UNIVERSITAT DE
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Regulació de l'activitat inflamatòria per IFN-γ en l'Arteritis de Cèl·lules Gegants (ACG)

Marc Corbera Bellalta

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Regulació de l'activitat inflamatòria per IFN-γ en l'Arteritis de Cèl·lules Gegants (ACG)

Tesi presentada per

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per optar al grau de Doctor per la Universitat de Barcelona

Tesi dirigida per

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Facultat de Medicina

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Al meu avi i a les meves iaies...

"Sempre he vist la vida com un camí. Un serpentejant camí de carro entre les muntanyes en el que sovint camines acompanyat i rares vegades sol. Una ruta amb pedres al camí, amb pujades i baixades, amb uns dies de pluja i altres de sol radiant, un camí on canviar de ritme, i fins i tot córrer si es vol. Un camí on la motxilla que portes a l'esquena a dies pesa que més que d'altres, però mai deixa d'omplir-se.

El segment del meu camí que he recorregut aquests darrers anys m'ha donat l'oportunitat de compartir la ruta amb molts altres caminants, alguns dels quals, han emprès ja altres camins separant-nos en bifurcacions llunyanes. A tots ells, i especialment al meu avi, dedico aquest llibre."

Agraïments

AGRAÏMENTS

Una vegada li vaig preguntar a una persona molt sabia per on havia de començar a escriure la tesi, i en un atac de franquesa i obvietat sense precedents em va dir: "comença per on et vingui més de gust!".

Així que lluny de començar a escriure com presentar-vos els resultats o els èxits científics, el que em venia més de gust era començar per escriure els agraïments. Un espai on poder deixar anar les emocions acumulades en aquest camí d'anys de feina. Un lloc on poder dir el que volia dir a qui volia dir-ho.

Disculpeu si m'ha servit com excusa per desfogar-me i potser si m'he estès una mica massa, però l'ocasió ho requeria, ja que en les següents 10 pàgines no he volgut obviar ningú, ni cap dels missatges que guardava per cada ú. I m'haureu de disculpar també per voler parlar aquí de totes aquelles persones que lluny de tenir res a veure amb la meva feina, han significat coses importants per mi durant aquest temps. També a ells els dedico aquesta tesi i l'esforç que hi hem invertit entre tants.

I no puc començar d'una altra manera que no sigui agraint a la Mariona el seu recolzament.

Suposo que forma part del protocol d'uns agraïments d'una tesi començar pel director de la mateixa, però vull que quedi ben clar abans de res, que la Mariona mereix aquest lloc d'honor encara que el protocol digués que havia d'anar a l'apèndix. De fet, dir que només m'ha recolzat durant aquest temps és una manera molt pobra i injusta de dir-ho. No és només que m'hagi recolzat, és que m'ha guiat, ajudat, educat, encoratjat, i acompanyat en aquest camí. Perquè és una persona extraordinària, una *jefa* que sap ser-ho malgrat les dificultats, que sap rectificar i rectificar-te, i que té una vessant humana d'inesgotable paciència; un pou d'on quan creus que ja no en pot sortir més saviesa encara en segueixen brollant noves idees, una persona que sap llegir entre línies el que cada ú necessita i s'angoixa amb el que cada ú pateix; algú que creu que té la sort d'estar rodejada de la millor gent, i no s'adona que és ella qui fa treure el millor de cadascú. Gràcies per ser algú a qui recordar per sempre.

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Aquest llibre és per tu amor meu. Gràcies Ca.

“Donde no falta voluntad, siempre hay un camino”

DernHelm (Éowyn)

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Abreviatures

ABREVIATURES

ACG: *Arteritis de Cèl·lules Gegants*

AMP: *Adenosina Monofosfat*

ANCA: *Anticossos Citoplasmàtics contra Neutròfils (Anti-Neutrophil Cytoplasmic Antibody)*

APC: *Cèl·lula Presentadora d'Antigen (Antigen Presenting Cell)*

AT: *Arteritis de Takayasu*

ATP: *Adenosina trifosfat*

Bcl-2: *Proteïna del limfoma de cèl·lules B 2 (B-cell lymphoma 2)*

CCL: *Quimiocina tipus C-C, β-quimiocina (C-C motif chemokine Ligand)*

CD: *Grup de diferenciació (Cluster of Differenciation)*

CREB: *Proteïna d'unió a l'element de resposta a AMP cíclic (cAMP Response Element Binding protein)*

CS: *Síndrome de Churg-Strauss (Churg-Strauss Syndrome)*

CSF-1: *Factor estimulador de colònies (Colony Stimulating Factor 1)*

CXCL: *Quimiocina tipus C-X-C, α-quimiocina (C-X-C motif chemokine Ligand)*

CX_3CL1: *Fractalquina (Fractalkine)*

CX_3CR1: *Receptor de la Fractalquina*

DNA: *Àcid desoxiribonucleic (DesoxyriboNucleic Acid)*

FOXP3: *Forkhead Box protein 3*

GAF: *Factor activador gamma (γ-Activator Factor)*

GAS: *Seqüències activades gamma (γ-Activated Sequence)*

GATA3: *Factor de transcripció 3 de la família GATA (Guanina-Adenina-Timina) (GATA binding protein 3)*

GTP: *Guanosina trifosfat*

HLA: *Antígens leucocitaris humans (Human Leukocyte Antigen)*

ICAM-1: *Molècula d'adhesió intercelular 1 (InterCelular Adhesion Molecule 1)*

IFNγ: *Interferó gamma (Interferon gamma)*

Ig: *Immunoglobulina*

IL: *Interleucina (Interleukin)*

IP-10: *Proteïna induïda per Interferó-10 (Interferon inducible Protein 10) (CXCL10)*

IRF: *Factor Regulador de l'Interferó (Interferon Regulatory Factor)*

ISGF3: *Factor de transcripció estimulat per interferó 3 (Interferon-stimulated Gene Factor-3)*

ISRE: *Elements de resposta estimulats per interferó (Interferon-Stimulated Response Elements)*

I-TAC: *Quimioatraient de cèl·lules T induït per interferó (Interferon–inducible T Cell Alpha Chemoattractant) (CXCL11)*

JAK: *Serina Tirosina Quinasa (Janus Tyrosine Kinase)*

KO: *Noquejat (Knock-out)*

MCP-1: *Proteïna Quimioatraient de Macròfags 1 (Macrophage Chemoattractant Protein 1) (CCL2)*

MHC: *Complex major d'histocompatibilitat (Major Histocompatibility Complex)*

MIG: *Monoquina Induïda per Interferó Gamma (Monokine Induced by Gamma interferon) (CXCL9)*

MIP-1α: *Macrophage Inflammatory Protein 1 alpha (CCL3)*

MIP-1β: *Macrophage Inflammatory Protein 1 beta (CCL4)*

MMP: *Metal·loproteassa de Matriu* **(***Matrix MetaloProtease)*

MPA: *Poliangiïtis Microscòpica (Microscopic Polyangiitis)*

NK: *Natural Killer*

NOS2: *Sintasa de Òxid Nítric (Nitric Oxid Sintase 2) (iNOS)*

PAN: *Poliarteritis nodosa*

PBMC: *Cèl·lula mononuclear de sang perifèrica (Peripheral Blood Mononuclear Cell)*

PBS: *Tampó fosfat salí (Phosphate Buffered Saline)*

PCR: *Reacció en cadena de la polimerasa (Polymerase Chain Reaction)*

PDGF: *Factor de Creixement Derivat de les Plaquetes (Plateled Derived Growth Factor)*

PIAS: *Proteïnes inhibidores dels STATs activats (Proteins that Inhibit Activated STATs)*

PTP1B: *Proteïna fosforiladora de tirosines 1B (Protein-Tyrosine-Phosphatase 1B)*

RAN: *Proteïna Nuclear Ras (Ras-Related Nuclear Protein) (TC4)*

RANTES: *Regulated on Activation Normal T cells Expressed and Secreted (CCL5)*

RNA: *Àcid ribonucleic (RiboNucleic Acid)*

RORγ: *Receptor Orfe unit a RAR gamma (RAR-related Orphan Receptor C)*

SBE: *Elements d'Unió als STATs (Stat Binding Elements)*

SDS: *Dodecilsulfat Sòdic (Sodium Dodecyl Sulfate)*

SHP: *Fosfatasa amb domini SH2 (SH2-containing Phosphatase)*

SNP: *Polimorfisme d'un sòl nucleòtid (Single Nucleotid Polymorphism)*

SOCS: *Supressors de la senyalització de les citoquines (Suppressor Of Cytokine Signaling)*

STAT: *Transductor de Senyal i Activador de la Transcripció (Signal Tranducer and Activator of Transcription)*

t-Bet: *Factor de transcripció T-Box expressat en cèl·lules T (T-box transcription factor expressed in T-cells) (Tbx21)*

TGFβ: *Factor Transformant del Creixement beta (Transforming Growth Factor beta)*

TNF-α: *Factor de Necrosi Tumoral alfa (Tumor Necrosis Factor alpha)*

VCAM-1: *Molècula d'Adhesió de Cèl·lules Vasculars (Vascular Cell Adhesion protein-1)*

VEGF: *Factor de Creixement de l'Endoteli Vascular (Vacular Endothelial Growth Factor)*

VSMC: *Cèl·lula del Múscul Llis Vascular (Vascular Smooth Muscle Cell)*

WG: *Granulomatosis de Wegener*

Introducció

INTRODUCCIÓ

MALALTIES AUTOIMMUNES I INFLAMACIÓ

El procés inflamatori, que en termes mèdics s'etiqueta amb el sufix "-*itis*", es defineix habitualment com una resposta complexa i inespecífica d'un teixit enfront una agressió, un intent protector de l'hoste per eliminar un patogen i/o cèl·lules danyades. Aquest procés, que consta d'un component cel·lular i un component molecular, comporta canvis hemodinàmics, alteracions de la permeabilització, modificacions cel·lulars, expressió de mediadors inflamatoris, i en general una mobilització de recursos del teixit per lluitar contra l'agent infecciós (1).

En la autoimmunitat però, aquest agent és moltes vegades desconegut, i la cascada inflamatòria s'inicia sense aparent infecció.
Les malalties autoimmunes per tant, es caracteritzen per presentar una disfunció del sistema immunològic que ataca de manera anòmala teixits del propi organisme, generant el que ja coneixem com el procés inflamatori.

Aquestes malalties autoimmunes es poden classificar de manera genèrica en sistèmiques o específiques d'òrgan. Aquelles malalties en les què el teixit diana de l'atac immunològic i la inflamació són els vasos sanguinis, es coneixen amb el nom de vasculitis.

Vasculitis

Les vasculitis inclouen un petit ventall de malalties autoimmunes, que contemplen un ampli espectre de manifestacions clíniques. I a grans trets poden separar-se en vasculitis infeccioses (conseqüència directa de la infecció per part d'organismes patògens) i vasculitis no infeccioses (aquelles no causades directament per la invasió de patògens, malgrat la infecció pugui guardar relació indirecta amb algunes d'elles).

A la conferència de Chapell Hill de 1994 es van consensuar els criteris de classificació dels diferents tipus de vasculitis no infeccioses, segons els quals, es van dividir les mateixes en

vasculitis de gran, mitjà i petit vas. Aquest consens fou revisat al 2012, establint una nomenclatura definitiva i delimitant categories de classificació adequades. D'aquesta manera les vasculitis de gran vas (LVV) inclouen l'arteritis de Takayasu (TAK) i l'arteritis de cèl·lules gegants (ACG). Les vasculitis de vas mitjà (MVV) són la poliarteritis nodosa (PAN) i la malaltia de Kawasaki (KD). Dins de les vasculitis de petit vas (SVV) s'inclouen les denominades vasculitis associades a ANCA (que també poden afectar vasos mitjans) i les vasculitis amb immunocomplexes. En les vasculitis associades a ANCA trobem la granulomatosis amb poliangiïtis, o malaltia de Wegener (GPA), la síndrome de Churg-Strauss (CS), i la poliangiitis microscòpica (MPA); mentre que en les de immunocomplexes trobem altres vasculitis com la púrpura de Henoch-Schönlein (IgAV), la vasculitis crioglobulinèmica (CV) i la vasculitis hipocomplementèmica urtical (HUV).

A aquesta classificació primària de les vasculitis, s'han d'afegir aquelles vasculitis de vas variable, que es caracteritzen per no tenir afectacions específiques segons la mida dels vasos diana, com la síndrome de Cogan (CS) o la malaltia de Behçet (BD); vasculitis d'òrgan específic, com l'arteritis cutània; vasculitis associades a malalties sistèmiques com el lupus o la sarcoïdosis; i finalment un darrer grup de vasculitis associades a probables etiologies diverses. (2, 3)

Una de les vasculitis més importants per la seva elevada freqüència, sempre dins del marc de les malalties rares que impliquen les vasculitis, és l'arteritis de cèl·lules gegants (ACG), vasculitis de tipus sistèmic, de caràcter granulomatós, i amb típica afectació pels grans vasos, i especialment de les branques de les artèries caròtida i vertebral.

L'ARTERITIS DE CÈL·LULES GEGANTS (ACG)

Història de l'ACG

L'arteritis de cèl·lules gegants fou descrita per primera vegada per J. Hutchinson al 1890 amb el nom d'arteritis trombòtica dels ancians, tot i que no va ser fins al 1932, on es va conèixer per primera vegada com arteritis temporal gràcies a Bayard Taylor Horton. Anys després se la va anomenar arteritis de Horton en honor a l'autor de la seva primera descripció histopatològica,

fins que posteriorment, al 1941, Gilmour li va donar el nom d'arteritis de cèl·lules gegants (ACG), que és el més emprat en l'actualitat (4).

Descripció

L'ACG és una malaltia inflamatòria crònica d'etiologia desconeguda, que constitueix actualment la vasculitis més freqüent al món occidental. Es tracta d'una malaltia amb una lleugera major incidència en el sexe femení i que afecta sempre a persones majors de 50 anys. En individus a partir d'aquesta franja d'edat té una incidència de aproximadament 1-2 casos/any per cada 10000 habitants i una prevalença de 1 cada 500 (5-7).

Clínica de l'ACG

La simptomatologia de l'arteritis de cèl·lules gegants pot classificar-se en tres grans grups de manifestacions clíniques:

- En primer lloc podem parlar de **símptomes locals**, que són aquells pròpiament lligats a la inflamació a nivell local principalment de les branques supraòrtiques de l'àrea craneocervical. Donen lloc a manifestacions com cefalea, claudicació mandibular o la ceguera ocasionada per la hipòxia del nervi òptic (que és la complicació isquèmica més freqüent en els pacients amb ACG).
- També tenim una **simptomatologia sistèmica**, com poden ser la presència en els malalts de astènia, anorèxia, pèrdua de pes o febre**.**
- I finalment aquella **simptomatologia associada a la polimiàlgia reumàtica**, com el dolor o la rigidesa en les articulacions.

Lligat amb aquesta amplitud de símptomes, tenim un ampli espectre de possibles respostes inflamatòries dels pacients, que comprèn des dels pacients amb resposta inflamatòria més dèbil, propensos a desenvolupar fenòmens isquèmics, però més receptius a la remissió dels símptomes mitjançant el tractament amb glucocorticoides; fins els pacients de resposta inflamatòria intensa, més refractaris, propensos a tenir relapses o recaigudes de la malaltia, i que requereixen dosis més perllongades de medicament, en alguns dels quals no acaba mai de remetre del tot la malaltia.

Diagnòstic

El mètode més usualment emprat per al diagnòstic de l'ACG, i que representa el *Gold Standard* en aquest punt, és la biòpsia de l'artèria temporal. A través d'aquesta es pot observar la presència d'infiltrats inflamatoris que provoquen l'engruiximent de la paret vascular, la hiperplàsia de la làmina íntima i per tant la formació de la neoíntima que obtura la llum del vas, o la característica presència en alguns casos de cèl·lules gegants originades a partir de la fusió de macròfags a l'interior de les formacions granulomatoses.

Com es pot veure a la figura 1, una artèria es composa de diverses làmines concèntriques separades per dues membranes elàstiques: l'externa i la interna. En condicions normals, no patològiques ni inflamatòries, a la part més propera a la llum del vas trobem l'endoteli vascular i la fina làmina íntima Aquesta està formada per cèl·lules miointimals i proteïnes de matriu extracel·lular i resta separada de la làmina mitja per la membrana elàstica interna. La làmina mitja està formada per teixit muscular llis i representa el component cel·lular majoritari de l'artèria. A continuació, a la part més exterior i separada per la membrana elàstica externa, trobem la làmina adventícia, en la qual descansen els *vasa vasorum* (arterioles i vasos sanguinis colaterals) i terminacions nervioses i està formada per teixit connectiu i fibres de col·lagen.

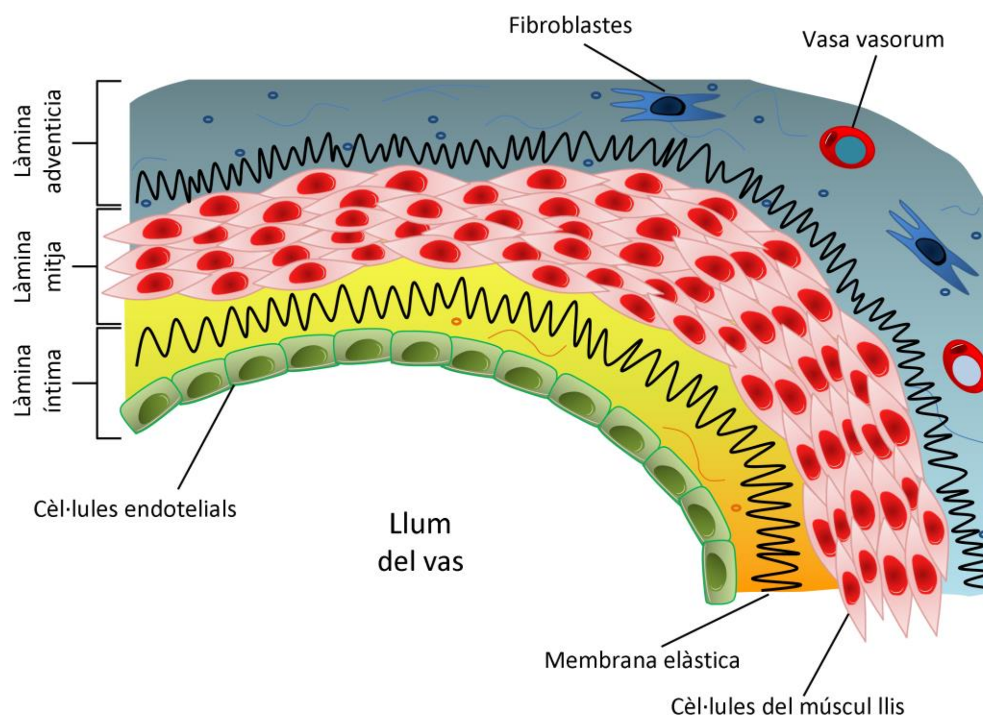

Figura 1. Estructura de la paret arterial

En la figura 2 s’observen les diferències evidents entre una biòpsia negativa sana i una biòpsia positiva per ACG. En aquesta s’aprecien la llum vascular pràcticament obturada, la presència de lesions granulomatoses amb cèl·lules gegants i la desestructuració de les làmines internes de l’artèria.

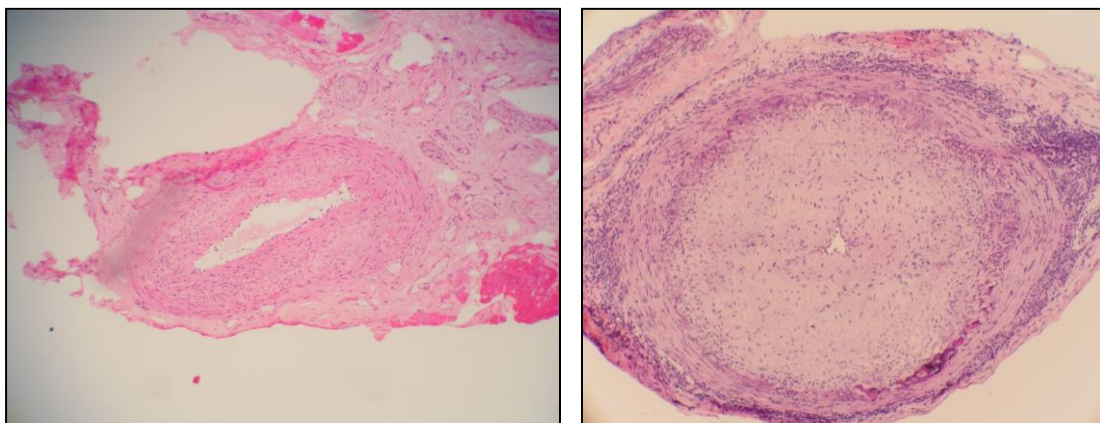

Figura 2. Artèries temporals, negativa i positiva per ACG.

Patogènesi de l'ACG

El coneixement actual sobre els processos patogenètics que tenen lloc en l’ACG és fonamentalment hipotètic, està basat principalment en treballs observacionals que mesuren l'expressió de marcadors inflamatoris i cel·lulars dels que s'extrapolen i es suposen funcions ja conegudes, i que correlacionen amb l'evolució dels pacients. És a dir, sobre evidències experimentals dèbils en línies generals (8). No és d’estranyar per tant que encara calgui aprofundir molt els pròxims anys en el coneixement de la patogènesi de la malaltia.

No podem oblidar que l'ACG és una malaltia més recurrent en dones i que afecta exclusivament persones majors de 50 anys. Així doncs la presència de determinades hormones, l'impacte de l'etapa reproductiva o l'efecte de l'envelliment sobre el sistema immune i el teixit vascular, semblen estar relacionats amb l'aparició de la malaltia.

No obstant, malgrat sembla que els estrògens podrien influir en les necessitats corticoidees de les pacients, i l'edat avançada està associada a major probabilitat de manifestacions isquèmiques, el coneixement de com aquests dos factors influeixen en l'aparició de l'ACG és per ara insuficient i està lluny de clarificar perquè tenen impacte sobre la seva incidència (9, 10).

Pel que fa a estudis de susceptibilitat genètica que ajudin a explicar el procés patogenètic de la malaltia, s'han dut a terme molts treballs que mostren associació de determinats polimorfismes a la probabilitat de desenvolupar ACG. Aquestes alteracions genètiques han estat associades sempre en primer lloc, a molècules dels MHC, responsables de la presentació antigènica, fet que reforça la hipòtesi que l'ACG és una vasculitis originada per un antigen.
Així doncs, trobem estudis sobre polimorfismes (SNPs) i altres alteracions genètiques com la presència de microsatèl·lits, associats als gens HLA (HLA-DR3, 4 i 5 i HLA-DRB1, i HLA-B15), però també als gens d'algunes citoquines i els seus receptors (TNF-α, IFN-γ, IL-4, IL-6, IL-18, IL12Rβ2), o en molècules associades a la funció endotelial i a la matriu extracel·lular (VEGF, MMP9) (11, 12).

Actualment, en la literatura, gràcies a la susceptibilitat associada a aquests polimorfismes del complex major d'histocompatibilitat tipus II (MHC II), s'ha consensuat que l'ACG s'inicia a nivell histològic a partir de l'aparició d'un antigen, d'origen encara desconegut, presentat per les cèl·lules dendrítiques a la làmina adventícia de la paret arterial (13, 14). Aquest antigen provoca l'activació i formació d'un infiltrat inflamatori, constituït majoritàriament per macròfags i limfòcits T (majoritàriament CD4+ col·laboradors, però també amb presència d'alguns CD8+ citotòxics), que progressa cap a l'interior de la paret arterial. Durant aquest procés, es dóna el trencament de les làmines elàstiques de l'artèria, gràcies a la síntesi de metal·loproteïnasses (MMPs) i l'alliberament de radicals lliures, i es donen processos de canvi de fenotip de les cèl·lules musculars llises (VSMCs) que conformen la làmina mitja, així com fenòmens apoptòtics. Aquestes cèl·lules canvien el seu patró d'expressió a un patró més proliferatiu i migratori i s'infiltren cap a la làmina íntima. És en aquest punt, on l'infiltrat inflamatori i les pròpies cèl·lules musculars expressen gran quantitat de proteïnes de matriu extracel·lular i factors de creixement i es dóna l'engruiximent o hiperplàsia de la làmina íntima. Un engruiximent que desemboca en la progressiva obturació de la llum del vas i que per tant acaba generant hipòxia i els processos isquèmics en el malalts. Aquest procés final es coneix amb el nom de la formació de la *Neoíntima* (13, 15). I és aquest procés hiperplàsic a la làmina

íntima el principal mecanisme d'oclusió vascular en l'ACG, tot i que n'hi ha d'altres de minoritaris, com la trombosi o l'espasme vascular (16, 17).

A continuació, en la figura 3, es mostra un esquema del procés fisiopatològic complet (18).

