in delayed disease progression. Although Freel et al. (41) found that Nef-specific cells could mediate VIA to the same extent as Gag-specific cells in acutely infected subjects, no association between these activities and clinical parameters was studied by these authors, as it is the case of the present work. Also, it is important to denote that both studies differ in definition of the specificity of VIA-mediating cells, which may account for the different results obtained. Regarding the functionality of VIA-mediating cells, we found that, within PHI subjects, those showing substantial $CD8^+$ T-cell VIA (>25%) had a higher frequency of $CD107A/B^+$ IFN-γ^+ $CD8^+$ T cells (even comparable to EC) than those having weaker VIA. Moreover, a significant correlation between the percentage of specific $CD107A/B^+$ IFN-γ^+ $CD8^+$ T cells and the $CD8^+$ T-cell VIA was found for PHI subjects (Fig. 6). In regard to the expression of CD107A/B, this result is in line with previous data obtained for EC, chronic, acute/early infected subjects and simian vaccinees (35, 41, 50). However, in our study, the simultaneous coexpression of both markers (CD107A/B and IFN-γ) is necessary to maintain the association. Other groups reported that MIP-1β secretion is also associated with VIA both in chronic and acute infection (20, 41). Because of technical constrains, we could not include the evaluation of this cytokine in our multicolor flow cytometry assay. However, it is presumable that a similar association would have been found due to the high contribution of MIP-1β to the total response during acute infection and its high rate of coexpression with CD107A/B and IFN-γ (6, 25). What we did observed was a nonsignificant yet border correlation between plasma MIP-1β (see further discussion below) and the proportion of bifunctional degranulating cells, i.e., the proportion of $CD107^+$ IFN-γ^+ plus $CD107^+$ IL-2^+ HIV-specific $CD8^+$ T cells out of the total $CD107^+$ HIV-specific $CD8^+$ T cells (Spearman's $r = 0.4368$, $P = 0.0699$; data not shown), suggesting an association between VIA, degranulation, and expression of MIP-1β. Finally, as it has been documented for chronic infection (35), no association was found between VIA magnitude and viral load. However, we could observe an association between VIA and the $CD4^+$ T-cell set point (Fig. 4D). This result, together with the higher VIA observed in EC (in our and other studies mentioned above) and an observation made in vaccinated rhesus monkeys where strong VIA was related to enhanced virus control in breakthrough infections (50), clearly indicates that $CD8^+$ T-cell-mediated VIA is a desirable feature to be elicited by prophylactic or therapeutic interventions aimed at delaying disease progression.

Early reports comparing the capacity of $CD8^+$ T cells to degranulate and secrete multiple soluble mediators upon stimulation, between individuals with progressive versus long-term nonprogressive HIV infection, have suggested that $CD8^+$ T-cell polyfunctionality would be a functional correlate of virus control (17, 18). However, these studies also raised the question of whether polyfunctionality was not the cause but the consequence of virus control. Results presented here argued in favor of the latter hypothesis: in our PHI cohort, polyfunctional HIV-specific $CD8^+$ T cells could be measured very early during infection (in consonance with our previous observation [25]), and the proportion of these cells seemed to slightly increase over time to the 12-month sample (Fig. 5), as also reported by Ferrari et al. (6). Although significant differences in the proportion of polyfunctional cells were observed in EC versus viremic chronic subjects (as expected, based on previous reports [17, 51]), no significant differences were observed when PHI individuals were segregated as rapid (PHI < 350) or regular (PHI > 350) progressors. Moreover, polyfunctional profiles were similar in PHI individuals with different immunodominant targets, indicating that polyfunctionality was not related to specificity (Fig. 5). These findings shed more light into the notion that, instead of being a marker of antiviral function, T-cell polyfunctionality would be directly affected by viral persistence. In other words, lack of T-cell polyfunctionality would be the consequence of constant antigen stimulation dur-

ing viremic chronic infection, which ultimately may lead to cell exhaustion and functional impairment, as postulated previously (6, 21). In this sense, $CD8^+$ T-cell phenotypic characterization in terms of exhaustion and memory markers in our cohort is being currently performed. On the other hand, the demonstration that, out of the many $CD8^+$ T-cell function combinations studied, only the frequency of specific bifunctional CD107A/B^+ IFN-γ^+ $CD8^+$ T cells correlated with the magnitude of $CD8^+$ T-cell VIA (Fig. 6) would indicate that not all the functions exerted by a polyfunctional cell would be equally relevant in mediating antiviral T-cell effector functions.

T-cell functional outcomes upon antigen engagement depend on the strength of the stimulus, which can be directly affected by the level of antigen sensitivity or cell avidity (52, 53). These parameters, in turn, can be indirectly inferred by computing the mean spot size in the IFN-γ ELISPOT assay (33) or the rMFI of a given function evaluated by flow cytometry (52). Here, we found that the analysis of IFN-γ mean spot size and IFN-γ rMFI gave comparable results, together indicating that HIV-specific cells showed less quality in all groups analyzed compared to CEF-specific cells (in EC the difference was not significant) (Fig. 2F and 7). CEF-specific cells were analyzed for comparison since they represent cells that target pathogens that were either cleared (influenza) or controlled (CMV and Epstein-Bar viruses), thus their analysis could provide putative signatures associated with virus control. This result suggests again that constant HIV antigen stimulation may be driving cell exhaustion. Moreover, and regardless of the specificity, cells from PHI subjects showed the lowest quality among all the groups analyzed, suggesting that, during acute/early HIV infection, there exists an overall deterioration of the $CD8^+$ T-cell compartment. In regard to CD107A/B mobilization, it was found that rMFI for Gag-specific cells was higher than for Nef-specific cells and comparable to that of CEF-specific cells in all three groups of subjects analyzed (Fig. 7). A relation among the magnitude of CD107A/B mobilization (evaluated by flow cytometry as in this work), antigen sensitivity, and antiviral activity has previously been established in another setting (52), supporting our association between the stronger ability of Gag-specific cells to degranulate and their stronger capacity to mediate viral inhibition.

A large amount of data indicate that the expression of certain HLA-I molecules is related to HIV control. Part of the evidence came from cohort studies where some alleles were overrepresented in EC and long-term nonprogressors (7, 8), while other studies provided insights into functional performance of the "protective alleles" regarding specificity, proliferation, polyfunctionality, antigen presentation, immune pressure, among other factors (8, 54–61). However, it has also been demonstrated that these protective alleles perform qualitatively different in controllers compared to progressors (60), indicating that a combination of T-cell function and phenotypes should coexist in order to achieve virus control. Conversely, some individuals reach EC status in the absence of protective alleles (as it can be observed in our cohort), indicating that they are not a requirement for virus control. For instance, $CD8^+$ T-cell VIA can be observed both in the presence and absence of protective HLA-I alleles (20, 35). Although in this work a simultaneous evaluation of multiple $CD8^+$ T-cell functions (in different terms such as magnitude, breadth, immunodominant targets, polyfunctionality, and VIA) was performed in HLA-typed individuals with known disease status, both the cohort size and the heterogeneity of alleles and responses preclude us from unequivocally establishing the relationship between $CD8^+$ T-cell function and phenotype and disease outcome. Further studies aimed at developing a model involving these three parameters will be needed.