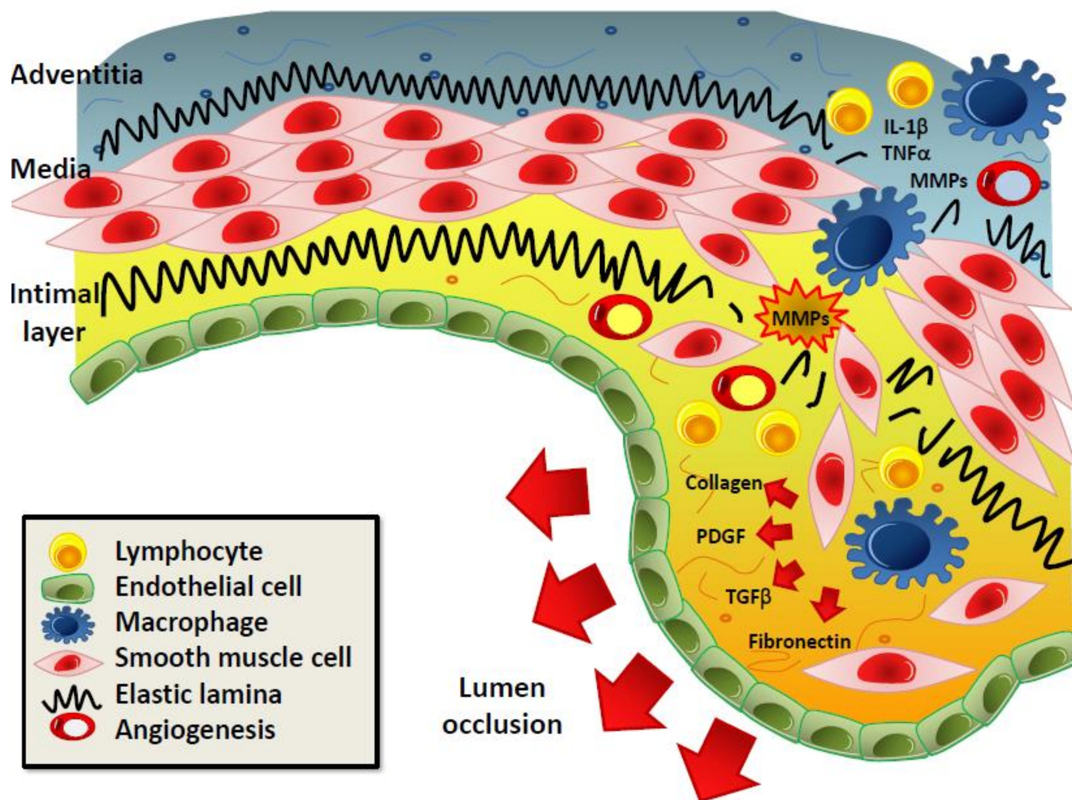

Figura 3. Fisiopatologia de l'ACG

Malgrat s'ha provat d'aprofundir en la identificació d'aquest antigen que inicia la cascada inflamatòria i la expansió oligoclonal dels limfòcits T (19), per ara desafortunadament cap treball ha obtingut conclusions rellevants i només aconseguim parlar d'associacions, però no de causalitats.

En aquest sentit alguns grups han estudiat en primer lloc l'associació de la malaltia a les fluctuacions cícliques epidemiològiques que corresponen a les malalties de caràcter infecciós, trobant èpoques de l'any amb elevada freqüència de diagnòstic respecte altres, cosa que suggeriria la participació d'agents patògens externs (20). Altres treballs en canvi, han provat d'aprofundir en la identificació del patogen presentant alguns candidats com *Chlamydia*

pneumoniae, *Mycoplasma pneumoniae*, Parvovirus B19 o diferents tipus de Herpes Virus que, o bé no han mostrat diferències significatives, o bé aquestes no han pogut ser confirmades en treballs posteriors (21, 22).

Així doncs, per ara, cap microorganisme en particular ha pogut ser descrit encara com a causant de la malaltia.

Tractament

Com ja he citat anteriorment, el tractament establert per l'ACG són els glucocorticoides, que en la major part dels casos condueixen els pacients a la remissió dels símptomes.

No obstant això, els tractaments perllongats amb glucocorticoides presenten una sèrie important d'efectes secundaris no desitjats i limitacions que afecten al 86% dels pacients i que repercuteixen en la qualitat de vida dels malalts de manera significativa (23, 24). Alguns d'ells són els següents:

- Entre un 12 i un 17% dels pacients segueixen patint pèrdua de visió durant les primeres setmanes de tractament amb glucocorticoides.
- Aproximadament un 20% dels pacients desenvolupa dilatacions aòrtiques secundàries que el tractament no aconsegueix evitar.
- Un 10-15% dels pacients també pateixen estenosis de grans vasos i claudicació de les extremitats durant el seguiment a llarg termini.
- El mateix percentatge de pacients presenta resistència als glucocorticoides, requerint dosis dels mateixos superiors a 10 o 15 mg/kg per dia.
- La meitat d'ells no toleren la retirada completa del tractament antiinflamatori en períodes inferiors a 2 anys
- El tractament perllongat amb glucocorticoides presenta efectes secundaris addicionals com l'aparició de hipertensió, miopatia, osteoporosi, cataractes, glaucoma, úlceres gastrointestinals, i en ocasions pot arribar a conduir a la mort del pacient.

Teràpies alternatives i investigació en l'Arteritis de Cèl·lules Gegants

Malauradament, malgrat els glucocorticoides presenten aquesta sèrie important de inconvenients, els tractaments adjuvants testats fins a dia d'avui han resultat tenir una eficàcia limitada (25, 26).

Són diverses les molècules, els medicaments contrastats i els anticossos monoclonals amb finalitat terapèutica que s'han assajat a dia d'avui en l'ACG. Alguns dels exemples més significatius són els que es relaten a continuació:

- *Infliximab*: Es tracta d'un anticòs quimèric específic contra el Factor de Necrosi Tumoral (TNF-α). Malgrat han estat reportats alguns casos de millora en pacients amb ACG de manera puntual, en els assajos clínics més rellevants no ha aconseguit reduir la freqüència de rebrots de la malaltia (27).
- *Methotrexate*: L'administració d'aquest medicament antiproliferatiu, antitumoral i immunosupressor ha demostrat tenir una eficàcia moderada sobre la probabilitat de relapses dels pacients amb ACG, i també un moderat efecte sobre la reducció de la dosi acumulada de corticoides i els seus efectes secundaris (28, 29).
- *Estatines*: Són un fàrmac antiinflamatori i anticolesterolèmic, adequat en pacients amb aterosclerosi. S'han administrat a pacients de ACG amb un tractament perllongat amb glucocorticoides sense mostrar millora en la probabilitat de relapses ni la duració del tractament (30).
- *Aspirina*: Amb l'objectiu d'evitar complicacions isquèmiques que ocasionalment es produeixen durant el tractament amb glucorticoides, s'ha administrat aspirina als pacients amb ACG, amb moderada eficàcia en la prevenció de fenòmens isquèmics en pacients amb alt risc cardiovascular i sempre sota exàmens individualitzats (31).
- *Cyclophosphamide*: Aquest agent immunosupressor ha tingut un efecte positiu modest sobre pacients amb ACG amb alts requeriments de glucocorticoides, malgrat que el nombre de casos estudiat és insuficient per extreure resultats clars (32).

A part dels exposats aquí, s'han realitzat o s'estan duent a terme actualment altres assajos clínics amb inhibidors del TNF-α com *Etanarcept* o l'*Adalimumab*, bloquejants de la IL-6 com el *Tocilizumab*, inhibidors de l'activació de limfòcits T com l'*Abatacept*, agents immunosupressors com l'*Azathioprine*, o teràpies anti-CD20 amb *Rituximab*. Desafortunadament en la majoria dels casos, els resultats no mostren millores significatives en els pacients, estan en fases primerenques de l'assaig o s'han realitzat amb un número de pacients encara massa reduït com per extreure'n conclusions (26).

A més, els assajos clínics no poden iniciar-se després d'un assaig funcional amb un model murí, ja que així com en d'altres vasculitis disposem de models animals d'estudi, com en les vasculitis lligades a ANCA (33-35), en l'ACG específicament no es disposa de cap model animal real per al disseny d'estudis funcionals.

Aquest dèficit de models experimentals ha condicionat fortament durant molt temps l'avanç en l'estudi dels processos patogenètics en l'ACG.

Una de les aproximacions més important de la literatura en aquest sentit, és el model de ratolí quimera de Cornelia Weyand *et al* (36), en el qual s'implanten fragments de biòpsies provinents de pacients amb ACG, a ratolins immuno-deprimits. Aquests ratolins són tractats amb glucocorticoides durant períodes controlats i, transcorregut aquest temps, les artèries són recuperades i analitzades a nivell d'expressió d'RNA de marcadors inflamatoris clàssics com la IL-1β, IL-6 o IFN-γ, entre d'altres.

COMPONENTS MOLECULARS DE LA INFLAMACIÓ

Com ja s'ha comentat a l'inici de la introducció, el procés inflamatori consta d'un component cel·lular (principalment limfòcits, monòcits i en el cas de l'ACG en molta menor mesura, neutròfils) i d'un component molecular, i és un fenomen complex de resposta d'un teixit generat pels agents inflamatoris. Aquests mediadors, són petites molècules de diferents grups (lípids, aminoàcids modificats o proteïnes), que són capaces de lliurar informació a les cèl·lules mitjançant receptors específics, i per tant generar respostes cel·lulars.

Un dels grups més importants dins dels mediadors inflamatoris són les Citoquines.

Les Citoquines

El terme citoquina defineix un ampli grup de molècules d'origen proteic en general amb un baix pes molecular, habitualment solubles i que modulen la comunicació intercel·lular mitjançant la seva unió a receptors específics de membrana. Aquesta unió pot desencadenar activacions o inhibicions d'altres molècules lligades a processos complexes de comportament cel·lular, tan diferents com la migració, la proliferació o la diferenciació funcional de les cèl·lules (18).

En general, els processos de regulació de les citoquines són complexes i presenten múltiples solapaments i contradiccions, segons la concentració de la pròpia citocina, la cèl·lula sobre la que actua, o quina és la cèl·lula alliberadora.

Durant els processos inflamatoris per tant, en rares ocasions, les citoquines actuen de manera lineal en una cadena de fosforilacions que conclogui amb l'activació d'uns factors de transcripció relacionats amb un mateix procés. Contràriament, es donen reforços positius i negatius sobre si mateixes, bifurcacions de les vies, solapament de senyal i molt sovint sinèrgies o estimulacions inesperades i antagonismes.

La complexitat d'aquesta xarxa d'interaccions dificulta la interpretació dels estudis funcionals i els mecanismes compensatoris atenuen possibles bloquejos o estimulacions- Malgrat això, el fet que moltes citoquines puguin ser considerades en un moment o un altre com dianes terapèutiques, és la única via per explicar processos fisiopatològics encara desconeguts i es converteix en la millor eina per millorar el tractament dels pacients.

Les citoquines constitueixen l'òrgan regulador de la inflamació en tots els punts dels que consta aquest procés.

Així doncs, tenim citoquines encarregades de la diferenciació funcional dels limfòcits T, com la IL-12, la IL-4 o la IL-17, que regulen la diferenciació cap a les respostes immunes Th1, Th2 i Th17 respectivament; citoquines activadores d'altres cèl·lules inflamatòries, com el TNFα i la IL-8 (CXCL8), o relacionades amb l'activació i permeabilitat endotelial, com l'IL-1β; citoquines

relacionades amb el remodelat vascular o amb l'angiogènesi, com els factors de creixement VEGF o el PDGF; o finalment citoquines reguladores del trànsit de leucòcits a l'interior de la paret vascular, les quimiocines.

Quimiocines:

La quimiotaxi és el fenomen segons el qual, organismes unicel·lulars o cèl·lules d'un organisme dirigeixen el seu moviment en funció de les concentracions de substàncies químiques del seu ambient.

Les quimiocines conformen una petita família d'una cinquantena de molècules, inductores de la quimiotaxis i altres canvis funcionals en leucòcits *in vitro*. S'han classificat en 4 superfamílies definint-se dos grans superfamílies i uns altres dos grups menors, segons la disposició espacial dels dos primers residus de Cisteïna de l'extrem N-terminal. Es defineixen així en C, CC, CXC o CXXXC (CX_3C) en funció dels graus de separació peptídica d'ambdues cisteïnes (37, 38).

El primer dels grups està constituït tan sols per dues molècules, la més important de les quals és la Limfotactina (XCL1). És una quimiocina específica de limfòcits (tant limfòcits B com limfòcits T) però no macròfags ni monòcits i actua a través del receptor XCR1 (39). També forma part d'aquest grup SCM-1β (XCL2).

Dins les quimiocines CC trobem en primer lloc a CCL-2 (MCP-1), un potent agonista i atraient de cèl·lules dendrítiques, limfòcits T de memòria, basòfils i sobretot de monòcits, i que realitza la seva funció a través d'un únic receptor de membrana, CCR2 (40).

Altres quimiocines importants dins d'aquest grup són CCL-3 (MIP-1α), CCL-4 (MIP-1β), i CCL-5 (RANTES), molècules amb afinitat quimiotàctica per limfòcits T i monòcits, i en menor mesura per eosinòfils, que exerceixen la seva funció principalment a través de CCR5, però que poden unir-se també a altres receptors, com CCR1 o CCR3 (40).

Pel que fa a les quimiocines del grup CXC, destaquen principalment en primer lloc CXCL-8 (IL-8), quimiocina que originàriament es va associar a una major afinitat per la quimiotaxi dels

neutròfils però posteriorment s'ha descrit que pot participar activament també en l'atracció de monòcits cap a l'endoteli vascular (41).

També formen part d'aquest grup, tres quimiocines directament induïdes per IFN-γ, CXCL9 (Mig), CXCL-10 (IP-10) i CXCL-11 (I-TAC), associades al receptor de quimiocines CXCR3, i amb activitat quimioatraient preferent per limfòcits T i neutròfils (40), però que en algun cas també han mostrat tenir efectes sobre monòcits (42).

En el grup de les quimiocines CXXXC o CX_3C, tenim un únic membre, la Fracktalkine (CX3CL1 o Neurotactina) i el seu receptor CX3CR1. Aquesta quimiocina, que es pot trobar en forma soluble o ancorada a membrana, s'expressa en la superfície endotelial i té una potent activitat quimiotàctica per limfòcits T i monòcits (43).

En la taula de la següent pàgina trobem una llista detallada de totes les quimiocines descrites en l'actualitat, i els seus receptors associats, en la què es pot comprovar la complexitat i el solapament d'aquestes interaccions (37, 44).

En la literatura recent trobem molts exemples de la implicació de les quimiocines en la inflamació vascular. En els pacients amb síndrome de Churg-Strauss, per exemple, trobem elevats nivells en sèrum de CCL26 (eotaxina-3) (45), mentre que en l'ACG, s'han descrit elevats nivells en sèrum de CCL2 (46). Aquesta elevada expressió en les lesions de CCL2 s'ha associat a pacients amb elevada persistència de activitat de la malaltia (47), suggerint així una possible implicació d'aquesta quimiocina en el manteniment de la inflamació en l'ACG.

Altres exemples del paper de les quimiocines o els seus receptors i la seva associació en vasculitis són: elevades concentracions de Fractalkine en pacients amb MPA (paper Fractalkine Kasama), producció de les quimiocines CCL18, CCL19 i CCL21 per part de cèl·lules dendrítiques diferenciades en lesions d'ACG (48), o elevats nivells d'expressió de CCR5 en limfòcits CD4+CD28- en la malaltia granulomatosa de Wegener (WG) (49).

Molts membres d'aquests quatre grups de molècules mostren homologies estructurals importants i, tal i com hem vist en la taula 1, sovint veuen sobreposades i redundants les seves funcions quimioatraients específiques.

Nom sistemàtic	*Nom comú*	*Receptor(s)*
Quimiocines i receptors família CXC		
CXCL1	GROα/MGSA-α	CXCR2
CXCL2	GROβ/MGSA-β	CXCR2
CXCL3	GROγ/MGSA-γ	CXCR2
CXCL4	PF4	CXCR3
CXCL4L1	PF4V1	CXCR3
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2 , PPBP	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	Mig	CXCR3
CXCL10	IP-10	CXCR3
CXCL11	I-TAC	CXCR3, CXCR7
CXCL12	SDF-1α/β	CXCR4, CXCR7
CXCL13	BLC/BCA-1	CXCR5
CXCL14	BRAK/bolekine	*Desconegut*
CXCL16	SR-PSOX	CXCR6
CXCL17	BMC	*Desconegut*
Quimiocines i receptors família C		
XCL1	Lymphotactin/SCM-1α/ATAC	XCR1
XCL2	SCM-1β	XCR1
Quimiocines i receptors família CX3C		
CX3CL1	Fractalkine	CX3CR1
Quimiocines i receptors família CC		
CCL1	I-309	CCR8
CCL2	MCP-1/MCAF	CCR2
CCL3	MIP-1α/LD78α	CCR1, CCR5
CCL3L1	LD78β	CCR1, CCR3, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
CCL7	MCP-3	CCR1, CCR2, CCR3
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5
CCL9/10	MRP-2, CCF18	*Desconegut*
CCL11	Eotaxin	CCR2, CCR3
CCL12	MCP-5	CCR2
CCL13	MCP-4	CCR1, CCR2, CCR3
CCL14	HCC-1	CCR1, CCR3, CCR5
CCL15	HCC-2/Lkn-1/MIP-1δ	CCR1, CCR3
CCL16	HCC-4/LEC	CCR1, CCR2, CCR5
CCL17	TARC	CCR4
CCL18	DC-CK1/PARC	*Desconegut*
CCL19	MIP-3β/ELC/exodus-3	CCR7, CCRL1, CCRL2
CCL20	MIP-3α/LARC/exodus-1	CCR6
CCL21	6Ckine/SLC/exodus-2	CCR7, CCRL1
CCL22	MDC/STCP-1	CCR4
CCL23	MPIF-1	CCR1
CCL24	MPIF-2/Eotaxin-2	CCR3
CCL25	TECK	CCR9, CCRL1
CCL26	Eotaxin-3	CCR3, CX3CR1
CCL27	CTACK/ILC	CCR10
CCL28	MEC	CCR3, CCR10

Taula 1. Quimiocines i els seus receptors

Altres molècules inflamatòries

A més de les citoquines i les quimiocines, existeixen altres molècules amb papers importants durant les cascades inflamatòries que es donen en l'ACG, i que convé conèixer i tenir en compte pel desenvolupament d'aquesta tesi.

Entre elles trobem aquelles molècules relacionades amb la matriu extracel·lular, com les Metalloproteïnases (MMPs). Els processos inflamatoris troben el seu antagonisme en els processos fibròtics i de reparació del teixit, i és en aquest procés on les proteïnes de matriu són més rellevants. Les MMPs, per exemple, són enzims encarregats de la degradació de les proteïnes que conformen la matriu extracel·lular i juguen un paper important durant el remodelat vascular (50). També formen part de les proteïnes de matriu els Col·làgens, que suposen la matèria prima del procés fibròtic.

Altres molècules importants són les molècules d'adhesió (ICAM-1 i VCAM-1), imprescindibles en el trànsit leucocitari i en la comunicació cel·lular, i per tant en el procés de migració de les cèl·lules de l'infiltrat inflamatori.

I finalment, en aquest grup de altres molècules relacionades amb els processos inflamatoris, trobem factors de creixement com el PDGF o el VEGF, potents estímuls proliferatius per molts tipus cel·lulars, entre ells per les VSMCs, i que poden participar de l'oclusió vascular i per tant trobar-se associats a fenòmens isquèmics en els malalts amb ACG (51).

Els interferons

Finalment, i també dins de les citoquines, un grup fonamental de molècules relacionades amb multitud de processos inflamatoris i d'activació de leucòcits, són els interferons.

Tot i que en un principi van ser descrits com agents que interferien en la replicació viral (52) i es van classificar segons el tipus cel·lular que els secretava, actualment la família d'interferons es classifica en tres grans grups: tipus I, tipus II i tipus III, segons l'especificitat del receptor, la homologia en la seqüència i la funció biològica (53, 54).

Els interferons de classe I, comprenen IFN-β, IFN-ω, IFN-κ IFN-ε i tots els subtipus de IFN-α, i s'uneixen a un únic receptor heterodimèric: IFNAR (compost per les cadenes IFNAR1 i IFNAR2). Són secretats per la major part dels tipus cel·lulars però en baixes concentracions i són responsables de la inducció d'una potent resposta antiviral (55).

Els interferons de classe III, o IFN-*lambdas* (IFN-λ1, 2 i 3, també anomenats IL-29, IL-28A i IL-28B) són els més recentment incorporats al grup d'interferons. Descrits per primer cop al 2003, tenen com a receptor el dímer de proteïna IFNλR1-IL10R2, es caracteritzen per compartir vies de senyalització amb els interferons classe I, activant el complex d'histocompatibilitat tipus I (MHC I), i tenint un paper rellevant en la defensa enfront dels virus i en l'activitat antitumoral (54, 56, 57).

L'únic interferó descrit com a tipus II és l'interferó-*gamma* (IFN-γ). Té una estructura diferent als tipus I i III. Està codificat en un *locus* cromosòmic separat i s'uneix a un receptor també diferent. A més, al contrari que en les altres classes d'interferons, l'activitat antiviral s'ha demostrat que no és la seva principal funció biològica (58).
En l'ACG sembla tenir un paper destacat donada la diferència en la seva expressió entre pacients i controls, sobretot si tenim en compte la rellevància de la via Th1 en aquesta malaltia. L'IFN-γ mostra, a més, certa resistència a la disminució de l'expressió en les lesions durant les primeres setmanes del tractament amb glucocorticoides (59).

L'interferó gamma (IFN-γ)

L' IFN-γ és una petita molècula peptídica de 143 aminoàcids i que pesa aproximadament uns 20kDa. Es tracta d'una citoquina dimèrica, cadascun dels monòmers de la qual es conforma per un nucli de 6 α-hèlix i un extrem C-terminal no plegat (60).
És produït per diferents tipus cel·lulars: limfòcits T CD4+, limfòcits CD8+ citotòxics, cèl·lules *Natural Killers* (NK), cèl·lules B, monòcits, macròfags i cèl·lules dendrítiques.

Aquesta producció d'IFN-γ és controlada per citoquines secretades per les cèl·lules professionals presentadores d'antígens (APCs), principalment les interleucines (IL)-12 i IL-18.

Entre els reguladors negatius de l'IFN-γ es troben altres interleucines (IL-4 i IL-10), TGF-β i els glucocorticoides (61, 62), malgrat com ja hem dit, s'ha demostrat que l'IFN-γ es manté elevat en les lesions més temps que altres citoquines durant les primeres setmanes de teràpia corticoidea (59).

El receptor de l'IFN-γ (IFNGR) és un tetràmer format per dues cadenes IFNGR1 associades a dues cadenes IFNGR2, totes elles amb activitat quinasa/fosfatasa intrínseca associades a maquinària de transducció de senyals. IFNGR1 té dos *locus* d'unió a Jak-1 i STAT1 (*Janus Tyrosine Kinase* i *Signal Transducer and Activator of Transcription*), que es requereixen per la fosforilació del receptor i la inducció de la resposta biològica.
Aquest receptor, es troba expressat en tots els tipus cel·lulars exceptuant els eritròcits madurs (63).

Vies de senyalització

Quan un dímer (no covalent) d'IFN-γ s'uneix al seu *locus* d'unió de la part extracel·lular del dímer de IFNGR1 (64), es creen uns llocs d'unió intercel·lular per a IFNGR2. Totes dues cadenes porten associades una subunitat de Jak-1 i Jak-2 respectivament, i la proximitat d'ambdues molècules en permet a la vegada la auto-fosforilació primer de Jak-2 i posteriorment la trans-fosforilació de Jak-1. Aquestes fosforilacions es segueixen de la fosforilació de les Tirosines de l'extrem C-terminal de la part citoplasmàtica dels IFNGR1, mitjançant Jak-1, i aquests residus són reconeguts pel domini SH2 de dues molècules STAT-1, que s'hi uneixen. Aquesta unió en provoca a la vegada la seva fosforilació mitjançant quinases d'unió a receptor, el seu desanexament i el seu viatge al nucli en forma de homodímer d'STAT-1. Aquest homodímer s'anomena GAF (*Gamma Activated Factor*). Les molècules d'STAT-1 fosforilat també poden formar heterodímers amb STAT-2 o STAT-3, que en menor mesura, també es veuen fosforilats per IFN-γ.
La família dels STATs està formada per set membres (STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b, STAT-6) (65), i la dimerització de tots ells també es dóna gràcies al seu domini SH2.

Un cop translocat al nucli mitjançant activitat GTPasa de RAN/TC4, gràcies a la unió de STAT-1 a les importines $\alpha5$ i $\beta1$ (66), GAF s'uneix de manera canònica a les seqüències GAS (*Gamma activated sequence*) del promotor de determinats gens, provocant-ne l'inici de transcripció. És el cas de IRF-1 o IRF-9, per exemple (67). Aquests elements GAS, poden contenir petites variacions en la seva seqüencia que els confereixen diferent afinitat i especificitat pels diferents tipus d'STATs, constituint d'aquesta manera un fenomen regulador de la transcripció.
El propi promotor de IFN-γ per exemple, conté una seqüencia GAS en el seu promotor, específica per STAT-4, factor de transcripció induït per IL-12 (67).