The cytokine network elicited after HIV/simian immunodeficiency virus (SIV) infection has been studied in order to shed light on the early pathogenic events of the infection and to understand how these events trigger the future course of the disease. Moreover, the level of different plasma cytokines and/or chemokines during acute infection has been proposed as a potential biomarker to predict the rate of disease progression (37–39, 62). However, to our knowledge, no report has established so far the relationship between plasma molecules and the HIV-specific cellular immune response. Here, a correlation between the relative immunodominance of the anti-Gag response and the levels of IL-2 and MIP-1β was found (Fig. 8). As mentioned before, these molecules are produced by $CD8^+$ T cells upon stimulation, and the percentage of cells expressing IL-2 and MIP-1β either alone or in combination with other functions were previously associated with virus control (4, 11, 14, 43, 49). IL-2 is an important molecule for T-cell homeostasis and immune system activation (63). Although different levels of plasma IL-2 during PHI infection have not been previously associated with HIV/AIDS disease outcome, decreased levels of other molecules also involved in T-cell homeostasis and T-cell phenotype determination, such as IL-15 and IL-12, respectively, have been associated with progressive infection in a cohort of chronically infected African-American women (38). On the other hand, it was determined that MIP-1β-producing $CD8^+$ T cells represent an elevated proportion within the HIV-specific $CD8^+$ T-cell response during acute infection and that they exert considerable immune pressure over viral replication (6). At the functional level, it was suggested that the production of MIP-1β is rapidly elicited upon stimulation on the specific cells (52), which may account for an enhanced activity of the MIP-1β-producing $CD8^+$ T cells over cells exerting other functions. Moreover, other authors have found an association between MIP-1β plasma levels and lower VL and higher $CD4^+$ T-cell counts during chronic infection (38). Taken together, all this evidence provides a rationale for the association between the protective role of immunodominant Gag-specific cellular response and plasma levels of IL-2 and MIP-1β. As already mentioned, and although further investigations will be needed to determine the nature of this relationship, these results are in line with the evidence indicating that early relative Gag immunodominance is somehow related to the generation of an effective antiviral immune response leading to slower disease progression.

In summary, multiple aspects of the HIV-specific $CD8^+$ T-cell subset (specificity, *ex vivo* viral inhibitory capacity, and polyfunctionality) arising early after infection were characterized in a cohort of acute/early infected subjects, in comparison with that found in viremic chronics and elite controllers. Importantly, the association of these functions with disease progression was studied. In the first place, it was found that specificity of cells arising early after the transmission event is critical for immune control: early relative immunodominance of Gag-specific cells was associated with delayed disease progression, in terms of $CD4^+$ T-cell count preservation, during the first year postinfection, in consonance with Gag immunodominance in EC and viremic controllers. Further insights into the functionality of $CD8^+$ T cells from

enrolled subjects allowed us to establish that the advantage of Gag-specific $CD8^+$ T cells would rely on their ability to mediate both lysis of infected cells (evidenced by higher capacity to degranulate and to mediate viral inhibition activity *in vitro*) and soluble inhibition of viral replication by the simultaneous secretion of IFN-γ. Also, higher relative contribution of anti-Gag-specific cells to the total anti-HIV cellular immune response correlated with plasma levels of IL-2 and MIP-1β, molecules that further contribute to viral inhibition.

The identification of phenotypic and functional properties of the $CD8^+$ T-cell subsets associated with viral control is urgently needed to aid in the design and performance evaluation of an effective HIV vaccine. In this scenario, data presented in the manuscript underscore the importance of considering both cell specificity and quality, in terms of both cytotoxic and noncytotoxic mechanisms, to boost preventive and therapeutic anti-HIV immune-based strategies.

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Acute meningoencephalitis due to human immunodeficiency virus type 1 infection in 13 patients: clinical description and follow-up

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The objective of this study is to describe a series of cases of severe meningitis caused by human immunodeficiency virus type 1 (HIV-1) occurring during primary infection or after antiretroviral treatment interruption. In an observational cohort study, 13 patients with clinical diagnosis of meningitis or meningoencephalitis were reviewed. Ten cases occurred during primary HIV-1 infection and 3 after antiretroviral therapy (ART) withdrawal. Demographic parameters, clinical presentation and outcome, and laboratory and cerebrospinal fluid (CSF) parameters were recorded. The risk factor for HIV-1 infection acquisition was sexual transmission in all cases. The most frequent systemic symptoms were fever (12/13) and headeache (9/13). Among neurologic symptoms, focal signs appeared in seven patients (53.8%), confusion in six (46.2%), and agitation in five (38.5%). The median CD4 cell count was 434 cells/mm3. In all cases, CSF was a clear lymphocytaire fluid with normal glucose levels. Cranial computerized tomography was performed in seven patients, with a normal result in all of them; brain magnetic resonance in eight patients was normal in five cases and showing cortical atrophy, limbic encephalitis, and leptomeningeal enhancement in one patient each. The electroencephalographs (EEG) just showed diffuse dysfunction in three cases. ART was started in 11 patients. HIV RNA load at 12 months was <50 copies/ml in all treated patients. The 13 patients recovered without neurologic sequela. Meningitis or meningoencephalitis during primary HIV-1 infection or after ART cessation are unusual but sometimes a life-threatening manifestation. Although all patients tend to recover and the necessity of ART is not well established, some data suggest its potential benefit in these patients. *Journal of NeuroVirology* (2008) **14,** 474–479.

Keywords: Primary HIV infection; acute retroviral syndrome; central nervous system involvement; meningitis; meningoencephalitis; antiretroviral therapy; HIV-1

Introduction

It is estimated that 40% to 90% of patients with primary human immunodeficiency virus type 1 (HIV-1) infection experience an acute retroviral syndrome; however, the nonspecific nature of the acute symptoms in most cases results in under diagnosis of this entity (Bachmeyer *et al,* 1997; Li *et al,* 2002; Atwood *et al,* 1993; Schacker *et al,* 1996, 1998). Although neurological presentations, including aseptic meningitis, have been described in 17% to 24% of these patients, severe cases of meningitis

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have been rarely reported (Schacker *et al,* 1998; Newton *et al,* 2002). Some authors suggest that patients who present neurological features during primary HIV infection may experience faster progression of HIV-related disease (Boufassa *et al,* 1995; Wallace *et al,* 2001).

An acute retroviral syndrome similar to what occurs during primary HIV infection has also been described during treatment interruptions (Colven *et al,* 2000), and cases of acute meningitis have been reported in this setting (Worthington and Ross, 2003; Breton *et al,* 2003).

In this study, a case series is presented of patients with severe HIV-1 meningitis occurring during the primary infection or after interruption of antiretroviral treatment. The clinical presentation of these patients and their evolution after recovery are described.

Results

Acute meningitis or meningoencephalitis was diagnosed in 13 patients, 10 during primary HIV infection and 3 in chronic HIV-infected patients after antiretroviral treatment interruption.

There were seven (53.8%) women and six (46.2%) men, with a median age of 39 (IQR: 32–54) years. HIV infection was acquired by sexual transmission in all cases (men who had sex with men in six cases and heterosexual in seven). The patients' clinical manifestations are summarized in Table 1. Among the neurological symptoms, a focal neurological sign was present in 53.8% of the cases, the most striking of these being ataxia, dysarthria, unilateral Bell palsy, and paresthesia. One patient also presented with uveomeningitis. Nearly half the patients had confusion or obtundation (46.2%). In contrast, only two patients had meningeal signs.

Table 1 Clinical manifestations in patients with meningitis due to HIV-1 infection

	PHI[a]	CHI[b]	Total[c]
No. of cases	10	3	13
Systemic symptoms			
Fever	10	2	12 (92.3)
Headache	8	1	9 (69.2)
Gastrointestinal symptoms	7	1	8 (61.5)
Lymph adenopathy	5	2	7 (53.8)
Sore throat	7	0	7 (53.8)
Rash	6	0	6 (46.2)
Neurological symptoms			
Focal neurological signs	5	2	7 (53.8)
Confusion/obtundation	5	1	6 (46.2)
Agitation	4	1	5 (38.5)
Seizures	2	0	2 (15.4)
Meningeal signs	1	1	2 (15.4)

[a]PHI = primary HIV infection.
[b]CHI = chronic HIV infection. Cases after antiretroviral therapy withdrawal.
[c]No. (%).