No obstant, en la via no canònica, STAT-1 fosforilat ja sigui en forma de homodímer, o heterodímer amb altres STATs, també és capaç d'interaccionar amb multitud d'altres factors de transcripció translocats al nucli.
És la variabilitat d'aquestes interaccions el segon fenomen que regula la activació diferencial d'uns paquets de gens o uns altres, segons les necessitats i dinàmiques cel·lulars. Així per exemple, un dímer d'STAT1 (o una molècula d'STAT1 i una d'STAT2, en menor mesura), units a p48 (IRF-9), conformen el factor de transcripció conegut com ISGF3, que no s'uneix als diferents models de seqüències GAS, com GAF, sinó que ho fa a les seqüències ISRE, encarregades d'activar grups de gens diferents (68-70).
IRF-1 i IRF-9 a més, regulats positivament per GAF, participen en la perpetuació de senyal de dues maneres. En primer lloc IRF-1 estimula la producció de STAT1, unint-se al seu promotor. I IRF-9 per la seva part, forma part del complex ISGF3 que participa de la via de senyalització no canònica (53).
ISGF3 regula per exemple, entre d'altres, IRF-2, o IFN-β, (53) i podria participar en la regulació de l'expressió de les quimiocines clàssicament descrites a la literatura com directament induïdes per IFN-γ (CXCL9, 10 i 11).

Quins són els factors de transcripció a través dels quals l'IFN-γ controla l'expressió d'aquestes quimiocines és una qüestió pobrament abordada a la literatura. No en tots els articles s'assumeix que GAF regula totes tres quimiocines (71). Així, els ratolins *knock-out* (KO) per STAT1 presenten dèficit en la producció d'algunes quimiocines, però aquest fet no permet

distingir si es dóna una estimulació d'STAT1 de manera directa via GAS, o de manera indirecta via ISRE, ja que STAT1 forma part tan d'un factor de transcripció com de l'altre. En el promotor de CXCL9 per exemple, s'ha descrit la presència d'una seqüència γ-RE1, homòloga de GAS, diana del factor de transcripció γ-RF1, que en regularia la transcripció de manera independent de GAF i ISGF3, però també sota l'estimulació de IFN-γ (72, 73).

Un altre exemple de combinació de factors de transcripció, és el que ocórre en la regulació de ICAM-1, on es requereix la unió del factor de transcripció SP1 a una molècula d'STAT1 per induir l'inici de la transcripció (74).

A més de la dualitat GAS / ISRE en el control de la transcripció gènica a través dels STATs induïts per IFN-γ, trobem altres seqüències homòlogues, com els SBE (*stat binding elements*) (75, 76), també subjectes a la regulació mitjançant factors de transcripció derivats de la combinació dels diferents STATs.

En la següent figura podem observar una il·lustració esquemàtica de la via de senyalització canònica de l'IFN-γ.

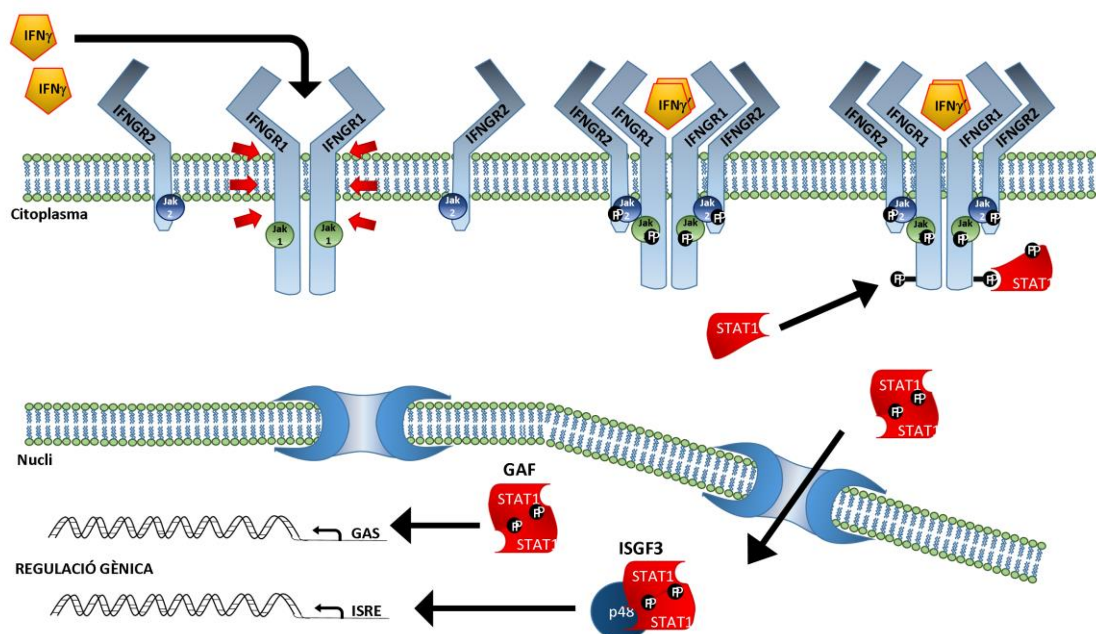

Figura 4. Via senyalització de l'IFN-γ.

Així doncs, com ja hem indicat, STAT1 no és la única via de senyalització de l'IFN-γ. Una evidència n'és que ratolins KO per STAT1 són significativament més resistents a algunes infeccions que ratolins KO pels receptors d'IFNs (77).

En absència de STAT1 de fet, l'IFN-γ fosforila i activa la síntesi de STAT3 de manera molt més significativa que en presència de STAT1 (78).

D'aquesta manera, podem parlar de dues vies de senyalització, STAT1 i STAT3 que, malgrat tenir paral·lelismes i sobreposicions de gens, i compartir mecanismes de senyalització amb dianes gèniques similars però no idèntiques, en alguns casos acaben tenint efectes oposats sobre el comportament cel·lular i poden presentar múltiples antagonismes.

Els STATs 1 i 3 en realitat, competeixen pel mateix locus d'unió a la Tirosina fosforilada de IFNGR1 a través dels seus dominis SH2, però s'ha descrit que STAT1, que representa la via canònica, té molta més afinitat per aquesta unió que STAT3. Aquesta competència i diferència d'afinitat es dóna també en els altres receptors de citocines associats a la maquinària d'activació d'STATs, cadascun dels quals té afinitat específica per unes tirosines fosforilades o unes altres (79).

Així doncs, mentre STAT1 actua com una proteïna supressora de tumors i inhibidora de la proliferació, STAT3, en absència de STAT1, actua com un oncogen antagonitzant les accions proapoptòtiques que duria a terme STAT1 (78).

Sembla per tan, i moltes línies d'investigació apunten en aquest sentit, que tots dos STATs (1 i 3) estarien implicats en la supervivència de diferents components cel·lulars de la paret vascular, i presentarien rols oposats tan en la regulació de la inflamació com en la de la proliferació en la gran majoria de tipus cel·lulars.

Les vies d'inducció de la mort cel·lular programada i inhibició de la proliferació de STAT-1, passen per l'activació de nombrosos gens relacionats amb el procés apoptòtic, com caspases, receptors de mort cel·lular, o la sintasa d'Òxid nítric, i la inhibició de senyals de supervivència cel·lular com Bcl-2.

STAT-3 en canvi, que té un nivell d'activació més elevat en contextos no inflamatoris, promou la proliferació a través de la inducció d'oncogens i gens reguladors del cicle cel·lular, com c-

myc, pim-1 o la ciclina D1, i inhibeix l'apoptosi a través de l'activació de Bcl-2. Malgrat això, aquestes activacions presenten algunes excepcionalitats segons l'estadi patològic de certs tipus cel·lulars en alguns tumors, com el glioblastoma o algunes línies cel·lulars mieloides, on probablement, STAT-3 actua en concert amb altres factors de transcripció, co-activadors i co-repressors simultàniament (80).

Pel que fa als processos inflamatoris, STAT-1 actua com a molècula proinflamatòria de manera directa activant gens que afavoriran el reclutament de cèl·lules del sistema immune com les quimiocines o les molècules d'adhesió, activant les vies dependents de NFκB, la producció d'espècies reactives d'oxigen que són en part responsables de la vasodilatació de la zona inflamada, i incrementant l'expressió de molècules dels complexes majors d'histocompatibilitat I i II.

STAT-3 per la seva part regula IL-10, citocina antiinflamatòria que juga un paper fonamental en el balanç d'activació i desactivació de les cèl·lules mononuclears, i que inhibeix l'expressió de gens de superfície de les APCs (80).

Finalment, un últim mitjà de regulació d'aquests efectes antagònics entre STAT1 i STAT-3 és la formació d'heterodímers. Malgrat es desconeix en gran mesura quina funció porten a terme aquests heterodímers STAT-1/STAT-3, si que sembla clar que tenen un efecte antiinflamatori, suggerint que les molècules d'STAT-3 segresten les d'STAT-1 bloquejant la resposta inflamatòria dels homodímers d'STAT-1, però que la formació d'aquests heterodímers també inhibeix l'estimulació de la proliferació pròpia dels homodímers d'STAT-3, potenciant la mort cel·lular (81).

En el control de regulació per altres vies sobre els diferents STATs trobem dos tipus de molècules diferents, aquelles que actuen en el citoplasma, impedint el mateix procés de fosforilació, o les que actuen a l'interior del nucli bloquejant els llocs d'unió al DNA.

En el primer del tipus trobem la família de molècules anomenades SOCS, que consta de vint membres (classificats en funció del seu tipus de domini central) i realitzen la seva funció adherint-se de manera competitiva als llocs d'unió dels receptors o a la subunitat catalítica dels Jaks bloquejant l'activació i fosforilació dels STATs. Així doncs per exemple, SOCS1 (que té

una diana de IRF-1 al seu promotor) es troba expressat en cèl·lules Th1 regulant la diferenciació d'aquesta via limfocitària i l'expressió d'IFNγ, mentre que SOCS3 (que en el seu promotor conté una seqüència GAS) ho fa en la via Th2, regulant l'expressió de IL-4 (82, 83).

En el segon grup, tenim les proteïnes anomenades PIAS, que inhibeixen l'activitat dels dímers fosforilats d'STATs a l'interior del nucli segrestant-los bloquejant els seus llocs d'unió al DNA.
A aquesta regulació, s'hi ha d'afegir el control sobre la fosforilació dels STATs, que a més, regula el seu trànsit cap a l'interior nuclear. Les fosfatases PTP1B o les del grup SHP (1 i 2), regulen el procés de desfosforliació dels dímers d'STAT a l'interior del nucli, procés que un cop complert es converteix ràpidament en una senyal d'exportació a citoplasma (82).
Aquesta regulació cobra més rellevància si tenim en compte que s'ha descrit que les molècules d'STAT tenen certa capacitat de regulació gènica independent del procés de fosforilació. És a dir, que poden viatjar a nucli en forma dimèrica no fosforilada, units a altres proteïnes d'unió del citoplasma, i actuar com a factors de transcripció de paquets de gens diferents.
L'IFN-γ estimula l'expressió dels STATs 1 i 3 també en aquesta forma no fosforilada (84).

Funcions de l'IFN-γ en la inflamació i en l'autoimmunitat:

En els processos immunològics, la secreció d'IFN-γ per part de les cèl·lules NK i les APCs (monòcits, macròfags i cèl·lules dendrítiques) regula la defensa innata de l'organisme enfront la infecció, mentre que la secreció per part dels limfòcits T s'encarrega de la resposta immune adquirida o adaptativa (61, 85).

Alguns treballs han demostrat que els ratolins KO per IFN-γ presenten baixa activitat basal de cèl·lules NK (86), i també se sap que el dèficit en la producció d'IFN-γ en nadons (al primer mes de vida) provoca dèficit en la mobilitat dels neutròfils i baixa activitat de les cèl·lules NK. Això posa de manifest que, malgrat l'IFN-γ està menys lligat que els IFNs tipus I a la defensa de l'hoste contra microorganismes, la importància d'aquesta molècula en la resposta inflamatòria lligada a la resposta immune innata és significativa (87).

D'aquesta manera, mutacions que provoquen pèrdua de funció en la cadena de IFNGR1 o 2, estan associades a una pobra resposta immune enfront infeccions de micobacteris o altres microorganismes (88, 89).
En aquest sentit, és interessant l'associació que s'ha trobat entre longevitat i presència de polimorfismes en l'IFN-γ. Es proposa que la pèrdua mínima d'activitat inflamatòria que provoquen els polimorfismes, no té un impacte significatiu en la capacitat de fer front a les infeccions per part de l'hoste, però en canvi, pot resultar un factor de prevenció en malalties relacionades amb la inflamació, com les malalties cardiovasculars, neurodegeneratives, osteoartritis, osteoporosi, o diabetis (90).

Pel que fa a la resposta adquirida, l'IFN-γ estimula l'expressió del complex major d'histocompatibilitat (MHC) tipus I, que conclou amb l'activació dels limfòcits CD8+ citotòxics, i exerceix aquest control a través d'una seqüència ISRE (83).
També en la resposta adquirida, deixant de banda la resposta citotòxica, quan parlem dels efectes que l'IFN-γ pot tenir *in vitro* en termes de biologia vascular cal destacar en primer lloc que *per sé* és capaç d'activar la via de senyalització que porta l'expressió de MHC de classe II, i promoure per tant l'activació pèptid-específica de les cèl·lules T CD4+ col·laboradores (53). Un dels mecanismes que exerceix en aquesta regulació és incrementar l'expressió de CIITA, el regulador principal de les molècules MHC de classe II (91).

A més, pel que fa al control sobre el sistema immune, a part de la regulació sobre l'expressió dels dos tipus de MHCs, l'IFN-γ és també responsable de la regulació en uns altres tres punts.

En primer lloc, participant en l'activació de la via de diferenciació limfocitària Th1 i promovent el desenvolupament de les cèl·lules Th1 a diferents nivells.
L'IFN-γ activa la síntesi de IL-12 en les APCs, i aquesta IL-12 estimula el propi promotor de l'IFN-γ mitjançant STAT-4. A més l'IFN-γ també estimula la síntesi de la subunitat β2 de IL12R en els limfòcits T CD4+, mantenint activa la resposta a IL-12 i facilitant-ne la diferenciació Th1.
Paral·lelament, l'IFN-γ també inhibeix els components de la via Th2 de diferenciació limfocitària, mitjançant l'estimulació de IRF-1 de manera directa, i IRF-2 de manera indirecta (via IRF-9).

Aquests dos factors de transcripció s'uniran al promotor de IL-4 bloquejant-lo. Això és coherent amb el fet que en les altres vies també existeixen elements inhibidors. Així doncs, el factor de transcripció propi de la via Th2, GATA3, és un inhibidor de STAT-4 (53, 68, 83).

Malgrat això, és important recordar en aquest punt que, tal i com hem comentat abans, l'IFN-γ també indueix (via STAT-1) l'apoptosi dels limfòcits a llarg termini.

Aquest complex sistema de regulació sobre la diferenciació dels limfòcits Th0 *naive* es troba detallat en la figura 5 (a continuació).

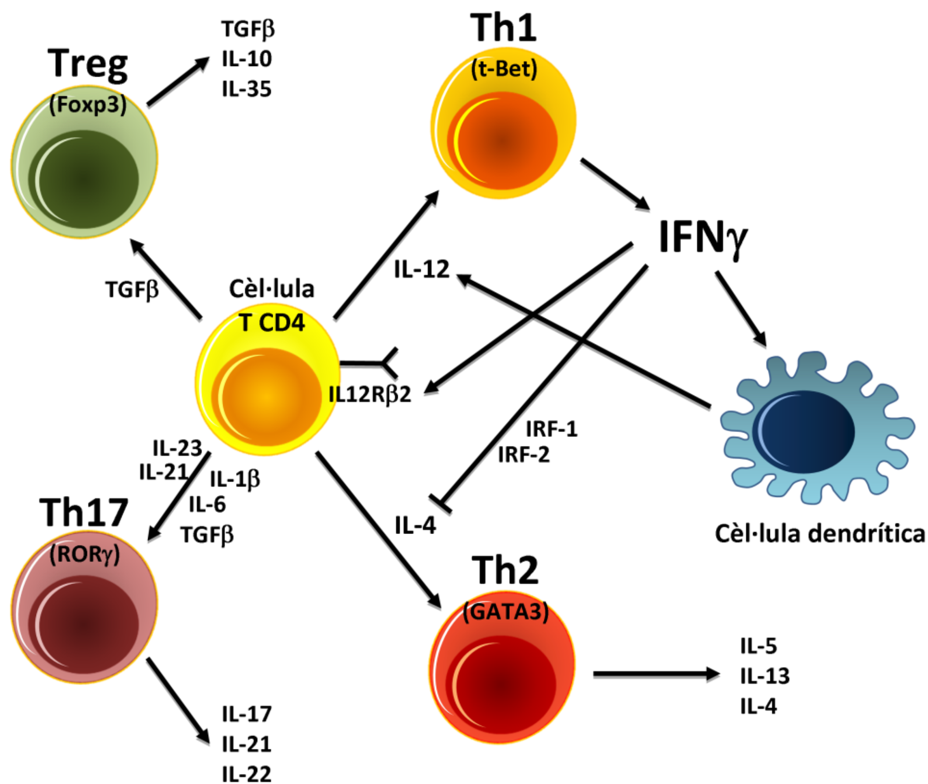

Figura 5. Esquema diferenciació cèl·lules Th0.

En segon lloc, l'IFN-γ és el factor principal promotor de l'activació dels macròfags. Aquest procés, que es defineix com unes alteracions funcionals i bioquímiques, reversibles, que donen activitat citotòxica (92), és específic i exclusiu del tipus II d'IFNs. Així doncs, ratolins KO per IFN-γ, IFNGR1 o STAT-1, són altament susceptibles a infeccions.

Els macròfags activats, tenen diverses vies per conduir a la mort microbiana o cel·lular, i una de les més important és la generació d'espècies reactives d'oxigen i nitrogen, que són activats de manera directa per IFN-γ, i activen la mort cel·lular produint dany mitocondrial i estimulant l'activitat oxidativa (68, 83).

Per acabar, en tercer lloc, l'IFN-γ participa activament en la regulació de les cèl·lules B i la immunitat humoral.

Així doncs, l'IFN-γ estimula el desenvolupament i la proliferació de les cèl·lules B inhibint la fosforilació de CREB, l'inhibidor de la proliferació de cèl·lules B induïda per la unió d'antigen-receptor. També estimula la secreció d'immunoglobulines (Igs), però el procés en el que participa activament i està més àmpliament descrit, és en l'intercanvi de cadenes pesades de les pròpies Igs. L'IFN-γ estimula el canvi de IgM a IgG2a i IgG3, a la vegada que l'expressió de t-Bet facilita l'eficàcia del procés. A més d'aquesta inducció de IgG2a, l'IFN-γ també actua de manera indirecta a través de la inhibició de IL-4, que activa el canvi de IgM a IgE i IgG1. La inhibició d'aquest canvi es dóna probablement via SOCS1, que redueix la fosforilació de STAT6, inhibint la via de senyalització de la IL-4 (68, 83).

Pel que fa als efectes de l'IFN-γ *in vivo* sobre el control de la inflamació, s'ha descrit que tot i que ratolins KO per IFN-γ o pel seu receptor no presenten defectes en el desenvolupament i tenen un sistema immune aparentment normal (62), si que la infecció d'aquests ratolins KO amb un *Herpes Virus* fa que desenvolupin formes greus necrosants (però no granulomatoses) de vasculitis de grans vasos (35). Això suggereix que l'IFN-γ pot tenir un paper dual en la inflamació, i per tant també en l'autoimmunitat i l'ACG, per una banda limitant de la destrucció vascular, i per l'altra essent l'eix de l'activació dels macròfags en l'inici de la resposta immunològica.

Un altre exemple en l'autoimmunitat del paper proinflamatori de l'IFN-γ, són els elevats nivells en plasma d'aquesta citocina que trobem a la sang de pacients amb Lupus (93). En el model de ratolí de lupus de fet, els ratolins KO per IFN-γ o per IFNGR1 tenen menys afectació per la

malaltia, i bloquejant IFN-γ amb tractaments específics s'observa una millora significativa en els ratolins malalts (94).
Però en altres casos, si ens centrem en aquesta conducta bipolar de l'IFN-γ, tenim l'exemple del model murí d'Artritis induïda per col·lagen, en el què el tractament bloquejant l'IFN-γ millora els ratolins malalts, però els empitjora en estadis més tardans de la malaltia (95).

Una possible explicació d'aquesta bipolaritat és que l'IFN-γ sembla promoure l'inici de la resposta immune però redueix la inflamació a temps tardans exercint el seu rol antiproliferatiu i proapoptòtic sobre les cèl·lules T.

Lligant amb aquest concepte dual de la molècula, en alguns models animals, el dèficit d'IFN-γ no sembla tenir cap efecte significatiu sobre la producció d'aneurismes aòrtics abdominals, fet que sí succeeix si els ratolins tenen dèficit de IL-4 (96), mentre que en altres models el bloqueig de l'IFN-γ facilita el desenvolupament d'aneurismes aòrtics induïts per transplantament al·logènic (97).

Malgrat això, en l'ACG, com ja hem dit abans, IFN-γ es troba elevat en les lesions durant les primeres setmanes de tractament (36). Si aquests alts nivells són la conseqüència d'una acció compensatòria o protectora de la pròpia citoquina, o es tracta d'un fenomen associat a la supervivència de la inflamació a la paret arterial, és una de les qüestions no resoltes a la literatura, i que la present tesi pretén dilucidar.
I és que la fina línia que separa l'autoimmunitat de la lluita contra la infecció, atorga a l'IFN-γ un rol dual cabdal en tots els processos inflamatoris, un rol subjecte a una complexa regulació per múltiples factors, i que dificulta la interpretació dels estudis funcionals en els que n'és l'objectiu.

Sembla clar en tot cas, que l'IFN-γ promou, via activació dels macròfags, la formació de granulomes, la presència dels quals és una de les lesions característiques en l'ACG. En absència de IFN-γ en canvi, observem lesions de caràcter necrosant. Aquest fet lliga amb la capacitat proapoptòtica de la via canònica de senyalització de l'IFN-γ, STAT1, que es caracteritza per ser antitumoral i antiproliferativa.

No obstant, aquest paper protector és només una cara de la moneda. A l'altra, tenim una citoquina capaç de engegar el procés immunològic a diferents nivells, provocant la diferenciació limfocitària i activant el component molecular i cel·lular de la maquinària inflamatòria que a la llarga pot conduir a la perpetuació de la lesió a la paret arterial.

Hipòtesi

HIPÒTESI

El tractament amb glucocorticoides és per ara la única via terapèutica viable per les persones que pateixen Arteritis de Cèl·lules Gegants.

No obstant, els glucocorticoides han demostrat generar en els pacients una quantitat important d'efectes secundaris, a la vegada que no eviten complicacions tardanes durant el seguiment del pacient tractat. En un percentatge considerable dels casos a més, no aconsegueixen prevenir els rebrots de la malaltia, ni efectes de les complicacions isquèmiques com la ceguera. Resulta necessari per tant, aprofundir en la recerca de teràpies alternatives que o bé minimitzin els efectes secundaris dels glucocorticoides i els inconvenients que es deriven del seu ús, o bé els substitueixin de manera parcial o total.

En aquesta línia de treballar en la millora de la teràpia corticoidea, l'IFN-γ s'ha postulat en la literatura, com una molècula responsable de multitud de processos relacionats íntimament amb les cascades inflamatòries, la diferenciació de limfòcits, i l'activació de macròfags. No obstant això, el seu paper a l'interior de l'artèria durant la formació i el manteniment de l'infiltrat inflamatori en l'ACG no ha estat encara del tot descrit.

A més, malgrat en altres vasculitis si que existeixen certs models animals per a l'estudi experimental, en l'Arteritis de cèl·lules Gegants no es disposa cap model animal real per a la realització d'aquests necessaris estudis funcionals.

Les nostres hipòtesis de treball per tant són:

- El model de cultiu d'arteria temporal serà un bon model experimental per a realitzar estudis funcionals que permetin obrir el ventall d'agents terapèutics per l'ACG.
- L'IFN-γ suposa una diana terapèutica interessant degut a la seva implicació en els fenòmens inflamatoris en la resposta Th1, descrita com la principal via de diferenciació limfocitària en l'ACG.