Table 2 shows the laboratory findings in blood and CSF of all patients. Median CD4 lymphocyte count was 434 (IQR: 289.5–577.5) cells/mm^3. Plasma HIV RNA ranged from 5100 (3.71 $\log_{10}$) to 22,000,000 (7.34 $\log_{10}$) copies/ml. In all cases, CSF was a clear fluid with normal glucose levels and a predominance of lymphocytes. ADA levels determined in 10 patients yielded a median of 7.5 (IQR: 4.75–10.25). In two patients, ADA levels were >10 IU/L.

In 7 of the 13 cases, we also recorded CSF HIV RNA load. In five patients with meningitis during primary infection, mean HIV viral load in CSF was 5.6 $\log_{10}$, as compared to 4.1 $\log_{10}$ in those who presented neurological symptoms after withdrawal of antiretroviral therapy.

Cranial computerized tomography was performed in seven patients, with normal results in all of them. Brain magnetic resonance was performed in eight cases, with the following findings: normal in five cases, cortical atrophy, limbic encephalitis and leptomeningeal enhancement in one patient each. EEG was performed in four patients, and diffuse dysfunction was observed in three of them.

All patients recovered from meningitis without neurological sequelae. The median hospital stay was 12 (range 6–22) days, and all patients were completely recovered at discharge. On the diagnosis of HIV meningitis or meningoencephalitis, antiretroviral therapy was started in 11 of the 13 patients. The different antiretroviral regimens and the CNS Penetration-Effectiveness Score for each patient are shown in Table 3. Plasma HIV RNA viral load at 12 months was <50 copies/ml in all the treated patients. Figure 1 shows the evolution of CD4 lymphocytes and plasma HIV RNA viral load during follow-up.

Discussion

The clinical presentation of primary HIV-1 infection varies from asymptomatic seroconversion to a severe symptomatic illness resembling infectious mononucleosis that can result in hospitalization (Schacker *et al,* 1996; Kassutto and Rosenberg, 2004; Huang *et al,* 2005). The most frequent neurological manifestation during primary HIV-1 infection is aseptic meningitis, although other disorders as cranial nerve VII palsy and radiculopathy have been described (Kassutto and Rosenberg, 2004). Although many patients seek medical attention during acute HIV-1 infection, the diagnosis is often missed (Rosenberg *et al,* 1999). Between 1997 and 2003, 75 patients with primary HIV infection were diagnosed in Hospital Clínic in Barcelona; 5 of these patients required hospital admission because of neurological symptoms and 3 (4%) had viral

Table 2 Blood and CSF laboratory findings

	Patients													
	1#	2#	3#	4*	5*	6*	7*	8*	9*	10*	11*	12*	13*	Median (IQR)
Blood														
CD4 lymphocytes (cells/mm^3)	315	200	570	551	591	264	434	585	391	319	487	709	190	434 (289.5–577.5)
HIV RNA load (copies/ml; log10)	7800	5100	140000	900000	4100000	—	$22x10^6$	1300000	$>10^6$	$>10^6$	151542	16600	259200	205371 ($14400–2 \times 10^6$)
	3.89	3.71	5.15	5.95	6.11		7.34	6.11	6.0	6.0	5.18	4.22	5.41	
CSF														
Glucose (mg/dl)	45	52	62	43	35	49	52	46	91	147	61	45	51	51 (45–61.5)
Proteins (mg/dl)	84	51	161	169	185	139	102	142	109	—	80	206	150	139 (82–165)
Leucocytes (cells/ml)	40	38	40	25	58	85	52	100	90	270	40	40	90	52 (40–90)
Lymphocytes (%)	89	95	27	—	72	95	71	95	50	99	—	92	—	90.5 (65.75–95)
ADA (IU/L)	9	10	6	11	16	—	4	10	5	—	—	5	4	7.5 (4.75–10.25)
HIV RNA load (copies/ml; log10)	2900	21000	—	100000	730000	—	560000	360000	—	250000	—	—	—	
	3.46	4.32		5	5.86		5.75	5.56		5.4				

#Meningoencephalitis after antiretroviral therapy interruption
*Meningoencephalitis during primary HIV-1 infection