Objectius

OBJECTIUS

1. Desenvolupar i establir un model experimental validable per a la realització d'estudis funcionals, basat en el cultiu d'artèria temporal humana de pacients amb Arteritis de Cèl·lules Gegants sobre Matrigel.
2. Confirmar el potencial i identificar les limitacions del model de cultiu d'artèria, avaluant la sensibilitat a la manipulació farmacològica, ja sigui mitjançant l'ús de fàrmacs clàssics o de molècules complexes o agents biològics.
3. Analitzar i aprofundir en el paper de l'IFN-γ en l'Arteritis de Cèl·lules Gegants, buscant mitjançant estudis funcionals en teixit i en cèl·lules aïllades el rol d'aquesta citoquina en els processos patogenètics de la malaltia, i el seu paper en la producció d'altres molècules i factors de transcripció, i en el manteniment i persistència de l'infiltrat inflamatori.
4. Analitzar la capacitat per inhibir respostes rellevants d'un anticòs monoclonal completament humà bloquejant de l'IFN-γ (Clone A6), definint per tant la possibilitat d'establir l'IFN-γ com una diana terapèutica en aquesta malaltia.

Materials i mètodes

MATERIALS I MÈTODES

Pacients, avaluació histopatològica

Durant el període de 2007 fins al 2013, 272 pacients van ser avaluats a la nostra institució i dirigits a la prova diagnòstica de biòpsia d'artèria temporal. Finalment només 95 d'aquests exemplars de biòpsia van presentar lesions d'ACG.

En el nostre primer treball del model de cultiu d'artèria es van incloure 50 pacients, 28 amb ACG i 22 controls.

Totes les dades relatives als pacients del primer estudi, es troben detallades més endavant en els *Material i Mètodes* de l'article.

El segon treball va ser dissenyat incloent 68 pacients, 38 dels quals presentaven biòpsia positiva per ACG. Entre les biòpsies negatives trobem un ampli ventall de patologies, incloent-hi polimiàlgia reumàtica (PMR) síndrome constitucional, artritis reumatoide (AR), infart cerebral, cefalea, distonia cranio-cervical, anèmia crònica, sarcoïdosi, demència vascular, poliarteritis nodosa (PAN), o pneumònia organitzativa croptogènica.

Un important factor limitant per a la inclusió dels pacients a l'estudi, és que el segment d'artèria sigui massa curt al moment de la biòpsia per motius quirúrgics, ja que l'artèria té l'objectiu diagnòstic en primer terme i, de recerca, en segon.

Un cop superada aquesta limitació, els criteris excloents de l'estudi són que el diagnòstic final resulti en altres vasculitis, o que la biòpsia, de ser negativa, presenti una temporal inflamada o inflamació de les artèries colaterals.

Aquests criteris per tant, expliquen la diferència entre el total de pacients diagnosticats de ACG a l'Hospital Clínic via biòpsia d'artèria temporal i el nombre total de pacients utilitzats en els estudis de cultiu d'artèria.

Els pacients inclosos al segon estudi (41 dones i 27 homes) tenen una mitjana d'edat de 77,33 anys i es comprenen en un rang entre 57 i 88 anys.

Pacients i controls estan aparellats i no mostren diferències en quant a edat i gènere tal i com es mostra a la següent taula.

	n	*Edat (±DS)*	*Gènere (% ♀)*
Pacients GCA	38	77,39 ± 6,72	60,53% (23/38)
Controls	30	77,25 ± 8,14	60% (18/30)
Total	68	77,33 ± 7,43	60,29% (41/68)

Taula 2. Dades pacients.

El 28,95% del total dels pacients amb ACG, estaven tractats al moment de la biòpsia, amb dosis superiors als 60mg/kg/dia, o almenys amb l'administració d'un Bolus de glucocorticoides.

Tots dos estudis han estat aprovats pel Comitè Ètic de l'Hospital Clínic de Barcelona i els pacients van firmar els consentiments informats corresponents.

Cultiu d'artèria temporal

Les seccions d'artèries temporals obtingudes a partir del material sobrant de les biòpsies amb finalitat diagnòstica, són processades segons el protocol descrit a l'article del Model de cultiu d'artèria temporal humana (98).

La viabilitat dels leucòcits que escapen de la biòpsia s'assaja amb *Trypan Blue* (Sigma, Ayrshire, UK).

Per el segon estudi, entre 3 i 4 seccions de cada biòpsia, en funció de la longitud de la mostra, es tracten amb l'anticòs anti-IFN-γ Clone A6 (10μg/mL, Novimmune, Switzerland), IFN-γ recombinant (100ng/mL, R&D Systems, Minneapolis, Minesota, USA), Immunoglobulina humana IgG1 (10μg/mL, Sigma), Dexametasona (0,5 μg/mL, Sigma), o es mantenen sense tractament, durant un període entre 5 i 10 dies.

Cultiu de cèl·lules del múscul llis

Les cèl·lules vasculars del múscul llis (VSMC) són extretes de biòpsies de pacients controls seguint el següent protocol:

Es cultiven les seccions de biòpsia sobre Matrigel amb medi de cultiu DMEM (Lonza), suplementat amb L-glutamina (1%), Sèrum Fetal Boví (10%), Fungizona (2.5μg/mL) i Gentamicina (200μg/mL).

Les cèl·lules són cultivades en les habituals condicions de temperatura i humitat, a 37ºC i un 5% de CO_2.

Aproximadament entre els 3 i 6 dies les primeres cèl·lules surten del gruix del teixit estenent-se pel Matrigel, i aconsegueixen arribar a la confluència al pou de la placa en un mes. Després d'això són sub-cultivades Tripsina-EDTA (Gibco, Life Technologies, Paisley, UK) a flascons de cultiu cada vegada més grans, fins que se-n'obtenen prou flascons de 150 cm2, i poden ser utilitzades per a realitzar experiments, sempre entre els passes 3 i 8 de creixement.

Cultiu de limfòcits, monòcits i cèl·lules sanguínies

Les cèl·lules mononuclears de sang perifèrica (PBMCs) són aïllades a partir de mostres de sang total utilitzant la tècnica per separació de gradient i segons densitat amb Lymphoprep (Axis-Shield, Oslo, Norway).

Aquestes PBMCs són cultivades amb medi RPMI (Lonza) també suplementat amb L-glutamina (1%), Sèrum Fetal boví (10%), Fungizona (2.5µg/mL) i Gentamicina (200µg/mL).

En el co-cultiu amb VSMCs s'utilitzen *Transwells* (Nunc), uns pous amb fons de membrana porosa que impedeixen el contacte directe entre els diferents tipus cel·lulars, però permeten la comunicació per molècules solubles.

Assajos de detecció múltiple d'expressió i secreció de citoquines

Amb l'objectiu de trobar molècules que puguin ser regulades per IFN-γ i dirigir el treball experimental, es van realitzar tècniques de detecció múltiple de molècules a nivell proteic en sobrenedants de cultiu, i d'expressió de mRNA amb les mostres d'artèries cultivades de dos pacients i dos controls.

Amb els sobrenedants de cultiu, es va realitzar un Human Cytokine Antibody Array de Bionova (Raybiotech, Norcross, GA) contra 40 molècules relacionades amb la angiogènesi i la inflamació, seguint el protocol de la pròpia casa comercial.

A nivell d'expressió de mRNA, es van utilitzar unes targetes mircofluídiques (de Applied Biosystems), amb 96 genes de molècules relacionades amb la inflamació (llistats a la taula 3 de la pàgina següent). Els resultats van ser quantificats i analitzats utilitzant un *software* de

Microfluidic card analyzed genes.

Gene Symbol	Gene Designation	Gene Symbol	Gene Designation
GUSB *	*Glucuronidase beta, MPS7*	CD68	*Macrophage antigen CD68, SCARD1*
RPL3L *	*Ribosomal protein L3-like*	CD80	*B-lymphocyte activation antigen B7*
18S *	*18S rRNA dimethylase*	CD86	*B-lymphocyte activation antigen B7-2*
GAPDH *	*G3P dehydrogenase*	CTLA4	*cytotoxic T-lymphocyte-associated protein 4, CELIAC3*
ACTB *	*Actin beta, PS1TP5BP1*	CD40LG	*CD40 antigen ligand, TNFSF5*
IL1A	*Interleukin-1 alpha*	HLA-DRA	*MHC class II, DR alpha chain*
IL1B	*Interleukin-1 beta*	HLA-DRB1	*MHC class II, DR-1 beta chain*
IL2	*Interleukin-2, TCGF*	TBX21	*T-box protein 21, T-bet*
IL3	*Interleukin-3, MCGF*	TNFRSF18	*TNF receptor superfamily member 18*
IL4	*Interleukin-4, BCGF-1*	ICOS	*Inducible T-cell co-stimulator, CVID1*
IL5	*Interleukin-5, BCGF-2*	NOS2A	*Nitric oxide syntase 2, INOS*
IL6	*Interleukin-6, BSF-2*	BCL2	*B-cell CLL/lymphoma 2, PPP1R50*
IL7	*Interleukin-7*	BCL2L1	*BCL2 like protein 1, PPP1R52*
IL8	*Interleukin-8, CXCL8*	BAX	*BCL2-associated X protein*
IL9	*Interleukin-9, HP40*	ICAM1	*Intercellular adhesion molecule 1*
IL10	*Interleukin-10, TGIF*	SELP	*P-selectin, CD62 antigen*
IL12A	*Interleukin-12 subunit p35, CLMFp35*	SELE	*E-selectin, ELAM-1*
IL12B	*Interleukin-12 subunit p40, CLMFp40*	HMOX1	*Heme oxygenase 1, HSP32*
IL13	*Interleukin-13, P600*	PTGS2	*Prostaglandin-endoperoxide synthase2, COX2*
IL15	*Interleukin-15*	REN	*Renin, Angiotensinogenase*
IL17	*Interleukin-17A, CTLA8*	LRP2	*Low density lipoprotein receptor-related protein 2, GP330*
IL18	*Interleukin-18, IFNg inducing factor*	MYH6	*Myosin heavy chain 6, alpha MHC*
CCL3	*C-C motif chemokine 3, MIP1 alpha*	CYP1A2	*Cytochrome P450 1A2, CP12*
CCL19	*C-C motif chemokine 19, MIP3 beta*	CYP7A1	*Cytochrome P450 7A1, CP7A*
CCL2	*C-C motif chemokine 2, MCP-1*	IFNG	*Interferon gamma*
CCL5	*C-C motif chemokine 5, RANTES*	PRF1	*Perforin-1*
CCR2	*C-C chemokine receptor type2, MCP1R*	GZMB	*Granzyme B, CTLA1*
CCR4	*C-C chemokine receptor type4, CKR4*	GNLY	*Granulysin, TLA519*
CCR5	*C-C chemokine receptor type5, CKR5*	FAS	*TNF receptor superfamily member 6*
CCR7	*C-C chemokine receptor type7, EBI-1*	FASLG	*Fas Ligand, TNFSF6*
CXCR3	*CXC chemokine receptor type3, IP10R*	TGFB1	*Transforming growth factor beta 1*
CXCL10	*Interferon inducible protein 10, IP-10*	SMAD3	*SMAD family member 3*
CXCL11	*Interferon inducible protein 9,IP-9, ITAC*	SMAD7	*SMAD family member 7*
CSF1	*Colony stimulating factor 1, MCSF*	SKI	*Proto-oncogene C-ski, SKV*
CSF2	*Colony stimulating factor 2, GMCSF*	FN1	*Fibronectin 1, MSF*
CSF3	*Colony stimulating factor 3, G-CSF*	COL4A5	*collagen type IV alpha 5, CA54*
STAT3	*Signal tranducer and activator of transcription 3, APRF*	C3	*Complement component C3, ASP*
NFKB2	*Nuclear factor Kappa-B subunit p100*	TNF	*Tumor necrosis factor-alpha*
IKBKB	*I-kappa B kinase 2, NFKBIKB*	LTA	*Lymphotoxin alpha, TNFSF1*
CD3E	*CD3 epsilon antigen, TCR-complex*	ACE	*Angiotensin I converting enzyme*
CD4	*CD4 antigen (p55), CD4mut*	VEGF	*Vascular endothilial growth factor A*
CD8A	*CD8 antigen alpha chain, LyT-2*	CD34	*Hematopoietic progenitor cell CD34 antigen*
CD19	*B-lymphocyte antigen CD19*	AGTR1	*Angiotensin II receptor type 1*
IL2RA	*Interleukin-2 receptor alpha, TCGFR*	AGTR2	*Angiotensin II receptor type 2*
CD28	*T-cell-specific surface glycoprotein CD28*	EDN1	*Endothelin-1, ET1*
CD38	*CD38 antigen (p45), T-10*	ECE1	*Endothelin converting enzyme 1*
CD40	*B cell surface antigen CD40, TNFRSF5*	TFRC	*Transferrin receptor, TRFR p90*
PTPRC	*CD45 antigen, protein tyrosine phosphatase receptor type C*	PGK1	*Phosphoglycerate kinase 1, MIG10*

Taula3. Gens presents en les targetes microfluídiques

Detecció de seqüències (SDS 2.3), considerant-se rellevants aquells canvis amb *ratios* superiors a 7 vegades.

També es va assajar la tècnica de Luminex enfront les següents molècules: CCL3, CCL4, CCL5, i CXCL8, utilitzant sobrenedants de cultiu d'artèria.

Quantificació de l'expressió gènica

La validació dels resultats de les proves de detecció múltiple a nivell de RNA s'ha dut a terme mitjançant PCR a temps real.

Després del cultiu, aproximadament unes 3 seccions d'artèria temporal per cada situació de tractament, són homogeneïtzades utilitzant un Polytron homogeneizer (IKA, Staufen, Germany) en reactiu de Trizol (Invitrogen). L'extracció de l'mRNA es realitza acord amb el mètode clàssic de separació de fases, precipitació i rentats utilitzant Clororform i Isopropanol, i Etanol al 70% (Sigma). El RNA resultant és quantificat per espectrofotometria (Quawell Technology, San Jose, CA, USA). 1µg d'aquest RNA és utilitzat en una transcripció reversa a cDNA mitjançant l'Archive Kit (de Applied Biosystems) en un volum final de 100µL. Les mostres són emmagatzemades a -80ºC fins el moment d'utilització.

Material i mètodes

S'ha analitzat en total l'expressió gènica de citoquines pro-inflamatòries (IL-1β, IL-6, TNFα, IFNγ), quimiocines i els seus receptors (CCL-2 (MCP-1), CCL-3 (MIP1α), CCL-4 (MIP1β), CCL-5 (RANTES), CXCL-8 (IL-8), CXCL-9 (MIG), CXCL-10 (IP-10), CXCL11 (I-TAC), CCR2, CXCR3, CX3CL1 (Fractalkine) i CX3CR1), factors de transcripció (T-Bet, GATA3, RORγ, STAT1, STAT3), molècules relacionades amb la matriu extracel·lular (COL I, COL III, FN1) metaloproteïnasses i els seus inhibidors (MMP-2, MMP-9, MMP-12, MMP-14, TIMP-1, TIMP-2), marcadors cel·lulars (CD3, CD20, i CD68) i factors de creixement (PDGF A i B, TGFβ) seguint les instruccions i protocols pre-dissenyats de Taqman probes de Applied Biosystems (Taqman^{R} Gene Expression Assays). La fluorescència i l'anàlisi dels resultats es realitzen utilitzant un ABI PRISM 7900 i el programa SDS v2.3 (Applied Biosystems). Finalment, l'expressió de tots els gens, es normalitza utilitzant un control endogen amb el gen GUSb, i se'n calcula l'expressió en unitats relatives mitjançant el mètode de ΔCt.

Quantificació de la secreció de Citoquines

Pel que fa al nivell proteic, la validació dels resultats d'expressió s'ha dut a terme mitjançant la detecció amb assajos immunològics d'ELISA, amb els quals quantifiquem el nivell de proteïna secretada al sobrenedant per les artèries cultivades, de les següents molècules: TNFα, IL-1β, IFNγ, CCL-2, CCL-3 (MIP1α), CXCL-9 (MIG), CXCL-10 (IP-10), CXCL-11 (I-TAC), i CXCL-8 (IL-8), segons els protocols subministrats per les cases comercials de R&D Systems i Bender MedSystems (Vienna, Austria) segons la molècula.

Quantificació de la fosforilació de STAT1 i 3 mitjançant Western Blot

Dues seccions per cada condició experimental del cultiu d'artèria van ser homogeneïtzades en 0,5mL de buffer de RIPA (Sigma-Aldrich, Ayrshire, UK) modificat amb els següents additius per tal de preservar els grups fósfor: Fenylmetilsulfonl fluorid (PMSF) (1mM), Benzamidina-HCl (1mM), Ortovanadat (2nM), Leupeptina, Aprotinina, i Pepstatina (0,5 µg/mL), Àcid Etilen-diamina-tetraacetic (EDTA) (1mM), Àcid Etilen-gliycol-tetraacetic (EGTA) (1mM), Florur de Sodi (NaF) (50mM), i detergent NP-40 (1%).

Per a la realització de l'assaig de Western Blot, s'utilitzen 15µg d'aquest lisat proteic que es sotmeten a electroforesi desnaturalitzant en un gel de poliacrilamida (10%), i són posteriorment transferits a una membrana de nitrocel·lulosa.

Les membranes són incubades amb els anticossos primaris anti-fosfoSTAT1 i 3, i anti- STAT1 i 3 total (clons D4A7 i 9H2 per STAT1, i D3A7 i 124H6 per STAT3 respectivament, Cell Signaling, Boston, MA) a dilució 1:1000, i són reutilitzades i reincubades mitjançant l'ús de solució de Stripping (Glycine 10%, SDS 1%, Tween). La immunodetecció de cada molècula es realitza amb anticossos secundaris lligats a un enzim colorimètric (HRP), contra les espècies en les què s'ha dissenyat el primari (ratolí o conill segons el cas) a dilucions 1:2000 (cell Signaling). La reacció colorimètrica es catalitza mitjançant el kit Supersignal West Dura Substrate (Thermoscientific), i la posterior quantificació es duu a terme en una cambra fosca ImageQuant LAS4000 (GE HealthCare Life Science, Pittsburgh, USA).

La quantificació de les bandes és normalitzada versus la senyal de un anticòs anti-β-Actina humana (ab75186 Abcam, Cambridge, UK) usat a dil·lució 1:500.

Anàlisi immunohistoquímic i microscòpia confocal

Les artèries processades per microscòpia confocal, són cultivades seguint els protocols anteriors, i seguidament fixades en paraformaldehid (4%) i rentades en concentracions decreixents de sacarosa (des de el 30% fins el 15%) durant 3 dies. Posteriorment són incloses en un bloc d'OCT (Sakura, Flemingweg, The Netherlands) i congelades a -80ºC.

Utilitzant un Cryotome Cryostat (Leica Microsystems, Wetzlar, Germany) obtenim talls d'entre 3 i 5µm de gruix, que són inclosos en portaobjectes i bloquejats amb X-Triton (Invitrogen).

Per a la immunohistoquímica amb l'anticòs humà anti-IFN-γ sobre mostres d'artèria humanes, hem utilitzat una versió biotinada del clon A6, ja que no podíem utilitzar un anticòs secundari anti-humà sobre mostres humanes sense obtenir inespecificitats.

Pel que fa a la immunofluorescència, els portaobjectes van ser fixats, bloquejats i incubats amb els anticossos primaris anti-CD3 (clone PS1, Novocastra, Leica Microsystems) i anti-CD68 (clone KP1, Dako) i anticossos secundaris conjugats amb els fluorocroms A488 i A555 (Molecular Probes, Life Technologies, Grand Island, NY). La immunodetecció es realitza al departament de Microscòpia mitjançant la utilització d'un Microscopi Confocal de fotons SP5 (Leica Microsystems).

Assajos funcionals de migració, proliferació i adhesió cel·lular

Cèl·lules mononuclears de sang perifèrica (PBMCs) obtingudes a partir de donants sans, són aïllades a partir de mostres de sang total utilitzant la tècnica de separació per gradient en reactiu de FICOLL, com ja hem explicat anteriorment. Les cèl·lules de múscul llis vascular (VSMCs) per la seva banda, són extretes de biòpsies negatives com s'ha citat també anteriorment.

La quantificació de la capacitat de migració de les PBMCs s'ha assajat mitjançant la utilització de cambra de Boyden, en experiments de 6 hores de duració. Les membranes de policarbonat que separen les dos seccions dels pous de la cambra, i a través dels porus de la qual (de 5µm de diàmetre) migren les cèl·lules, són fixades i tenyides amb metanol i hematoxilina, i el comptatge de cèl·lules es realitza sota microscopi.

La proliferació de les VSMCs es determina mitjançant espectrofotometria. Les cèl·lules són crescudes en plaques de 96 pous, en experiments sota estímuls entre 24 hores i 7 dies. Després d'això són fixades i tenyides amb Cristall violeta (CV) al 0,2%, en un 20% de metanol (Sigma Aldrich) durant 10 minuts. Posteriorment els pous són resuspesos afegint un 1% de Sodium Dodecyl Sulfate (SDS) (Sigma Aldrich) i la placa és quantificada en un lector de plaques a una longitud d'ona de 620nm.

Per tal d'inhibir la senyalització de les quimiocines dependents d'IFN-γ, hem utilitzat un agent químic antagonista del receptor CXCR3 (a 100mM) (500486, Calbiochem, San Diego, CA).

Pel que fa a l'assaig d'adhesió cel·lular, VSMCs extretes a partir d'artèries sanes es fan créixer en plaques de 96 pous en diferents concentracions d'estímuls i en absència d'aquests. Després de 24hores en cultiu, s'afegeixen PBMCs (aïllades amb FICOLL com hem descrit), a raó de $7{,}5\cdot10^4$ per pou, i la placa és incubada durant 30 minuts a 37ºC per permetre l'adhesió cel·lular. Posteriorment els pous són rentats amb PBS i fixats i tenyits amb Cristall Violeta al 0,2% en metanol durant 10 minuts. Després d'això el colorant es solubilitza amb SDS i l'adhesió és mesurada per espectrofotometria amb un lector de plaques.
Per l'assaig d'adhesió fluorescent les PBMCs són pre-tractades amb un kit Red fluorescent (de Sigma Aldrich) abans de la incubació amb les VSMCs durant 30 minuts. Tots dos tipus cel·lulars són fixats amb metanol, i les VSMCs són tenyides de manera fluorescent utilitzant un anticòs anti-Alpha Actina de múscul llis (ab54723, Abcam).
Les preparacions per microscòpia es completen amb el muntatge utilitzant medi amb DAPI (Duolink, Olink Bioscience, Uppsala, Suècia) per tal de tenyir els nuclis de totes les cèl·lules.

Estudi dels promotors gènics

Per a l'anàlisi de les dianes de factors de transcripció en els promotors de les quimiocines, es va fixar en primer lloc una longitud de cerca per cada promotor de 5000pb (5kb), 4kb *upstream* de l'inici de transcripció, i 1kb *downstream*, ja que alguns factors de transcripció es troben sovint inserits en el propi gen, i així s'evita la introducció de molts falsos positius, que es poden donar en anàlisis de territoris més amplis.

Un cop fixada la longitud es va utilitzar una base de dades, el UCSC genome, per descarregar-nos les seqüències de les àrees dels promotors dels gens, que van ser revisades fent un BLAT, com a control intern.

Les seqüències revisades van ser introduïdes al programa online Mapper database: http://mapper.chip.org/, que té una base de dades de seqüències diana de factors de transcripció (99). Introduint la seqüència del promotor de cada gen, s'obtenen quines dianes estan presents en cada segment, i quantes vegades. Posteriorment, amb la llista de factors de transcripció que regulen cada promotor, es va utilitzar un Diagrama de Venn per comparar els conjunts i saber quines dianes compartien les diferents quimiocines.

Anàlisi estadístic

En primer lloc, es determina la normalitat dels conjunts de mostres mitjançant un test de Kolmogorov-Smirnov. En funció de si es confirma la normalitat o no, es realitzen els assajos estadístics de T de Student (en cas de normalitat), o la prova no paramètrica del Test de Mann Whitney en el cas contrari, ja sigui tant per l'anàlisi de dades aparellades com per variables independents, i sempre utilitzant el software SPSS 2.0, versió PASW 18.0.

Resultats

Changes in biomarkers after therapeutic intervention in temporal arteries cultured in Matrigel: a new model for preclinical studies in giant-cell arteritis

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Resultats

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EXTENDED REPORT

Changes in biomarkers after therapeutic intervention in temporal arteries cultured in Matrigel: a new model for preclinical studies in giant-cell arteritis

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ABSTRACT

Background Search for therapeutic targets in giant-cell arteritis (GCA) is hampered by the scarcity of functional systems. We developed a new model consisting of temporal artery culture in tri-dimensional matrix and assessed changes in biomarkers induced by glucocorticoid treatment.

Methods Temporal artery sections from 28 patients with GCA and 22 controls were cultured in Matrigel for 5 days in the presence or the absence of dexamethasone. Tissue mRNA concentrations of pro-inflammatory mediators and vascular remodelling molecules was assessed by real-time RT-PCR. Soluble molecules were measured in the supernatant fluid by immunoassay.