Table 3 Antiretroviral regimen employed for each patient

Patient	Antiretroviral regimen	CNS Penetration-Effectiveness Score
1	zidovudine + lamivudine + efavirenz	2
2	zidovudine + lamivudine + lopinavir/ritonavir	2.5
3	zidovudine	1
4	no treatment	—
5	tenofovir + emtricitabine + lopinavir/ritonavir	2
6	zidovudine + lamivudine + lopinavir/ritonavir	2.5
7	zidovudine + lamivudine + lopinavir/ritonavir	2.5
8	tenofovir + emtricitabine + lopinavir/ritonavir	2
9	no treatment	—
10	stavudine + lamivudine + indinavir	2
11	tenofovir + didanosine + lopinavir/ritonavir	1
12	tenofovir + abacavir + lamivudine + lopinavir/ritonavir	2,5
13	stavudine + lamivudine + nelfinavir	1

meningitis (Sued *et al,* 2006). In a recent retrospective study of CSF samples, Hanson *et al*. reported that 3 of 57 (5%) patients with clinical signs and inflammatory findings on CSF study indicative of CNS infection had primary HIV infection, which had not been suspected or diagnosed (Hanson *et al,* 2007). To avoid delays in the diagnosis, these authors have suggested that assessment of patients with clinical and laboratory findings consistent with meningoencephalitis should include tests to detect acute HIV infection. In this report, we describe 10 patients with acute meningoencephalitis during primary HIV-1 infection and 3 patients who developed neurological impairment after interruption of antiretroviral therapy. This finding has been reported previously by Colven *et al*. in a series of three chronic HIV-1 infected patients presenting with retroviral rebound syndrome after cessation of suppressive antiretroviral therapy; one of these patients developed acute meningitis 3 weeks after the interruption (Colven *et al,* 2000).

The clinical presentation of patients with meningoencephalitis caused by HIV infection did not differ from that seen in other causes of meningoencephalitis. It is important to point out that most patients in our series presented with severe neurological manifestations, such as obtundation, focal neurological signs, agitation, and even seizures. The other neurological signs in our series consisted in uveomeningitis, ataxia, unilateral Bell palsy, dysarthria, and paresthesia. All these manifestations have been reported previously (Li *et al,* 2002; Pascual *et al,* 2005; Serrano *et al,* 2007). Moreover, 2 of our 13 cases presented generalized tonic-clonic seizures during the episode. In both cases, the electroence-

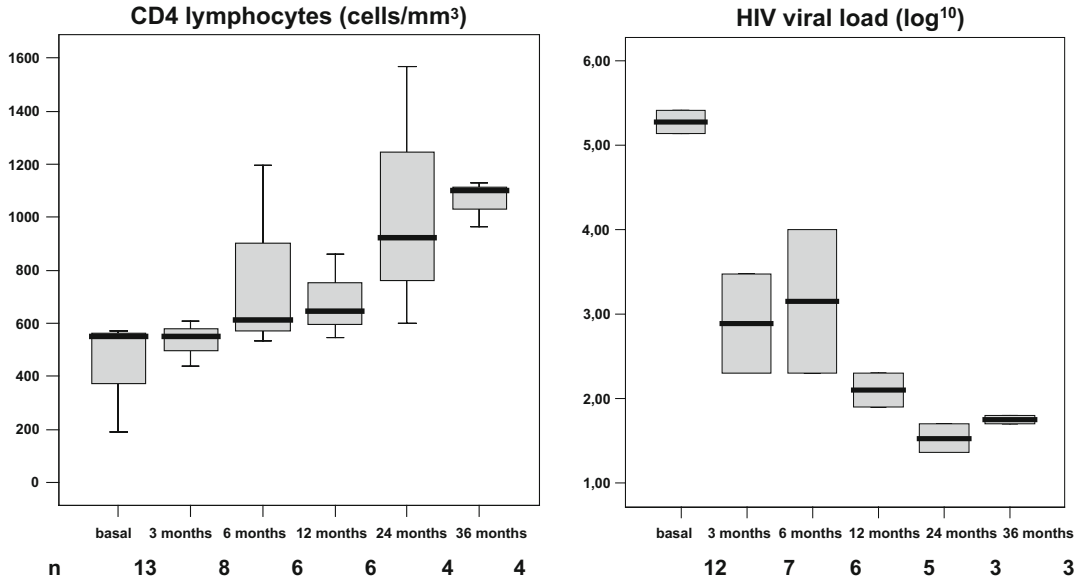

Figure 1 Evolution of CD4 lymphocytes (cells/mm^3) and plasma HIV-1 RNA viral load ($\log_{10}$ copies/ml) during follow-up.

phalogram demonstrated nonspecific global cortical dysfunction without paroxysms. Despite the presence of focal neurological signs, the radiological findings showed an absence of specific or focal abnormalities, except in one case, in which limbic encephalitis was detected in the cranial magnetic resonance study.

Two of the 10 patients in whom ADA levels were determined had an ADA value higher than 10 IU/L. In a previous report, this cut-off proved to be suggestive of tuberculous meningitis (Ribera *et al,* 1987). However, high ADA levels have been also described in patients with cytomegalovirus (CMV) neurological disease, and in cryptococcal, lymphomatous, and candidal meningitis (Corral *et al,* 2004). These findings show that acute HIV menigoencephalitis may be another cause of elevated ADA levels in CSF.

As has been reported, patients undergoing HIV-1 seroconversion with acute neurological symptoms had significantly higher mean CSF HIV-1 viral load than did patients without neurological syndromes (Tambussi *et al,* 2000; Mellgren *et al,* 2005; Wendel and McArthur, 2003). Tambussi *et al* compared HIV-1 viral load in CSF between patients with and without neurological syndrome; a strong correlation between neurological symptoms and viral load was found. Mean CSF HIV level was significantly higher in patients with neurological symptoms (4.12 log) than in those without (2.58 log) (Tambussi *et al,* 2000). In patients with acute meningitis after antiretroviral treatment interruption, HIV-1 viral load was considerably higher in CSF than in plasma (Colven *et al,* 2000).

Despite the presence of severe neurological manifestations, in the vast majority of cases reported in the literature, as well as in ours, the clinical outcome is favorable, with complete recovery and no after-effects.

Early diagnosis of acute HIV-1 infection often creates a dilemma for clinicians with regard to treatment. Potential benefits of early antiretroviral therapy have been suggested, but differences in morbidity and mortality have not been proven. The theoretical benefits that are frequently discussed must be weighed against the significant risk of long-term medication toxicity and cost (Kassutto and Rosenberg, 2004).

The rational basis to initiate antiretroviral treatment in patients with acute neurological manifestations during primary HIV infection derives from cohort studies. In a cohort of 277 adults enrolled more than 1 year after HIV-1 primary infection, Boufassa *et al* demonstrated that the relative risk of developing AIDS was 6.11 in patients with neurological manifestations during primary infection and 2.32 in those with non-neurological manifestations, as compared to a group of patients showing no clinical manifestations during primary infection (Boufassa *et al,* 19957). Apart from a reduced chance of developing acquired immunodeficiency syndrome (AIDS)-defining diseases, early antiretroviral therapy may result in more rapid resolution of symptoms, particularly in cases with severe neurological manifestations.

Eleven patients in our series initiated antiretroviral treatment following the neurological presentation. We cannot affirm that their clinical improvement was due to the treatment, because spontaneous resolution has also been described. However, as in other viral illnesses, resolution of the neurological symptoms may have been accelerated by antiretroviral therapy, which is particularly important in patients with severe manifestations