Results Histopathological features were exquisitely preserved in cultured arteries. mRNA concentrations of pro-inflammatory cytokines (particularly IL-1β and IFNγ), chemokines (CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) and MMP-9 as well as IL-1β and MMP-9 protein concentrations in the supernatants were significantly higher in cultured arteries from patients compared with control arteries. The culture system itself upregulated expression of cytokines and vascular remodelling factors in control arteries. This minimised differences between patients and controls but underlines the relevance of changes observed. Dexamethasone downregulated pro-inflammatory mediator (IL-1β, IL-6, TNFα, IFNγ, MMP-9, TIMP-1, CCL3 and CXCL8) mRNAs but did not modify expression of vascular remodelling factors (platelet derived growth factor, MMP-2 and collagens I and III).

Conclusions Differences in gene expression in temporal arteries from patients and controls are preserved during temporal artery culture in tri-dimensional matrix. Changes in biomarkers elicited by glucocorticoid treatment satisfactorily parallel results obtained in vivo. This may be a suitable model to explore pathogenetic pathways and to perform preclinical studies with new therapeutic agents.

INTRODUCTION

Giant-cell arteritis (GCA) is a granulomatous arteritis of the elderly, targeting the aorta and its branches with a striking tropism for the cranial arteries.[1] Although most patients with GCA experience a remarkable relief with high-dose glucocorticoids (GC), treatment has proven to be unsatisfactory. GC fail to prevent further sight deterioration in 10%–17% of patients presenting with visual impairment and are unable to avoid large vessel damage leading to aortic dilatation in about 22.5% of patients.[2 3] Moreover, more than 50% of patients relapse when GC are tapered[4 5] and GC-related adverse events occur in a more than 80% of patients with GCA.[6] There is an unmet need for more effective and specific therapies.

Search for therapeutic innovation in GCA is difficult due to the limited understanding of pathogenesis and the scarcity of functional models where the impact of therapeutic interventions can be assessed. The pathogenesis model of GCA is based on the identification of particular cell types (CD4T lymphocytes, macrophages, dendritic cells, endothelial cells),[7 8] cell activation and differentiation markers,[7–9] and inflammatory mediators in lesions.[9–13] The interpretation of immunopathology findings is often extrapolated from basic immunology principles, and the role of infiltrating cells and their products is assumed from their known biological activities and association with particular phenotypes,[10 11 13] histopathological changes or outcomes.[12–14] Proof of concept is weak for the majority of grounds on which the current pathogenetic model is sustained.

The frustrating experience with anti-tumour necrosis factor (TNF) therapy in GCA underlines the crucial need for functional systems. TNFα was considered a potential therapeutic target based on its strong upregulation in lesions[13] and correlation of tissue and serum TNFα levels with GC requirements and relapsing course.[13 15] In spite of these observations and in spite of the therapeutic efficacy of TNF blockers in other granulomatous diseases, neutralising TNFα with infliximab did not seem sufficient to abrogate inflammatory activity in GCA.[5] Blocking IL-6 receptor is currently being considered as a therapeutic option.[16] This and other interventions could benefit from preclinical functional testing.

A functional model was created by Brack *et al*[17] subcutaneously engrafting fragments of human temporal arteries into severe combined immunodeficiency (SCID) mice. This pioneer model has been useful to detect changes in cytokine expression in temporal artery tissue after pharmacological

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treatment of engrafted mice[18 19] or after selective depletion of specific cell types with antibodies injected to animals,[8 17] providing proof of concept of some of the basic pathogenic principles. However, this model is complex, expensive and not widely available. Moreover, monitoring of successful engraftment is difficult and the accessibility of therapeutic agents administered to the mice cannot be controlled. Due to its complexity, the majority of published experiments have been performed with only 1–3 temporal arteries split into several mice.[8 18 19]

The Engelbreth-Holm-Swarm sarcoma-derived tri-dimensional matrix, Matrigel,[20] provides anchorage and survival signals for vascular smooth muscle cells (VSMC).[21] Based on these findings, we developed a new model to assess changes in lesions after therapeutic intervention, consisting of culture of temporal artery sections embedded in Matrigel. We found that cultured arteries remained viable for at least 2 weeks with exquisitely preserved morphology. Moreover, this system was sensitive enough to demonstrate clear differences in cytokine expression between normal and inflamed arteries as well as changes induced by therapeutic intervention.

METHODS

Patients

Temporal artery biopsies were performed to 50 consecutive patients with suspected GCA for diagnostic purposes. A 5–15 mm segment was saved for this study and the remaining fragment was processed for histopathological diagnosis. The study was approved by the Ethics Committee of the Hospital Clínic of Barcelona and patients signed informed consent.

A total of 28 biopsies disclosed histopathological features of GCA and 22 revealed no inflammatory infiltrates. Patients with a negative temporal artery biopsy were eventually diagnosed with other conditions (see online supplementary methods).

Temporal artery culture

Temporal artery fragments were placed in RPMI 1640 medium (Lonza; Verviers, Belgium) supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, California, USA), 2 mM L-glutamine (Invitrogen), amphotericin B at 2.5 μg/ml (Invitrogen) and gentamycine (Braun, Germany) at 200 μg/ml. An average of 10.79±2.91 (mean±SEM) 0.8–1 mm sections per specimen were cut in a tissue culture hood. Matrigel (Collaborative Biomedical Products, Bedford, Massachusetts, USA) was allowed to thaw on ice and 24-well tissue culture plates were coated with a 25 μl Matrigel drop per well, which was allowed to solidify at 37°C for 30 min. One temporal artery section per well was dipped in the Matrigel coating and covered with 1 ml medium. Dexamethasone (Sigma, Ayrshire, UK) at 0.5 μg/ml was added to selected wells. Each condition was tested in 3–4 replicate wells. Sections were incubated al 37°C in 5% CO_2 for 5 days. Replicates of supernatant fluids and biopsies were respectively pooled. Biopsies were frozen in TRIzol reagent (Invitrogen) and stored at −80°C. Random specimens were cultured for 2 weeks in order to assess morphology preservation after extended culture periods and fixed in 10% formalin for H&E staining and histopathological examination.

Immunostaining

Cultured temporal artery sections were de-paraffinised, washed in phosphate-buffered saline (PBS) and endogenous peroxidase was blocked with H_2O_2. Slides were incubated with mouse antihuman CD3 (clone PS1, Leica Microsystems, Wetzlar, Germany, at 1:60 dilution) or undiluted mouse antihuman CD68 (clone KP1 from Dako, Glostrup, Denmark, ready to use). Optimal dilutions were tested on human tonsils (positive control). Isotype-matched mouse immunoglobulins served as negative controls. Immunodetection was performed with a HRP-labelled polymer conjugated to a secondary antibody (EnVision, Dako) using 3-3′-diaminobenzidine as a chromogen.

Cytokine mRNA measurement by real-time quantitative RT-PCR

Three to four temporal artery sections per condition were homogenised in TRIzol reagent. RNA extraction was performed according to the chloroform-isopropanol precipitation method. Total RNA (1 μg) was reverse transcribed to cDNA using Archive kit (Applied Biosystems, Life Technologies, Carlsbad, California, USA) in a final volume of 100 μl, employing random hexamer priming. Samples were stored at −80°C until use.

Gene expression of pro-inflammatory cytokines (IL-1 β, IL-6, TNFα, interferon (IFN)γ), chemokines (chemokine ligand (CCL)2/monocyte chemoattractant protein (MCP)-1, CCL3/MIP-1α, CCL4/MIP1β, CCL5/regulated upon activation normal T cell expressed and secreted (RANTES) and CXCL8/IL-8), metalloproteases (matrix metalloproteinases (MMP)-2, MMP-9) and their inhibitors (tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2), growth factors (platelet derived growth factor (PDGF) A and B) and vascular matrix components (collagen I, collagen III) was assessed using specific predeveloped Taqman probes from Applied Biosystems (Taqman Gene Expression Assays; see online supplementary methods). Fluorescence was detected with ABI PRISM 7900 Sequence Detection system and results were analysed with the Sequence Detection Software V.2.3 (Applied Biosystems). Comparative Ct method was used to assess the relative gene expression. All samples were normalised to the expression of the endogenous control GUSb and values were expressed as relative units.

Detection of inflammatory mediators in the supernatant fluid by immunoassay

Pro-inflammatory cytokines (IL-6, TNFα, IL-1β, IFNγ), chemokines (CCL2/MCP-1 and CCL3/MIP-1α), metalloproteases (MMP2 and MMP-9) and growth factors (PDGF AB) were detected by enzyme-linked immunoassay (Quantikine ELISA kits from R&D Systems, Minneapolis, Minnesota, USA) in the culture supernatants form all patient and control arteries.

CCL4/MIP-1β and CXCL8/IL-8 were assessed by the Multiplex Luminex system (Life Technologies, Paisley, UK) in the supernatant fluid from 10 patients and six controls.

Statistical analysis

Mann–Whitney test was applied for statistical analysis.

RESULTS

Viability of the system and preserved morphology

Arterial sections were daily monitored under an inverted microscope. In GCA arteries white blood cells were visible in the periphery of the artery and remained bright and viable as assessed by Trypan blue exclusion throughout the duration of the experiment (figure 1A). After 1 week, VSMC began to spread and extend towards the matrix, further supporting the viability of this system (figure 1B).

As shown in figure 1C and 1D histopathological examination of cultured GCA arteries disclosed that morphological details including distinct arterial layers, inflammatory infiltrates, internal elastic lamina fragments and giant-cells were perfectly preserved. Over time, the intensity of inflammatory infiltrates decreased in cultured arteries, as described in arteries engrafted into SCID mice.[17] As shown in figure 1E–H, inflammatory

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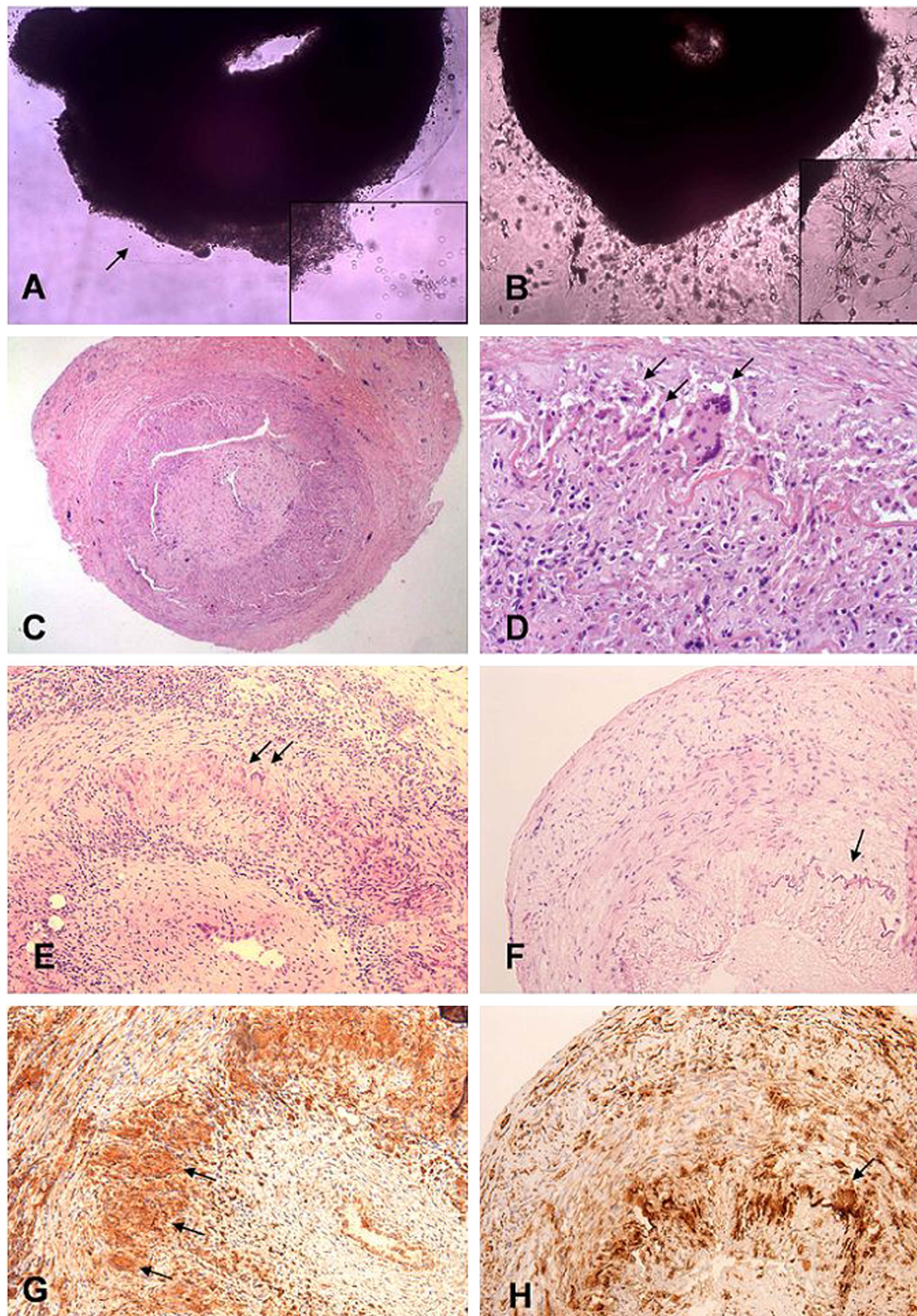

Figure 1 Histopathological findings in temporal artery sections from patients with giant-cell arteritis (GCA) cultured in Matrigel. (A) Temporal artery section from a patient with GCA cultured for 24 h and observed under an inverted, phase-contrast microscope. The arrow shows bright leukocytes cumulating in the periphery of the artery (inset shows a closer view). (B) Temporal artery section from a patient with GCA after 7-day culture. Vascular smooth muscle cells (VSMC) sprout from the artery and leukocytes migrate outwards (inset shows a closer view). Identity of these cells as VSMC has been previously demonstrated.[21] (C) H&E staining of a temporal artery section cultured for 2 weeks showing exquisite preservation of morphology. (D) Closer view of another temporal artery section cultured for 2 weeks displaying giant cells (arrows) along fragments of the internal elastic lamina. (E) H&E staining of a section of a freshly removed artery. Arrows show giant-cells. (F) Serial section of the artery disclosed in E cultured for 2 weeks showing a reduction in inflammatory infiltrates. The arrow indicates typical internal elastic lamina fragments. (G) Macrophages and numerous giant-cells (arrows) identified by anti-CD68 immunostaining in a freshly removed artery. (H) Anti-CD68 immunostaining of a serial section cultured for 2 weeks. Giant-cells (arrow) are dramatically reduced.

infiltrates, including giant-cells, decreased after 2-week culture. Examination of the cultured arteries under an inverted microscope disclosed that, over time, some inflammatory cells migrated along the outgrowing VSMC (figure 1B).

Differences in expression and release of relevant molecules between cultured GCA and control arteries

To assess the model reliability we investigated expression of pro-inflammatory cytokines, chemokines, metalloproteinases and

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growth factors largely known to be expressed in GCA lesions and thought to be relevant to pathogenesis. We also explored some additional chemokines, such as CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CXCL8/IL-8, not previously investigated in GCA.

After a 5-day incubation period, remarkable differences in the spontaneous expression and release of various relevant factors were detected between GCA and control arteries, underlining the accurate sensitivity of the system to distinguish between non-inflamed and inflamed arteries (table 1). Differences in gene expression were particularly significant for IL-1β, IFNγ, chemokines CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES, and MMP-9. Less marked or no differences were observed for other factors known to be upregulated in GCA lesions including IL-6, TNFα and CCL2. Intense expression by cultured control arteries probably minimised differences.

Of interest, MMP-9, mainly produced by inflammatory cells, was overexpressed in patient versus control specimens whereas MMP-2, constitutively expressed by VSMC, was similar between patients and controls, paralleling again what has been observed in uncultured temporal artery biopsies.[22] As previously observed in freshly removed arteries, TIMP-1 and TIMP-2 mRNAs were decreased in inflamed arteries, leading to increased proteolytic balance.[22] Vascular remodelling factors PDGFs, CCL2, MMP-2 and collagens were strongly expressed in cultured arteries with no relevant differences between patients and controls.

Variations in the secretion of various markers were observed. TNFα and particularly IL-6 were remarkably released in the supernatant fluid (table 1). However, IFNγ and IL-1β, markedly expressed at the mRNA level, were secreted in small amounts. This parallels what happens in vivo where circulating TNFα and IL-6 are increased in sera of patients whereas IL-1β and IFNγ are not easily secreted and remain around the detection threshold in human serum. Therefore, this system allows evaluation of cytokine expression and investigation of cytokine secretion.

Similarly, while there were significant differences in chemokines CCL3/MIP1-α, CCL4/MIP-1β and CCL5/RANTES between patients and controls at the mRNA level, differences in released chemokines were less apparent.

Effect of the culture system on gene expression in cultured arteries

Since control arteries notably expressed various mediators we next investigated whether the culture system itself influenced gene expression. Frozen tissue from the original artery was available for six of the GCA patients and five controls and the expression of selected markers was compared between sections of the same specimen before and after 5-day culture in Matrigel. With the exception of IFNγ, the culture system upregulated expression of pro-inflammatory cytokines, chemokines CCL2 and CXCL8, and MMP-9 in both patients and controls. PDGFs and collagen III were markedly increased in control arteries whereas IFNγ and collagens decreased in GCA specimens (figure 2). In general, the culture system minimised differences between patients and controls.

Effect of dexamethasone on inflammatory infiltrates and on the expression and release of inflammatory and vascular remodelling markers

To assess whether this ex vivo system allowed accurate detection of changes induced by pharmacological intervention, we compared expression and release of inflammatory markers between artery sections cultured with medium alone and sections from 10 patients cultured in the presence of dexamethasone. A marked decrease in cytokine production was observed (figure 3 and table 2). Chemokines were downregulated at the mRNA level but changes in chemokine release were, again, less apparent (table 2). Vascular remodelling factors such as CCL2, MMP-2 and PDGF as well as collagens I and III were not downregulated by dexamethasone.

Dexamethasone treatment for 2 weeks induced a decrease in macrophage infiltration as assessed by CD68 mRNA expression and immunohistochemistry (figure 4). No effect on T cells was observed during the same treatment period.

Table 1 Differences in biomarker mRNA expression (*relative units*) and protein secretion (*pg/ml*) between cultured temporal artery sections from GCA patients and controls

	mRNA concentration (relative units)			Protein concentration (pg/ml)		
	GCA biopsies	Control biopsies	p Value	GCA biopsies	Control biopsies	p Value
IL-1β	35.91±8.80	14.22±2.86	**0.047**	5.61±1.33	−0.85±0.58	**0.000**
IL-6	448.54±86.88	380.04±68.37	0.543	25583±9404	7805.4±2685	0.076
TNFα	4.70±2.26	6.69±1.24	0.420	21.25±5.09	8.42±2.59	0.053
IFNγ	0.805±0.257	0.012±0.011	**0.010**	7.75±2.41	7.75±2.41	0.764
CCL-2/MCP-1	648.72±155.21	729.5±201.42	0.758	11268±1903	5850.91±4316.1	0.227
CXCL-8/IL-8	2287.9±619.9	4346.5±1092.4	0.095	81403±25050	34778.2±18253.9	0.157
CCL-3/MIP-1α	86.31±16.9	20.16±.5.10	**0.002**	25.617±2.503	26.62±4.25	0.834
CCL-4/MIP-1β	28.21±6.13	5.36±1.13	**0.003**	12.61±3.1	6.15±1.3	0.087
CCL-5/RANTES	139.83±37.34	16.42±5.58	**0.007**	21.43±5.19	17.33±6.54	0.633
MMP-2	2097.4±276.9	3450.9±1143.4	0.297	39125±11144	15250±8280	0.192
MMP-9	1283.85±408.2	304.21±90.70	**0.039**	48913±10740	7825±3512.4	**0.006**
TIMP-1	11813±3550	15126.8±5893.7	0.613	Not done	Not done	–
TIMP-2	586.68±87.77	2798.1±1135.2	0.074	Not done	Not done	–
COL I	1545.6±284.61	1065.34±196.7	0.175	Not done	Not done	–
COL III	3674.4±637.07	3979.2±991.5	0.789	Not done	Not done	–
PDGF A	71.14±24.75	163.55±40.13	0.056	Not done	Not done	–
PDGF B	40.78±7.78	43.807±7.992	0.806	Not done	Not done	–
PDGF AB	Not applicable	Not applicable	–	23.375±3.245	41.50±15.34	0.325

Values in bold are statistically significant ($p<0.05$). mRNA expression was detected in the entire cohort of 28 GCA patients and 22 controls. CCL3/MIP-1α, CCL4/MIP-1β and CXCL8/IL-8 protein concentrations were detected by Luminex in 10 patients and six controls. The remaining proteins were detected by ELISA in the entire cohort.
COL, Collagen; GCA, giant-cell arteritis.

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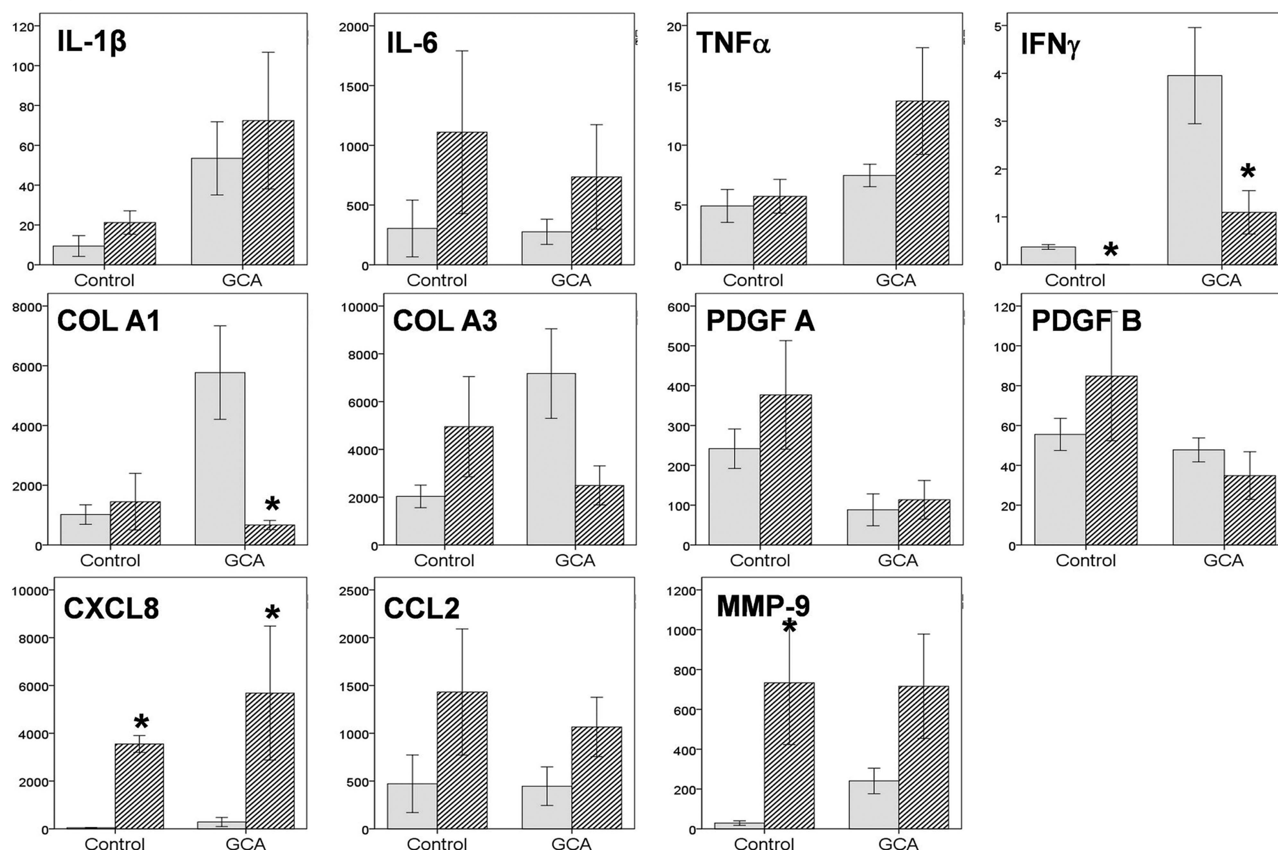

Figure 2 Effects of the culture system on biomarker expression. mRNA concentration (*relative units*) of pro-inflammatory cytokines, chemokines, vascular remodelling factors and matrix proteins in freshly removed (white bars) versus cultured, untreated, serial temporal artery sections (dashed bars) from six giant-cell arteritis patients and five controls (mean±SEM). *p<0.05 comparing fresh versus cultured arteries. Statistics are only indicative given the low number of samples studied.

DISCUSSION

Functional models are essential to explore pathogenic pathways and to test therapeutic intervention in diseases. We developed a new model of temporal artery culture in tri-dimensional matrix to perform functional studies in GCA. Short-term explant culture of involved tissue has been previously used in other conditions such as rheumatoid arthritis and has provided useful insights into involved immunopathogenic pathways.[23] A previous attempt of culturing temporal artery explants was tried by Blain *et al*.[24] However, without the use of a supporting matrix, the specimen remained viable for a short period of time. Specimens were cultured for 20 h only and the release of mediators in the supernatant fluid had to be induced with lypopolysaccharide which is an important exogenous manipulation.