such as seizures. The two patients who did not receive antiretroviral treatment presented spontaneous improvement; hence, therapy was deferred. Letendre *et al* demonstrated that a lower cerebral penetration-effectiveness score of antiretroviral regimen correlated with higher CSF viral loads (Letendre *et al,* 2008). Ranks less than 2 were associated with an 88% increase in the odds of detectable CSF viral load. Poorer penetration of antiretroviral (ARV) drugs into the CNS appears to allow continued HIV replication in the CNS, as indicated by higher CSF HIV viral loads. In our study, 8 of the 11 treated patients had a CPE ≥ 2, although we could not assess the evolution of HIV viral load in CSF. Because inhibition of HIV replication in the CNS is probably critical in treating patients who have HIV associated neurocognitive disorders, antiretroviral treatment strategies that account for CNS penetration should be considered in patients with neurological manifestations during primary infection. Recently Gasnault *et al* have observed in patients with progressive multifocal leukoencephalopathy that the use of an antiretroviral regimen, including drugs with high penetration into the CNS, lead to a better survival (Gasnault *et al,* 2008).

In conclusion, neurological impairment during primary HIV-1 infection or after interrupting antiretroviral therapy is a relatively uncommon manifestation that tends to recover spontaneously. Although initiation of antiretroviral therapy in these patients is still not well established, emerging data suggest its potential benefit.

Patients and methods

From 1999 to January 2007, we retrospectively identified and reviewed 13 patients with a clinical diagnosis of meningitis or meningoencephalitis during acute HIV-1 infection ($n = 10$) or after withdrawal of antiretroviral therapy ($n = 3$). The study was performed in two hospitals in Barcelona (Hospital Universitari Vall d'Hebron and Hospital Clínic i Provincial).

To be included in the study, patients had to meet the following criteria: (1) diagnosis of meningitis or meningoencephalitis defined by the presence of fever and/or headache, cerebrospinal fluid (CSF) leukocyte count >10 cells/mm^3, and CSF protein count >50 mg/dl. When confusion, seizures, or any other focal neurological signs were present, a diagnosis of meningoencephalitis was established; (2) exclusion of other potential causes of meningitis or meningoencephalitis; (3) diagnosis of acute HIV-1 infection defined by all the following criteria: negative or indeterminate Western blot tests, detectable plasma HIV RNA, and complete seroconversion after the episode. Meningoencephalitis after antiretroviral therapy withdrawal was established when the above-mentioned criteria manifested during a period of 1 to 10 weeks after stopping antiretroviral treatment.

The following data were recorded for all patients: gender, age, race, risk factors for acquiring HIV infection, clinical manifestations, neurological complications, and outcome. In patients with meningoencephalitis after antiretroviral therapy withdrawal, the years of HIV infection and days without antiretroviral therapy were reviewed. Antiretroviral therapy was recorded for each patient. Each antiretroviral was given a Cerebral Penetration-Effectiveness (CPE) rank of 0 (low: ddI, TNF, ddC, APV, NFV, RTV, SQV, SQV/r, TPV/r, T20), 0.5 (intermediate: 3TC, d4T, EFV, APV/r, FPV/r, ATZ/r, DRV/r), or 1 (high: ABC, FTC, AZT, DLV, NVP, IDV, IDV/r, LPV/r) based on their chemical properties, concentrations in CSF, and/or effectiveness in the central nervous system (CNS) in clinical studies (Letendre *et al,* 2008; Gasnault *et al,* 2008). Laboratory parameters included total leukocyte count, platelet count, CD4 lymphocyte count (cells/mm^3), plasma HIV RNA load (copies/ml), and the following CSF parameters: cell count, glucose (mg/dl), proteins (mg/dl), and CSF HIV-1 viral load (copies/ml). Microbiological determinations performed to exclude other diagnoses included Gram, Ziehl-Nielssen, and India-ink stains, bacterial, mycobacterial, and fungal cultures, latex cryptococcal antigen, herpes virus family polymerase chain reaction (PCR), and adenosine deaminase (ADA) (IU/L). In our laboratory, ADA levels >10 IU/L are considered to be highly suggestive of tuberculosis (Ribera *et al,* 1987).

Cranial magnetic resonance imaging and/or computerized tomography as well as electroencephalography (EEG) were performed at the discretion of the attending physician.

Quantitative variables are expressed as medians and interquartile range (IQR) and qualitative variables as frequencies and percentages.

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