The main innovation of our culture system is the embedding of the specimen in Matrigel which supports viability with active production of inflammatory mediators and their spontaneous release into the culture medium. In addition to provide an anchorage system for the wounded VSMC medial layer of the excised sections, Matrigel provides survival and proliferation signals for VSMC[21] which, in turn, may promote survival of infiltrating lymphocytes and macrophages. In this model, morphology was excellently preserved within 2-week culture.

There was a remarkable variability in the spontaneous production of inflammatory mediators, reflecting the notable differences in the density of inflammatory infiltrates and individual variation in cytokine production existing among patients with GCA. This observation underlines the need of a suitable model where testing specimens from multiple donors is feasible.

Spontaneous expression of IL-1β, IFNγ, MMP-9 and chemokines CCL3, CCL4 and CCL5 was significantly higher in explants from patients compared with controls and closely paralleled what has been described in immunopathology studies of freshly removed GCA arteries.

GC substantially reduced the production of pro-inflammatory cytokines IL-1β, IL-6 and TNFα both at the mRNA and protein level and also IFNγ mRNA. Expression of chemokines was also markedly decreased. These changes were similar to what has been observed in cross-sectional comparisons in biomarker expression between biopsies obtained from untreated patients and biopsies from patients who have already received GC,[7 22] in sequential biopsies obtained in four patients before and after 1 year of GC treatment,[14] or results obtained in temporal artery biopsies engrafted in the SCID mice.[18] GC treatment induced also a decrease in macrophage infiltration, whereas virtually no effect was observed on T cells, suggesting that T cell infiltration may be more resistant to GC therapy.

An interesting contribution of this study is that the expression of vascular remodelling factors such as CCL2/MCP-1, MMP-2, PDGFs and collagen I and III is not influenced by GC. A previous study comparing sequential biopsies obtained in four patients before and after 1 year of GC treatment showed, indeed, that vascular remodelling factors increased after long-term GC treatment.[14] This may explain why some patients

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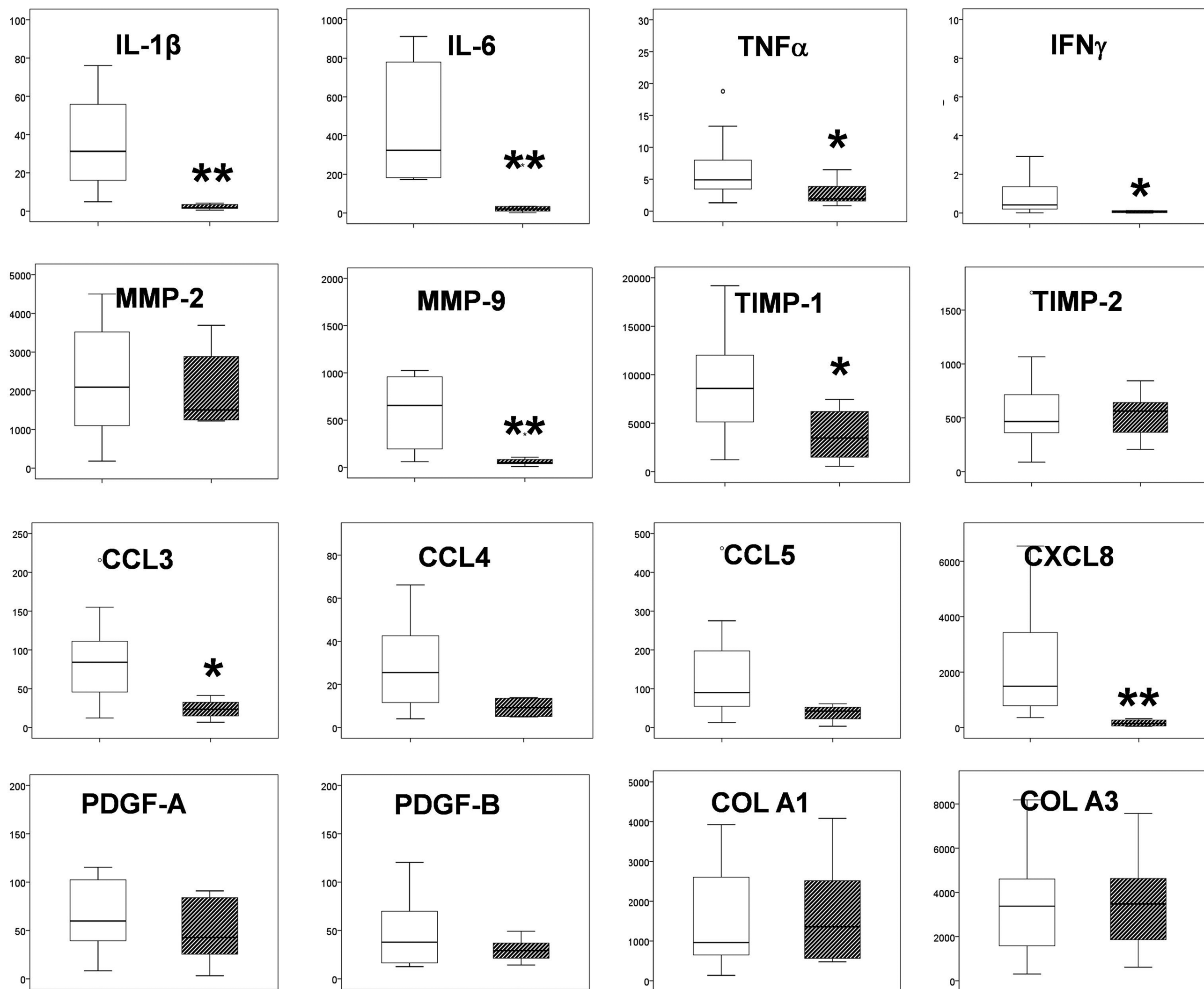

Figure 3 Changes in biomarker mRNAs induced by dexamethasone treatment. Comparison in mRNA concentration of selected biomarkers between untreated temporal artery sections from the giant-cell arteritis cohort (white box) and temporal artery sections from 10 of the patients subjected to dexamethasone at 0.5 mg/ml (grey box). *p<0.05; **p<0.005.

Table 2 Changes in biomarker protein concentration (*pg/ml*) in the supernatant fluid from untreated cultured GCA temporal artery sections and cultured GCA sections exposed to dexamethasone

	Protein concentration (pg/ml)		
	Untreated artery sections (mean ±SEM)	Dexamethasone-treated artery sections (mean±SEM)	p Value
IL-1β	6.06±1.32	2.41±0.76	**0.017**
IL-6	31059.8±10600.6	4796.5±1968.4	**0.020**
TNFα	27.043±6.398	2.22±2.104	**0.041**
IFNγ	4.901±1.8	4.792±1.66	0.961
CCL-2/ MCP-1	11759±2679.5	5130.2±598.83	0.921
CCL3/ MIP-1α	24.11±9.05	19.41±11.38	0.399
CXCL8/ IL-8	132670±41358.1	2465.5±631.76	0.056
MMP-2	39.125±11.14	27.5±7.79	0.204
MMP-9	48.91±10.74	8.64±1.89	**0.003**
PDGF AB	23.38±3.25	20±2.83	0.394

GCA, giant-cell arteritis.

continue to develop vascular occlusive events in spite of GC treatment.[2]

A limitation of this model is that Matrigel itself, by promoting survival and proliferation of smooth muscle cells, may directly influence the expression or detection of some products introducing a bias in the results. The culture system downregulated IFNγ and collagen I expression in GCA arteries and, conversely, upregulated the expression IL-6, CCL2/MCP-1, MMP-9, CXCL8/IL-8, PDGFs and collagen III in control arteries. These molecules may be part of vascular remodelling/repair programme stimulated by surgical injury and facilitated by attachment to the matrix. These observations indicate that some differences in gene expression observed between patients and controls are minimised by the culture system but, at the same time, enhance the significance of the differences observed. Furthermore, this finding underlines the need of investigating how the culture system influences the expression of any factor to be tested in this model.

Another limitation is that detection of some mediators such as chemokines in the culture medium may not accurately reflect their actual production. Chemokines act in an autocrine/paracrine manner and interact with matrix proteins to create a local

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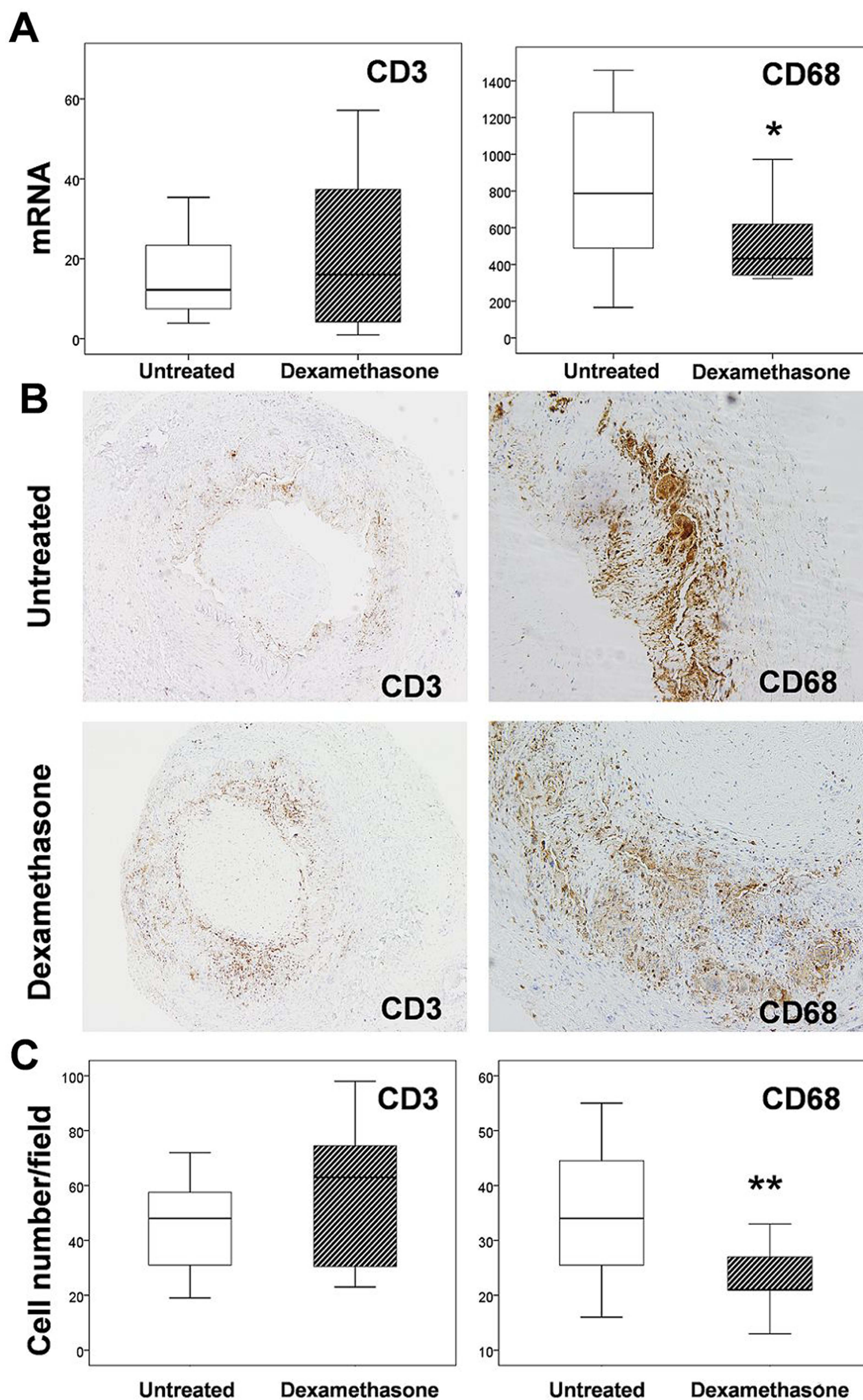

Figure 4 Effects of dexamethasone treatment on the density of infiltrating T lymphocytes and macrophages. (A) Differences in mRNA concentration of CD3 (T lymphocyte marker) and CD68 (macrophage marker) between 28 untreated giant-cell arteritis (GCA) temporal artery sections and 10 GCA sections exposed to 0.5 μg/ml dexamethasone. *p=0.059. (B) Changes in infiltrating T lymphocytes (identified by anti-CD3 immunostaining) and macrophages (identified by anti-CD68 immunostaining) upon dexamethasone treatment. (C) CD3 or CD68 cell number per field in three paired arteries cultured with or without dexamethasone. **p=0.004.

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gradient. Therefore, chemokines may be retained in the artery and surrounding proteoglycan-rich matrix, according to their physiological function.[25] Dissociation between tissue and serum concentrations of relevant chemokines has been observed in several chronic inflammatory conditions.[12]

Our model overcomes some of the limitations of the temporal artery engraftment into the SCID mice. It allows daily monitoring of viability, it ensures direct accessibility of the molecules tested, it allows serial detection of proteins secreted into the culture medium and morphology is better preserved. Since retrieval of the cultured specimens is direct and simple, very thin sections can be used, allowing the assessment of replicates to assure consistency, and the testing of various conditions per specimen. This is very important given the remarkable variability in the intensity of inflammatory infiltrates and cytokine production among patients. In addition, it is cheap, easy, spares animals and does not require special equipment besides tissue culture facilities. In fact, since the initial communication of preliminary results,[26 27] this model is being used by other investigators.[28] It shares with the SCID mice model the limitation that only changes in biomarkers can be assessed and true, clinically relevant, disease outcomes cannot be investigated.

In summary, we developed an artery explant culture system based on the unique properties of Matrigel in creating a tridimensional matrix support and promoting VSMC survival. This method is sensitive enough to detect changes after intervention and may be useful to explore pathogenic pathways and to assess the impact of new therapeutic agents.

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Contributors MCC and MUR designed the study. J-MD contributed important input to its design. MC-B, AG-M, EL, EP-R and PR-L performed the experimental work. GE-F, SP-G, MAA, JH-R and MB contributed to clinical selection and contributed to the experimental work, PLF supervised the immunopathology studies. All authors evaluated and criticised the data and J-MD provided important contributions to their interpretation. MC-B and MCC wrote the manuscript. All authors read, made improvements and approved the final version.

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Changes in biomarkers after therapeutic intervention in temporal arteries cultured in Matrigel: a new model for preclinical studies in giant-cell arteritis

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RESUM DE RESULTATS

1. Transcorregudes dues setmanes de cultiu, hem confirmat la viabilitat dels leucòcits que han escapat de la biòpsia, i als 7 dies trobem ja a la perifèria arterial les primeres cèl·lules musculars creixent per la matriu i estenent-se pel pou de la placa.

   Després d'aquestes dues setmanes de cultiu, la morfologia de la paret arterial està plenament preservada, sent visibles les làmines elàstiques, les diferents capes cel·lulars de l'artèria, les cèl·lules gegants, i el propi infiltrat inflamatori.
2. Amb el pas dels dies no obstant, l'artèria en cultiu es desinflama progressivament, probablement degut a la desvinculació física d'un sistema immune complet, i potser activa un programa de reparació del teixit que s'origina a partir de l'extracció quirúrgica. Falta determinar si les cèl·lules inflamatòries moren o escapen del teixit cap al Matrigel, ric en factors de creixement.
3. Analitzant l'expressió de mRNA i la secreció de proteïnes entre biòpsies provinents de controls sans amb biòpsies de pacients amb GCA, hem observat certes diferències pel que fa a l'expressió d'algunes citoquines proinflamatòries. Els pacients tenen en concret, nivells de mRNA més elevats de IL-1β, IFN-γ, CCL3, CCL4, CCL5 i MMP9 que els controls. Resultats que a nivell de secreció de proteïna al sobrenedant es confirmen només per IL-1β i MMP9. Faltaria determinar si la resta realment no es secreten o queden atrapats al Matrigel.
4. El propi sistema de cultiu té un efecte sobre les arteries i la seva expressió d'algunes molècules degut al que hem comentat abans. Exceptuant l'IFN-γ, el cultiu d'artèria tendeix a incrementar l'expressió de citoquines proinflamatòries, quimiocines (CCL2 i CXCL8) i MMP9, tant en pacients com en controls, minimitzant així les diferències observades.
5. Pel que fa a l'efecte dels glucocorticoides (administrats en forma de dexametasona a les artèries) s'observa una inhibició generalitzada de les citoquines de caràcter proinflamatori (IL-6, IL-1β, IFN-γ, TNFα , de totes les quimiocines estudiades (CCL3, CCL4, CCL5 i CXCL8) i també de MMP9.

6. Les molècules relacionades amb el remodelat vascular en canvi (PDGF, MMP2, i Col·làgens), no es troben disminuïdes pel tractament.
7. En l'assaig d'immunohistoquímica podem veure una clara disminució de l'expressió del marcador de macròfags (CD68) regulada pels glucocorticoides, que en canvi no semblen tenir cap efecte sobre el marcador de limfòcits T (CD3).

Blocking Interferon Gamma (IFN-γ) Reduces Expression Of Chemokines (CXCL9, CXCL10 And CXCL11) And Macropahge Infiltration In Giant-Cell Arteritis

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EXTENDED REPORT

Blocking interferon γ reduces expression of chemokines CXCL9, CXCL10 and CXCL11 and decreases macrophage infiltration in ex vivo cultured arteries from patients with giant cell arteritis

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ABSTRACT

Background Interferon γ (IFNγ) is considered a seminal cytokine in the pathogenesis of giant cell arteritis (GCA), but its functional role has not been investigated. We explored changes in infiltrating cells and biomarkers elicited by blocking IFNγ with a neutralising monoclonal antibody, A6, in temporal arteries from patients with GCA.

Methods Temporal arteries from 34 patients with GCA (positive histology) and 21 controls were cultured on 3D matrix (Matrigel) and exposed to A6 or recombinant IFNγ. Changes in gene/protein expression were measured by qRT-PCR/western blot or immunoassay. Changes in infiltrating cells were assessed by immunohistochemistry/immunofluorescence. Chemotaxis/adhesion assays were performed with temporal artery-derived vascular smooth muscle cells (VSMCs) and peripheral blood mononuclear cells (PBMCs).

Results Blocking endogenous IFNγ with A6 abrogated STAT-1 phosphorylation in cultured GCA arteries. Furthermore, selective reduction in CXCL9, CXCL10 and CXCL11 chemokine expression was observed along with reduction in infiltrating CD68 macrophages. Adding IFNγ elicited consistent opposite effects. IFNγ induced CXCL9, CXCL10, CXCL11, CCL2 and intracellular adhesion molecule-1 expression by cultured VSMC, resulting in increased PBMC chemotaxis/adhesion. Spontaneous expression of chemokines was higher in VSMC isolated from GCA-involved arteries than in those obtained from controls. Incubation of IFNγ-treated control arteries with PBMC resulted in adhesion/infiltration by CD68 macrophages, which did not occur in untreated arteries.

Conclusions Our ex vivo system suggests that IFNγ may play an important role in the recruitment of macrophages in GCA by inducing production of specific chemokines and adhesion molecules. Vascular wall components (ie, VSMC) are mediators of these functions and may facilitate progression of inflammatory infiltrates through the vessel wall.

INTRODUCTION

Giant cell arteritis (GCA) is a chronic inflammatory disease targeting large and medium-sized arteries in aged individuals.[1] In spite of the initial response to high-dose glucocorticoids (GCs), 40–60% of patients relapse when GCs are tapered and prolonged GC treatment results in significant side effects.[2]

Search for new therapeutic options requires better understanding of pathogenesis. GCA has been classically considered a Th1-mediated disease based on the granulomatous nature of inflammatory lesions with the presence of giant cells.[1 3] Moreover, while transcripts of several cytokines (ie, interleukin (IL)-6, tumour necrosis factor-α (TNFα) can be detected in unaffected temporal artery biopsies (TABs),[4–7] IFNγ, the distinctive cytokine produced by Th1 lymphocytes, is selectively expressed in GCA lesions.[4 7–12] Recently, Th17-mediated mechanisms are also emerging as a relevant component of GCA pathogenesis.[9–13]

IFNγ has important roles in innate and adaptive immunity. It is primarily expressed by Th1 and natural killer (NK) cells and also by plasmacytoid dendritic cells, B cells and macrophages.[14 15] IFNγ signals by inducing dimerisation of its receptor chains, which, in turn, induces phosphorylation of JAK 1 and JAK 2, creating binding sites for STAT-1.[14 16 17] STAT-1 phosphorylation results in STAT-1 dimerisation or formation of multimolecular complexes (ie, ISFG3 composed by STAT-1, STAT-2 and IRF9 molecules) subsequently inducing transcription of genes bearing gamma-activated sequences or interferon-stimulated responsive elements (ISREs), respectively, in their promoter regions.[14 16 17] Some of the STAT-1-induced target genes are themselves transcription factors (ie, IRFs), creating subsequent waves of inflammatory molecule expression.[18] Adding complexity, IFNγ may also induce STAT-3, particularly in conditions of STAT-1 paucity.[19] IFNγ promotes NK cell activity, macrophage activation, Th1 differentiation and expression of class I and class II major histocompatibility complex molecules on antigen-presenting cells.[14–21] Based on these functions, IFNγ is thought to have a major role in GCA. Its expression by adventitial infiltrates in early GCA lesions suggests a relevant role from the initial steps of vascular inflammation.[11 22]

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To date, no mechanistic studies assessing the role of IFNγ in the development of full-blown lesions in GCA have been performed. In this study, we explored functional roles of IFNγ on GCA lesions by exposing cultured temporal artery sections from patients with GCA to a neutralising anti-human IFNγ monoclonal antibody.

PATIENTS AND METHODS

Patient samples

TABs were performed to 55 patients with suspected GCA for diagnostic purposes. A 5–15 mm segment was saved for the present study. Thirty-four patients had histopathological features of GCA in their TAB. Thirteen of them had started GC treatment 3.6±3.2 days before TAB. Twenty-one patients had no inflammatory infiltrates and served as controls. Clinical data of GCA patients and final diagnosis in control patients are disclosed in online supplementary tables S1 and S2, respectively.

Neutralising, monoclonal antibody A6 against IFNγ

A6 is a fully human monoclonal antibody generated in the laboratories of Novimmune (Geneva, Switzerland) from human immunoglobulin libraries using in vitro display technologies. A6 binds to human IFNγ and neutralises its bioactivity.

Temporal artery culture

Temporal artery sections from patients with GCA and controls were embedded in Matrigel to ensure prolonged survival, cultured ex vivo as described[7] with or without A6 antibody (10 μg/mL), recombinant IFNγ at 100 ng/mL (R&D Systems, Minneapolis, Minnesota, USA), human non-immune immunoglobulin IgG1 at 10 μg/mL (Sigma, Ayrshire, UK) as a negative control or dexamethasone (0.5 μg/mL, Sigma). Each condition was tested in 3–4 replicate wells. Biopsies were frozen in TRIzol reagent (Life Technologies) for RNA extraction or in radioimmunoprecipitation assay buffer with protease and phosphatase inhibitors for western blot studies.

Cell culture

Vascular smooth muscle cells (VSMCs) were isolated from TABs as previously described[22] and used after 3–8 doubling passages. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood of healthy donors using Lymphoprep (Axis-Shield, Oslo, Norway) (see culture details in online supplementary methods).

In specific experiments, VSMCs were seeded in 12-well plates and cultured alone or with 0.5×10^6 PBMC/well. At the end of the co-culture period, PBMCs were isolated from supernatants by centrifugation. The underlying VSMCs were separately recovered after gentle treatment with EDTA (Versene, Life Technologies) to remove adherent PBMC.

Gene expression analysis

Total RNA was obtained from cultured arteries or cells and cDNA was obtained by reverse-transcription (see online supplementary methods). Specific pre-developed TaqMan probes from Applied Biosystems (TaqMan Gene Expression Assays) (see online supplementary table S3) were used for PCR amplification. Fluorescence was detected with ABI PRISM 7900 Hardware and results were analysed with the Sequence Detection Software V.2.3 (Applied Biosystems). Gene expression was normalised to the expression of the endogenous control GUSb using comparative ΔCt method.[5–7 11] mRNA concentration was expressed in relative units with respect to GUSb expression.

Chemokine secretion

CXCL9, CXCL10 and CXCL11 concentrations in supernatants were measured by immunoassay using Quantikine (R&D Systems).

STAT-1 and STAT-3 phosphorylation in cultured arteries

Lysates were obtained from cultured artery sections or VSMC and phospho-STAT-1/total STAT-1 and phosho-STAT-3/total STAT-3 were assessed by western blot (see online supplementary methods).

Immunohistochemistry and immunofluorescence staining

Detailed immunostaining of cultured temporal artery sections or VSMC, as well as primary and secondary antibodies used, are depicted in online supplementary methods.

Chemotaxis assay

PBMC chemotaxis was assessed using Boyden chambers with 5 μm pore polycarbonate filters (see specific details in online supplementary methods).

Cell adhesion assays

VSMCs were grown to confluence in 96-well plates and stimulated with recombinant IFNγ. After 24 h 7.5×10^4 PBMCs per well were added and incubated at 37°C for 30 min. Wells were gently rinsed with phosphate-buffered saline and cells were fixed and stained with 0.2% crystal violet in 20% methanol for 10 min. Plates were extensively washed and crystal violet was solubilised with 1% sodium dodecyl sulfate (50 μL/well). Optical density was assessed by spectrophotometry at 560 nm wavelength.

Statistical analysis

Student's t test, when applicable, or Mann–Whitney test was applied for independent or paired samples for statistical analysis using SPSS software, PASW V.18.0.

RESULTS

Clone A6, a neutralising human monoclonal antibody against IFNγ, recognises IFNγ in GCA lesions and interferes with IFNγ-mediated signalling

Since A6 was screened and selected by its potential to neutralise IFNγ in vitro, we assessed its ability to bind IFNγ expressed in GCA lesions. As shown in figure 1, biotinylated A6 antibody immunostained GCA-involved temporal artery sections, whereas immunostaining with biotinylated human IgG1 was negative. Moreover, immunostaining of non-inflamed temporal artery sections from a control individual was negative according to the absence of IFNγ expression in normal arteries.[4 7]

Temporal artery culture in 3-D matrix has been shown to be a suitable model to test the effects of easily diffusing molecules such as dexamethasone.[7] However, it is not known whether complex molecules such as monoclonal antibodies are able to elicit biological responses in this system. To test this point, we investigated whether A6 was able to interfere with IFNγ-mediated signalling in cultured arteries by exploring the phosphorylation status of the transcription factors STAT-1 and STAT-3. As shown in figure 1E, F, normal arteries in culture had a remarkable constitutive expression and phosphorylation of STAT-3 but not STAT-1. Involved GCA arteries in culture had increased expression and phosphorylation of STAT-1 and decreased expression and phosphorylation of STAT-3 compared with cultured normal arteries. A6 antibody decreased expression and phosphorylation of STAT-1, whereas STAT-3 expression/

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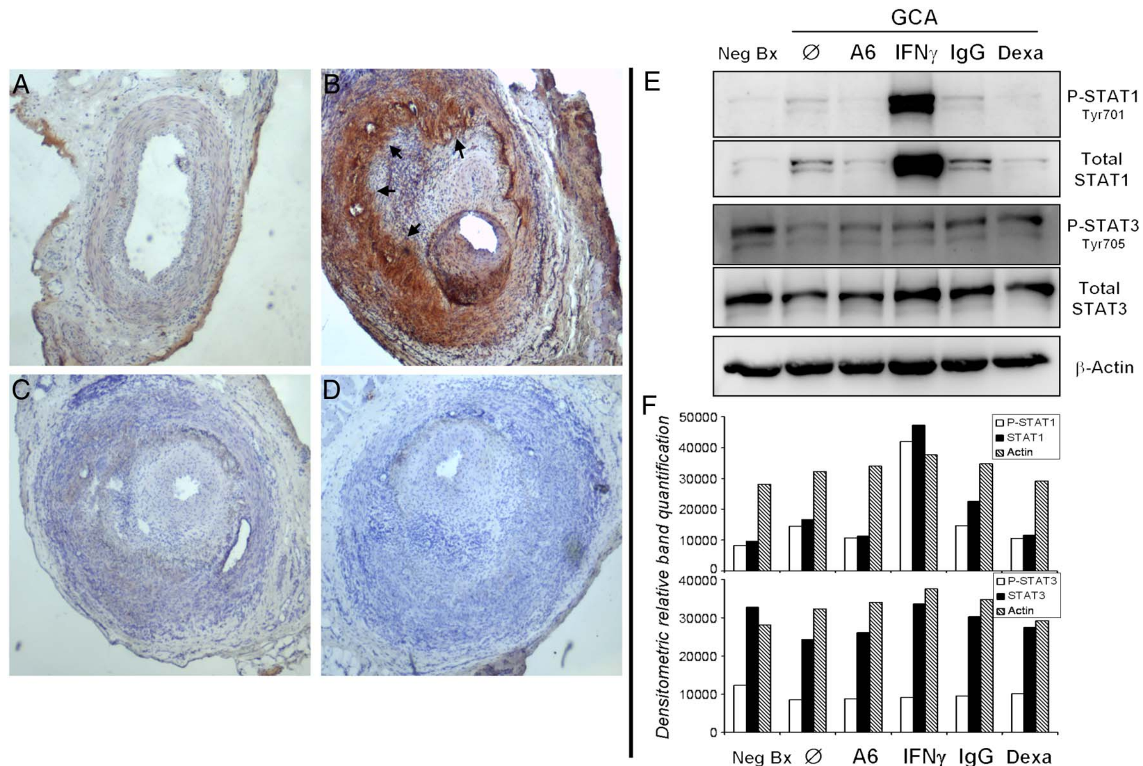

Figure 1 Interferon γ (IFNγ) expression in arteries with giant cell arteritis (GCA) and the effects of IFNγ on cultured temporal arteries from patients with GCA. (A–D) Immunostaining with A6 or control IgG1 on normal temporal arteries or GCA-involved arteries. (A) Histologically negative temporal artery from a control individual incubated with biotinylated A6 (negative control). (B) GCA-involved artery incubated with biotinylated A6 antibody (brown staining reflects IFNγ expression). (C) A GCA-involved artery incubated with biotinylated non-immune human IgG1 (isotype control). (D) A GCA-involved artery incubated with the detection system reagents but no A6 (negative control). (E) Western blot performed to assess phosphorylated or total STAT-1 and STAT-3 in protein extracts of a cultured histologically negative biopsy (Neg Bx) and a cultured GCA-involved biopsy (GCA) untreated (Ø), exposed to A6 (10 μg/mL), IFNγ (100 ng/mL), human IgG1 (IgG; 10 μg/mL) or dexamethasone (Dexa; 0.5 μg/mL) for 5 days. The experiment was repeated twice with consistent results and a representative blot is shown. β-Actin was used as a control for loading. (F) Densitometric analysis of bands obtained in western blot experiments is exemplified in (E).

phosphorylation was not substantially affected. Treatment with recombinant IFNγ strongly increased expression and phosphorylation of STAT-1.

Neutralising endogenous IFNγ production selectively downregulates CXCL9, CXCL10, CXCL11 chemokines and STAT-1 expression in cultured GCA arteries: adding exogenous IFNγ elicits opposite effects

We investigated the effects of neutralising IFNγ with A6 on the expression of a variety of candidate molecules relevant to the pathogenesis of vascular inflammation and remodelling in GCA.[3 5 11 22–30] Molecules investigated included transcription factors involved in T-cell functional differentiation, proinflammatory cytokines, chemokines/chemokine receptors, adhesion molecules, growth factors, metalloproteinases and their natural inhibitors, and extracellular matrix proteins (table 1 and figure 2). Since the temporal artery culture conveys by itself a downregulation of IFNγ expression with respect to the original fresh arteries, which may have minimised these results,[7] we sought to confirm the potential effects of IFNγ revealed by inhibition with A6 antibody by treating cultured GCA arteries with recombinant IFNγ.

Among the molecules tested, neutralising endogenous IFNγ with A6 mainly downregulated STAT-1 and chemokine CXCL9, CXCL10 and CXCL11 mRNAs (figure 2A). As shown in figure 2B, exposure of cultured GCA arteries to IFNγ elicited the expected opposite effects and induced strong expression of STAT-1 and chemokines CXCL9, CXCL10 and CXCL11 (figure 2B). CXCL9 and CXCL10 concentrations in the supernatant fluid were also reduced upon A6 antibody treatment and increased under exposure to recombinant IFNγ (figure 2C). Concentrations of CXCL11 were around the detection level and were not substantially modified by A6 antibody or recombinant IFNγ, suggesting that CXCL11 is not secreted or is retained in the extracellular matrix.

Table 1 shows the effect of blocking IFNγ with A6, as well as the effect of adding recombinant IFNγ on the additional molecules tested. Neutralising IFNγ with A6 tended to decrease HLA-DRA, TBX21, NOS-2, TNFα, IL-6, CCL2, CXCR3, intracellular adhesion molecule-1 (ICAM-1) and platelet-derived growth factor A mRNAs and, consistently, these tended to increase upon exposure to recombinant IFNγ. However, with the exception of TNFα, differences were not statistically significant, possibly due to the relatively low number of specimens analysed and the wide individual variability in expression of inflammatory products. Dexamethasone was able to markedly downregulate additional relevant molecules not influenced by A6 (table 1).

VSMCs contribute to chemokine production induced by IFNγ

Most of the effects of IFNγ have been investigated in T cells, monocytes and endothelial cells. To mimic vascular inflammatory infiltrates, we co-cultured PBMC from healthy donors with human temporal artery-derived VSMC, the main component of the arterial wall. Co-culture induced a variety of chemokines

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Table 1 Mean fold change in mRNA of selected representative genes related to T helper functional differentiation, inflammation and vascular remodelling in cultured temporal arteries from 34 patients with GCA and 21 controls subjected to different treatments (control IgG1, A6, recombinant IFNγ or dexamethasone)

Fold increase	GCA/control	GCA biopsies Clone A6/IgG1	IFNγ/untreated	Dexa/ untreated
Transcription factors				
TBX21	3.1154*	**0.7133**	**2.7776***	0.9522
GATA3	1.7611*	1.1780	1.3536	0.7716
RORC	3.6482*	0.8558	1.3571	0.8380*
STAT-3	0.5913	0.9232	1.6143	1.0565
Proinflammatory molecules				
Cytokines				
IL-1β	3.6162*	0.9041	1.1481	0.0342
TNFα	1.6621*	**0.7561***	**2.7333***	0.3047*
IL-6	1.4685	**0.7026**	**1.5621**	0.0757*
IFNγ	15.0840*	0.9119	0.6848	0.3869*
IL17A	103.5418*	1.4793	0.5557	0.0279*
Chemokines				
CCL2	1.6839	**0.8950**	**1.2292**	0.3424*
CCL3	6.3415*	0.9815	0.4381	0.2125*
CCL4	13.9455*	1.0598	0.1611	0.2058
CCL5	4.8446*	1.2153	1.3709	0.6600
CXCL8	1.3023	0.9805	0.9512	0.0748*
Chemokine receptors				
CCR2	6.9054*	1.3463	2.6116	0.5746
CXCR3	10.0717*	**0.6640**	**2.1125***	Not done
Adhesion molecules				
ICAM-1	1.7275	**0.8225**	**2.0204**	0.1137
VCAM-1	0.7031	0.9611	1.6861	0.0787
Other				
HLADRA	3.8304*	**0.7373**	**3.0312***	0.8810
NOS-2	1.7010	**0.2443**	**2.7067**	1.7251
Vascular remodelling-related molecules				
Growth factors				
PDGFA	0.4752*	**0.7690**	**1.6640**	0.4331
PDGFB	0.6820	0.8081	1.0921	0.6338
TGFβ	0.6106*	1.0119	1.2298	0.5117
Extracellular matrix proteins				
FN1	0.8757	1.0473	Not done	2.3182
COL1	1.9086	0.9953	Not done	0.9594
COL3	0.9037	0.8602	Not done	1.0790
Metalloproteases				
MMP-2	0.5738*	0.9101	0.5082	0.5827
MMP-9	2.3692*	0.8468	0.8204	0.0708*
MMP inhibitors				
TIMP1	1.8323*	0.9844	1.0956	0.3405*
TIMP2	0.5663*	0.9815	0.9614	0.7737

Number of specimens analysed: negative biopsies: 21; untreated GCA: 29; GCA treated with A6: 21; GCA treated with IgG1: 18; GCA treated with IFNγ: 9; GCA treated with dexamethasone: 11.
Bold values indicate consistent opposite results achieved by blocking IFNγ with A6 or by adding recombinant IFNγ.
*$p<0.05$.
Clone A6, anti-human IFNγ monoclonal antibody; GCA, giant cell arteritis; IgG1, isotype-matched control immunoglobulin; ICAM, intracellular adhesion molecule; IFNγ, recombinant interferon γ; MMP, matrix metalloproteinases; PDGF, platelet-derived growth factor.

not only in PBMC but also in VSMC, indicating that VSMCs are an active source of chemokines in an inflammatory microenvironment (figure 3A). IFNγ was produced and secreted by PBMC, basally and in co-culture (see online supplementary figure S1). In accordance with the previous results, neutralising IFNγ with A6 strongly and selectively inhibited CXCL9, CXCL10 and CXCL11 chemokine expression by all cell types (figure 3A). A slight, non-significant, reduction in STAT-1 and adhesion molecule ICAM-1 was observed. No effects of A6 were observed on the expression of STAT-3, VCAM-1 or other chemokines tested in this multicellular system (figure 3A and online supplementary figure S2).

To confirm that VSMC are an important source of chemokines upon IFNγ influence, we exposed human temporal artery-derived VSMC to IFNγ, which elicited a remarkable increase in STAT-1 (figure 3B). An induction of CXCL9, CXCL10 and CXCL11 and upregulation of constitutive CCL2 was obtained, whereas expression of other chemokines related to Th1 responses (ie, CCL3, CCL4 or CCL5) or CXCL8 was not significantly induced (figure 3B). Promoter analysis (4 kb upstream and 1 kb downstream of the transcription initiating sequence) of chemokine genes was performed using Chip Bioinformatics Mapper (http://snpper.chip.org/mapper/mapper-main).[31] The promoters of CXCL9, 10, and 11 as well as CCL2 shared ISRE sequences, whereas the remaining chemokines tested did not, supporting the exquisite sensitivity of these cytokines to IFNγ exposure. However, although IFNγ significantly upregulated constitutive CCL2 expression by cultured VSMC, A6 failed to downregulate CCL2 in multicellular systems such as PBMC/VSMC co-culture or whole GCA arteries where other inducers may be present (table 1 and figure 3A).

As previously demonstrated in other settings,[18 32 33] IFNγ also upregulated STAT-1 and adhesion molecule ICAM-1 expression by cultured VSMC and induced a slight expression of VCAM-1 and STAT-3 (figure 3B).

We next cultured temporal artery-derived VSMC from 8 patients and 11 controls and investigated chemokine production at passage zero to avoid phenotypic changes induced by prolonged culture. As shown in figure 3C, patient-derived VSMC conserved IFNγ signature and expressed significantly higher mRNA concentrations of IFNγ-induced chemokine CXCL9 and a tendency to increased expression of CXCL10 and CCL2 than VSMC derived from control individuals. Higher mRNA concentrations of other chemokines not clearly influenced by IFNγ in our experiments, particularly CCL4, were also observed. This may indicate VSMC exposure to stimuli other than IFNγ or to second-wave IFNγ-induced mediators in the complex multicellular and multimolecular microenvironment existing in GCA lesions.

Functional relevance of chemokine and adhesion molecule expression by VSMC

Treatment of cultured temporal artery-derived VSMC with IFNγ resulted in an increase in adhesion to PBMC (figure 4A).

The effect of IFNγ on ICAM-1 expression by VSMC was confirmed in GCA lesions. VSMC expressed ICAM-1 in cultured GCA arteries, particularly in the vicinity of inflammatory infiltrates. ICAM-1 expression was reduced in sections treated with A6 antibody and increased in sections treated with IFNγ (figure 4B). As already described,[25] ICAM-1 expression was also observed in *vasa vasorum* endothelial cells and inflammatory cells (figure 4B).

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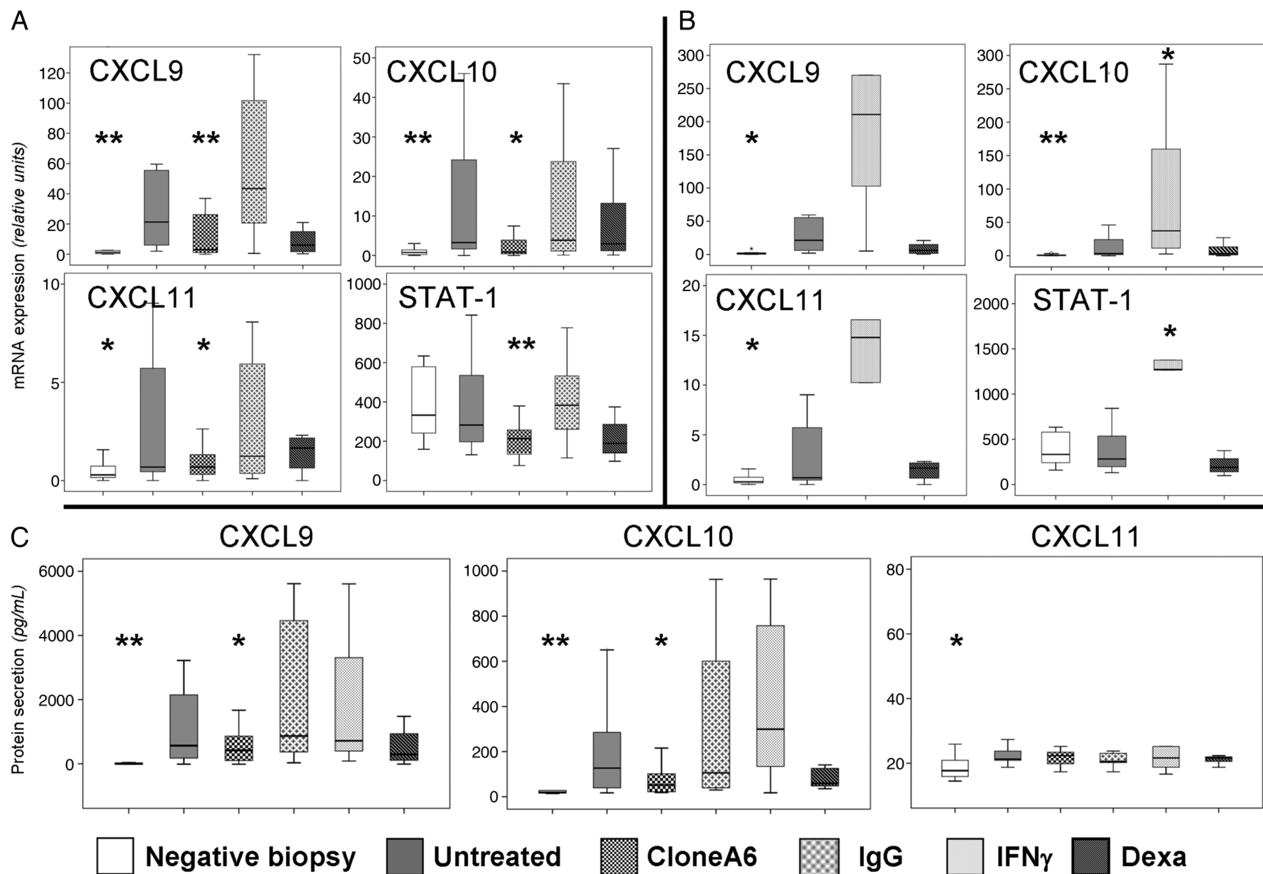

Figure 2 Changes in gene expression and protein secretion induced by blocking interferon γ (IFNγ) with A6 or adding additional IFNγ on cultured giant cell arteritis (GCA) biopsies. (A) mRNA concentrations (relative units) of CXCL9, CXCL10, CXCL11 and STAT-1 in cultured control arteries (negative biopsy) versus cultured GCA-involved arteries untreated or exposed to A6, human IgG1, or dexamethasone (Dexa) at the same concentrations as in figure 1. Statistical comparisons was performed between histologically negative and GCA-involved arteries and between IgG1-treated and A6-treated GCA involved arteries. *p<0.05; **p<0.005. Notice that the Y scale is different for each molecule. (B) mRNA concentrations (relative units) of CXCL9, CXCL10, CXCL11 and STAT-1 in cultured histologically negative arteries (negative biopsy) versus GCA-involved arteries untreated, or exposed to IFNγ or dexamethasone (Dexa) at the same concentrations as in figure 1 in a different set of experiments. Statistical comparison was performed between histologically negative and GCA-involved arteries and between GCA arteries untreated or treated with recombinant IFNγ. *p<0.05; **p<0.005. Notice that the Y scale is different for each molecule. (C) CXCL9, CXCL10 and CXCL11 concentrations (pg/mL) in the supernatants of cultured normal arteries and GCA-affected arteries untreated or exposed to A6, human IgG1, IFNγ or Dexa at the same concentrations as in figure 1. Statistical comparison was performed between histologically negative and GCA-involved arteries and between IgG1-treated and A6-treated GCA involved arteries. *p<0.05; **p<0.005.

Chemokine-rich supernatant of VSMC exposed to IFNγ stimulated PBMC chemotaxis in Boyden chambers (figure 4C), and this increase was abrogated by an antagonist of CXCR3, chemokine receptor common to CCL9, CXCL10 and CXCL11.

Effects of IFNγ neutralisation on infiltrating mononuclear cells in cultured temporal arteries from patients with GCA

The above findings suggest an important role for IFNγ in the initial recruitment of inflammatory cells in temporal arteries from patients with GCA and the participation of VSMC, the major component of normal arteries in this process. To confirm this hypothesis, normal temporal arteries were exposed to recombinant IFNγ and induction of CXCL9, CXCL10 and CXCL11 was confirmed (figure 5A). Incubation of IFNγ-treated normal arteries with PBMC from healthy donors resulted in infiltration of the artery wall by CD68 macrophages, which formed aggregates resembling giant cells (figure 5B). No CD3-positive cells penetrated the artery walls.

We next exposed cultured temporal arteries from patients with GCA to A6 and explored changes in the number of infiltrating T cells (CD3) and macrophages (CD68). Blocking IFNγ did not decrease the number of T cells (data not shown) but reduced the number of CD68-expressing cells and abrogated the presence of giant cells (figures 5C, D).

DISCUSSION

In this study, the first attempt to investigate the functional role of IFNγ in GCA, blocking endogenous IFNγ with a neutralising anti-IFNγ antibody, led to a significant reduction of STAT-1 and chemokine CXCL9, CXCL10 and CXCL11 expression in ex vivo cultured GCA arteries. Moreover, neutralising IFNγ resulted in decreased infiltration by CD68-expressing macrophages and reduced expression of TNFα along with a non-significant trend to decrease inflammatory molecules typical of a proinflammatory (M1-like) phenotype (HLA-DRA and inducible nitric oxide synthase).[20] These molecules, previously known to be expressed in GCA, are modulated by IFNγ in other

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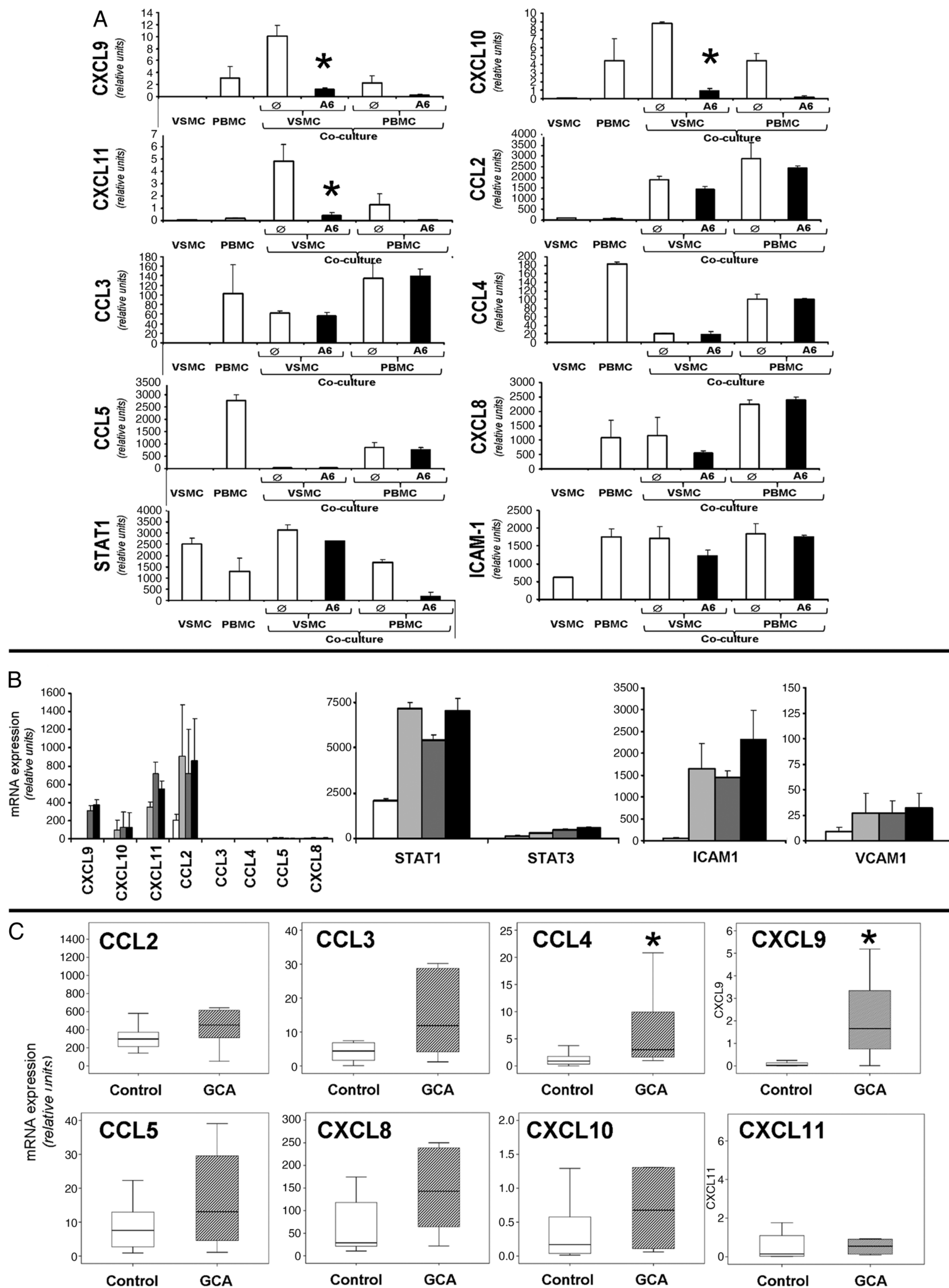

Figure 3 Effect of blocking the spontaneous interferon γ (IFNγ) production by co-cultured vascular smooth muscle cells (VSMCs) and peripheral blood mononuclear cells (PBMCs) on chemokine expression and effect of adding IFNγ on chemokine expression by temporal artery-derived VSMC. (A) VSMCs from normal temporal arteries were incubated alone or with PBMC (healthy donor) per well for 24 h. PBMCs were also cultured alone as a control. Cells were cultured with (A6) (filled bars) or without (Ø) (empty bars) the anti-IFNγ mAb A6 (10 μg/mL). After co-culture, PBMCs (adherent and non-adherent) were separated from VSMC. RNA was extracted from each individualized cell type: PBMC cultured alone, VSMC cultured alone, co-cultured PBMC (adherent and non-adherent) and co-cultured VSMC, and mRNA levels of various chemokines was determined. Notice that the Y scale is different for each molecule. The experiment was repeated three times with consistent results. *Significant reduction by A6 $p<0.05$ (B) Cultured VSMCs obtained from histologically normal temporal arteries were exposed to increasing concentrations of recombinant IFNγ(0-20-50-100 ng/mL) for 24 h and expression of transcription factors STAT-1 and STAT-3, chemokines and adhesion molecules intracellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 was assessed by real-time quantitative RT-PCR. All increases in expression of chemokines, ICAM-1, STAT-1 and VCAM-1 were statistically significant ($p<0.05$) compared to baseline. Increase in STAT-3 was significant ($p<0.05$) at the highest IFNγ concentration. (C) Spontaneous chemokine mRNA expression (relative units) by primary cultures of VSMC obtained from 11 normal arteries (empty boxes) or giant cell arteritis (GCA)-involved arteries (filled boxes). *$p<0.05$.

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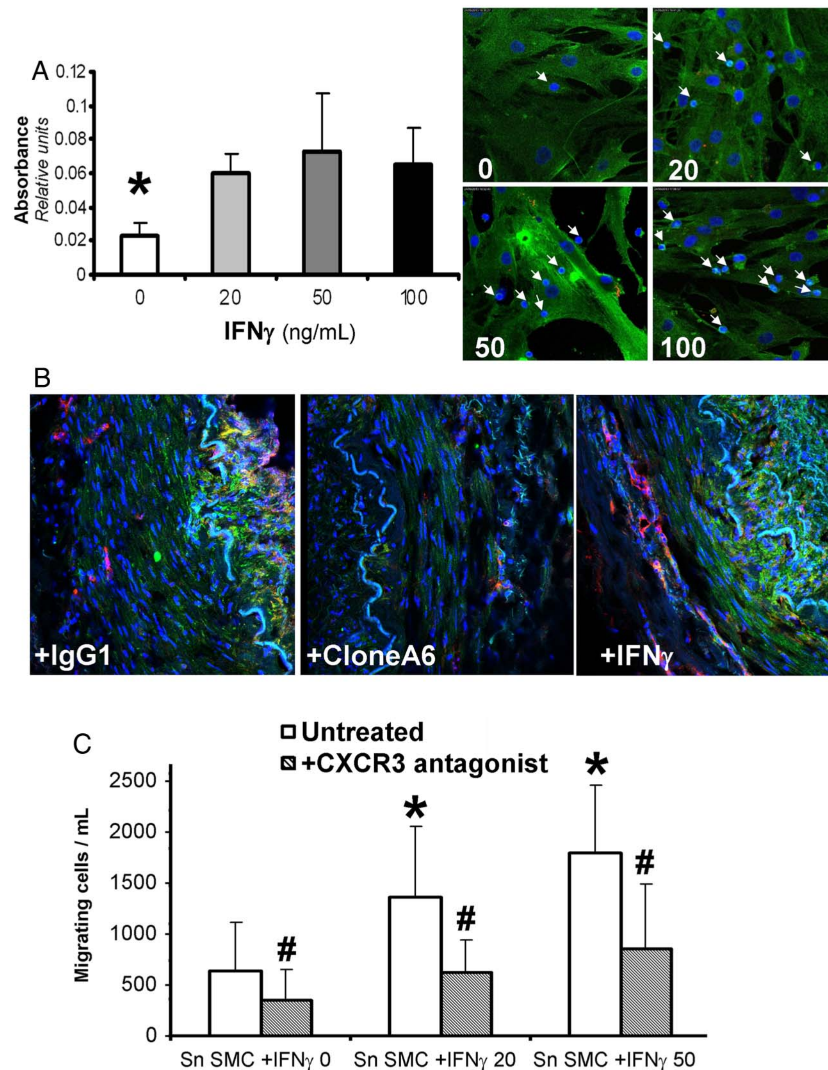

Figure 4 Changes in vascular smooth muscle cell (VSMCs) adhesiveness and chemoattraction to peripheral blood mononuclear cells (PBMCs) upon exposure to interferon γ (IFNγ). (A) PBMC adhesion to VSMC obtained from histologically normal temporal arteries and exposed to increasing concentrations of IFNγ. VSMC cultured in 96-well plates were exposed to increasing concentrations of IFNγ (0–20–50–100 ng/mL) for 24 h. PBMCs (75 000/well) were added and incubated for 1 h, washed and stained with crystal violet. Bars represent absorbance of solubilised dye in VSMC incubated with PBMC after subtraction of absorbance obtained from VSMC alone. *p<0.05. In parallel confirmatory experiments, VSMCs were seeded in chamber slides (Nunc, Waltham, Massachusetts, USA), and VSMCs were immunostained with a mouse monoclonal anti-human alpha smooth muscle actin antibody (ab54723, Abcam) (green). Nuclei were stained with DAPI (blue). The size of PBMC nuclei (indicated by arrows) are smaller than those from VSMC and can be easily distinguished. (B) Expression of intracellular adhesion molecule (ICAM)-1 (green) and CD31 (red) in temporal arteries from a patient with giant cell arteritis (GCA) exposed to IgG1 (10 μg/mL), A6 (10 μg/mL) or IFNγ (100 ng/mL)for 5 days. Nuclei were stained with DAPI (blue). VSMCs (elongated cells) express ICAM -1, particularly in the vicinity of infiltrating mononuclear cells. Co-expression of CD31 and ICAM-1 (merged, yellow) is observed on endothelial cells from adventitial vasa vasorum and neovessels. ICAM-1 expression is clearly reduced by A6 antibody. (C) Chemotactic activity of PBMC to the supernatant of VSMC exposed to IFNγ. Supernatants from VSMC obtained from histologically normal arteries incubated with increasing concentrations IFNγ (0–20–50 ng/mL) for 24 h were used to assess the induction of PBMC chemotaxis in the presence or in the absence of the CXCR3 inhibitor 500486. Bars represent the number of cells/mL that migrated to the lower chamber after 6 h incubation (mean±SD of three counts). *p<0.05 (increase in migration at any IFNγ concentration versus baseline). #p<0.05 (inhibition by CXCR3 antagonist).

settings.[5 19 20 23 26] Treatment of GCA arteries with exogenous IFNγ elicited opposite effects and tendencies, supporting the specificity of these findings.

Blocking IFNγ in our system led to a highly selective inhibition of ISRE-containing chemokine genes CXCL 9, 10 and 11. Although IFNγ also induced ISRE-dependent CCL2 in isolated VSMC, the effect of blocking IFNγ on CCL2 expression in GCA arteries was not apparent probably due to the remarkable constitutive expression of CCL2 in aged temporal arteries and the presence of potential additional inducers.[34] Since CXCL 9, 10 and 11 are powerful chemoattractants of mononuclear cells and these are able to interact with ICAM-1 expressing microvessels in inflamed arteries, our findings support a relevant role for IFNγ in the development and perpetuation of inflammatory infiltrates.

Based on the potent known effects of IFNγ on macrophages, and their predominance in GCA lesions, we expected that blocking IFNγ would have higher impact in the expression of downstream macrophage inflammatory products such as HLA-DR, NOS-2 and monokines.[20] In our model, IFNγ

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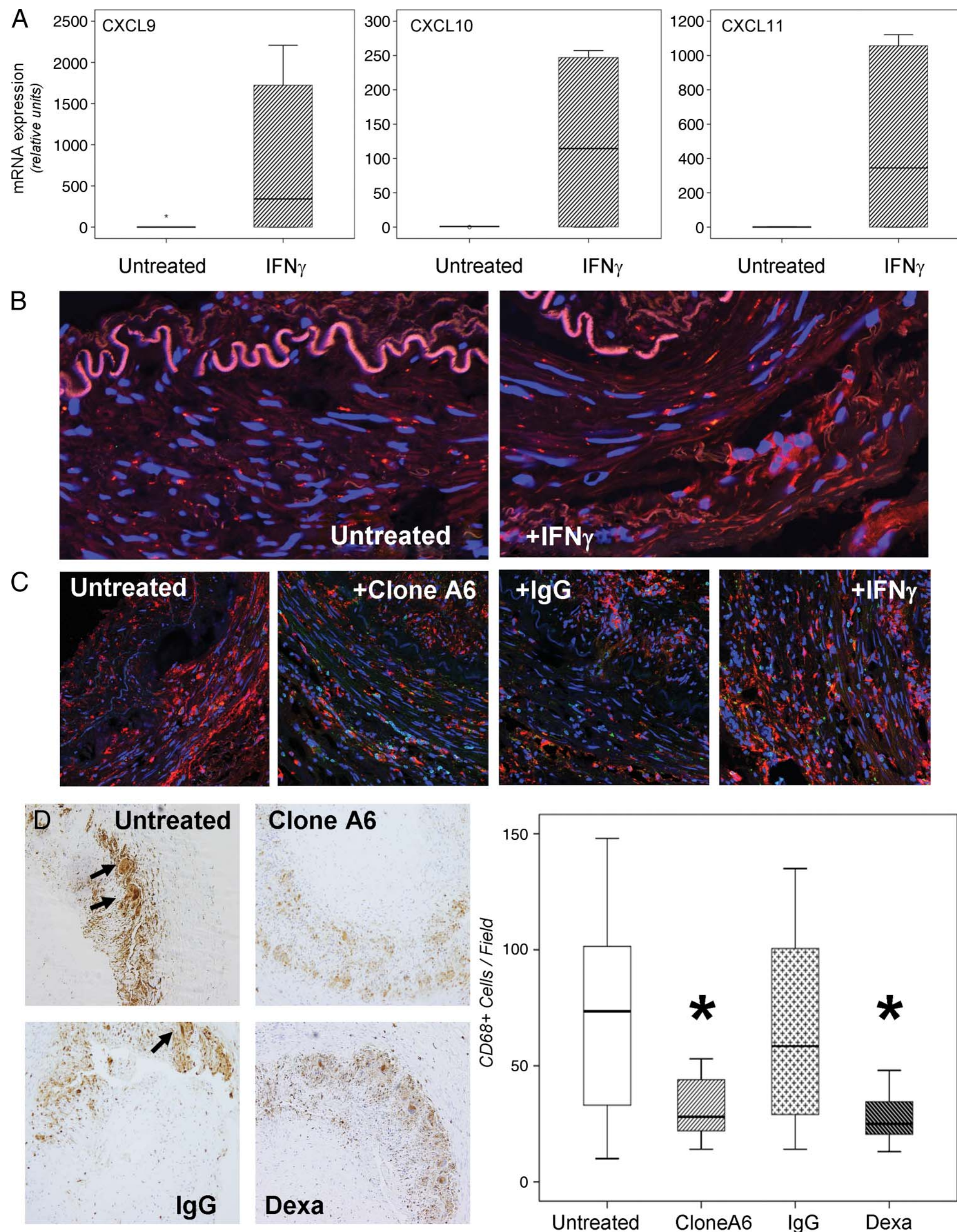

Figure 5 Effect of interferon γ (IFNγ) on chemokine expression and macrophage infiltration of histologically normal temporal arteries and reduction of macrophage infiltration in giant cell arteritis (GCA)-involved arteries following IFNγ blockade. (A) Histologically normal temporal arteries (N=6) were cultured on Matrigel with or without IFNγ (100 ng/mL) for 5 days and chemokine mRNA expression was assessed (relative units) by RT-PCR. (B) Histologically normal temporal arteries cultured in 24-well plates as above were incubated in medium alone or in medium containing IFNγ (100 ng/mL) for 4 days and were subsequently exposed to PBMC from a healthy donor (0.25×10^6/well) for 5 days. Cryosections of the retrieved arteries were processed for immunofluorescence, sectioned and immunostained with an anti-CD68 mAb (red). Nuclei were stained with DAPI (blue). (C) GCA affected temporal arteries were cultured in medium alone or in medium containing A6 antibody (10 μg/mL), control IgG1 (10 μg/mL) or recombinant IFNγ (100 ng/mL) for 5 days, washed and processed for immunofluorescence and immunostained with anti-CD68 mAb as in (B). Nuclei were stained with DAPI (blue). (D) Cryosections of GCA-affected temporal arteries, cultured as in (C), were immunostained with an anti-CD68 mAb. Notice reduction in immunostained CD68 cells and disappearance of giant cells (arrows) following anti-IFNγ or dexamethasone (Dexa) (0.5 μg/mL) treatment. Graph shows number of CD68+ cells/field (×100) in cultured GCA-involved temporal arteries untreated or exposed to A6, control IgG1 or dexamethasone (Dexa). Twelve fields/condition were assessed. *$p<0.05$.

neutralisation slightly modified or did not modify at all a number of relevant proinflammatory molecules that were, indeed, suppressed by GC. This may be determined by concomitant activation of IFNγ-independent pathways. In this regard, neutralisation of IFNγ did not substantially reduce expression and activation of STAT-3, which appears to be highly activated in normal arteries and in GCA lesions, in accordance with the remarkable production of IL-6 in normal and inflamed arteries.[4–6] Concomitant activation of STAT-3 and nuclear factor-κB may sustain expression of many inflammatory molecules in spite of IFNγ blockade.[35–37]

Most of the studies investigating IFNγ proinflammatory functions have explored its effects on macrophages and endothelial cells.[18 33] In vascular biology, the effects of IFNγ have been

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essentially investigated in the setting of atherosclerosis and graft vasculopathy.[38 39] In these models, IFNγ is expressed in lesions and production of IFNγ induced chemokines have been attributed to endothelial cells and inflammatory cells and only occasionally related to myofibroblasts.[38] In VSMC, the effects of IFNγ have been mainly related to vascular remodelling and neointima formation.[39] In recent years, it has become apparent that VSMC may acquire a strong proinflammatory phenotype in the appropriate context.[40 41] Our findings indicate that, in GCA, VSMC are also important targets for IFNγ, which renders them active producers of chemokines and adhesion molecules, especially ICAM-1. Consequently, VSMCs likely contribute to the progression of inflammatory infiltrates through the medial layer of the artery wall and to the development of full-blown granulomatous lesions in GCA.

GC, the cornerstone of current GCA treatment, rapidly downregulate the expression of a variety of inflammatory cytokines (ie, IL-1β, TNFα, IL-6, IL-17), adhesion molecules (ie, ICAM-1) and matrix metalloproteinases (ie, MMP-9).[7 11 27 28] However, GC do not repress IFNγ transcription.[35] Although prolonged GC treatment eventually results in decreased IFNγ expression by other mechanisms,[11 42–44] acute GC effects on IFNγ expression in GCA lesions are less dramatic than that observed with other cytokines.[7 27 35] This has led to the hypothesis that incomplete suppression of IFNγ accounts for GCA relapses during GC tapering or withdrawal,[29] and IFNγ has been considered a potential therapeutic target.[29 35] However, our findings indicate that reducing STAT-1 expression and activation by blocking IFNγ may not be sufficient to abrogate inflammatory activity in full-blown GCA lesions, which may require blockade of multiple pathways. However, interfering with IFNγ might be useful in preventing relapses, given the relevant role of IFNγ in the recruitment of inflammatory cells since the very early inflammatory stages.[11 45]

In considering IFNγ as a potential therapeutic target, it is important to consider that IFNγ may have a protective role by limiting tissue injury.[16 46] Blocking IFNγ worsens, indeed, experimental arthritis by promoting Th17 differentiation and exacerbation of IL-17-mediated inflammatory responses.[47] In addition, an infectious trigger of GCA has been postulated, although no causative agents have been consistently identified.[48] In this regard, IFNγ-deficient mice infected with murine herpesvirus HV68 develop necrotising large-vessel vasculitis, supporting the well-known role of IFNγ in host defence against viruses but also suggesting a role in limiting vascular injury.[46 49] Moreover, in some experimental settings, but not in others, IFNγ deficiency exacerbates aortic aneurysm development, which is one of the important delayed complications of GCA.[50–56]

Our study has several limitations. On the one hand, it explores functional activities of IFNγ in a target organ isolated from a functional immune system and variations in chemokine and adhesion molecule expression could not result in effective changes in leucocyte recruitment. It is likely that, in vivo, inhibition of lymphocyte and monocyte recruitment and subsequent macrophage activation would result in greater impact on the generation of downstream inflammatory products. In addition, as mentioned, the culture itself downregulates IFNγ expression,[7] which may have minimised the effect of IFNγ neutralisation in our model. GC treatment of some patients prior to the TAB may also have influenced results.[7]

In spite of these limitations, our findings indicate an important role for IFNγ in the recruitment and activation of macrophages, which may sustain and amplify subsequent waves of proinflammatory cascades in GCA. Moreover, our findings support the suitability of the temporal artery culture model to test functional activities not only of pharmacological agents or chemicals but also of complex molecules such as biological agents.

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Contributors MCC and MC-B designed the study. MHK-V and J-MD contributed important input to its design. MC-B, EP-R, EL and NT performed the experimental work. MAA, SP-G, AG-M, GE-F and JH-R contributed to clinical selection and contributed to the experimental work. RA and AE supervised in silico promoter regions studies. All authors evaluated and criticised the data and PR-L and MHK-V provided important contributions to their interpretation. MC-B and MCC wrote the manuscript. All authors read, made improvements and approved the final version.

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Competing interests MHK-V and WGF are full employees by Novimmune. MCC, J-HR, GE-F and SP-G are currently participating in a clinical trial of tocilizumab (anti-IL-6R) in giant cell arteritis sponsored by Hoffmann-La Roche.

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Blocking interferon γ reduces expression of chemokines CXCL9, CXCL10 and CXCL11 and decreases macrophage infiltration in ex vivo cultured arteries from patients with giant cell arteritis

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RESUM DE RESULTATS

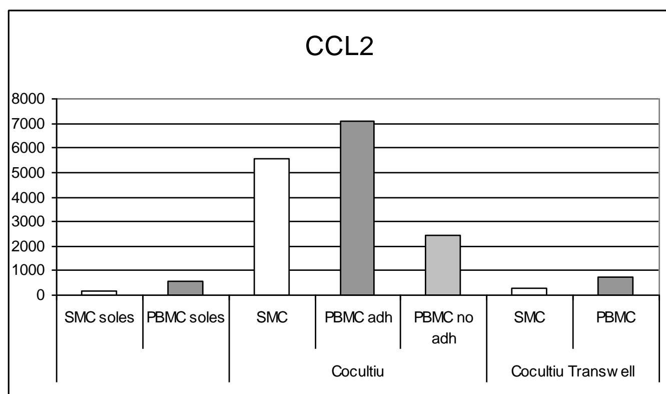
1. L'anticòs monoclonal humà contra IFN-γ Clone A6 s'uneix de manera específica en l'assaig de immunohistoquímica, a l'IFN-γ en les seccions d'artèria, i ens mostra una distribució de la mateixa citoquina que inclou gran part de les làmines mitja i adventícia de la paret arterial.
2. Utilitzant tècniques de detecció múltiple de molècules a nivell de mRNA (amb targetes microfluídiques) i de proteïna (amb *arrays* de proteïna) en les mostres del cultiu d'artèria, hem vist que el bloqueig d'IFN-γ inhibeix la concentració d'algunes quimiocines com CCL3, CCL5, CXCL8, entre d'altres citoquines rellevants com IL-1β, IL-6 o TNF-α.
3. L'expressió de STAT1 i STAT3 analitzada per Western Blot mostra les diferències existents entre els nivells d'expressió de tots dos STATs. Així, veiem STAT3 expressat de manera significativament més elevada en els controls que en els pacients. Pel que fa a STAT1, l'anticòs Clone A6 n'inhibeix clarament la secreció en les artèries positives cultivades, mentre que l'addició de IFN-γ recombinant al cultiu, estimula tant la expressió com l'activació via fosforilació de STAT1. Aquesta estimulació amb IFN-γ, també incrementa en menor mesura STAT3.
4. En el cultiu d'artèria, a nivell de mRNA hem vist significativament inhibides les expressions de CXCL9, 10 i 11, i STAT1 en les seccions tractades amb Clone A6, mentre que en les seccions estimulades amb IFN-γ l'efecte ha estat oposat, també de manera significativa. L'IFN-γ estimula també HLA-DRA i NOS2. En la taula 2 podem observar les diferències obtingudes en altres molècules estudiades, algunes de les quals mostren tendències a la inhibició o l'estimulació en totes dues situacions. Pel que fa a la secreció tant CXCL9 com CXCL10 mostren els mateixos resultats que en mRNA, mentre que CXCL11 es manté per sota del llindar de detecció de la tècnica, així que no podem saber si realment no es secreta o es queda atrapat al Matrigel.
5. El cocultiu de VSMC amb PBMCs activa de manera significativa l'expressió de totes les quimiocines estudiades en les cèl·lules musculars, mentre que el contacte entre cèl·lules només té un efecte estimulador sobre les PBMCs en CXCL9, 10 i 11, i CCL2.

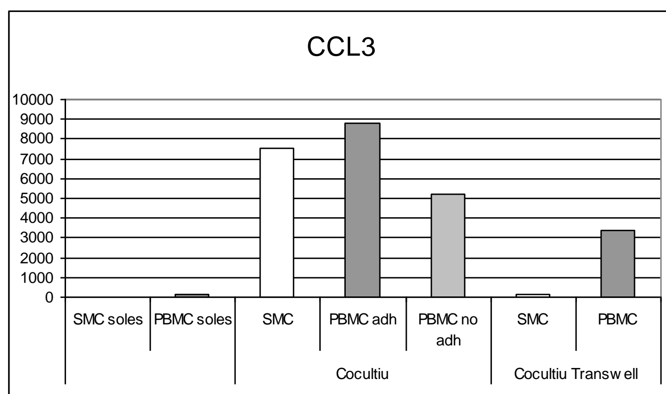
En presència de l'anticòs anti-IFN-γ, l'expressió de CXCL9, 10 i 11 es troba significativament disminuïda en tots dos tipus cel·lulars, però no s'observa efecte sobre les altres quimiocines.

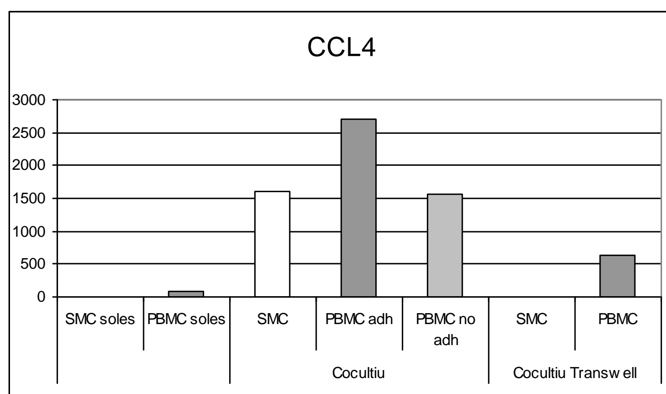
6. En el cultiu de VSMCs aïllades provinents de biòpsies de pacients control sans, concentracions creixents de IFN-γ, estimulen significativament l'expressió de CXCL9, 10 i 11, i també la quimiocina CCL2 (però no les CCL3, CCL4, CCL5, ni CXCL8), i de tots dos factors de transcripció STAT1 i STAT3.
7. Aquelles VSMCs que provenen de biòpsies de pacients amb ACG, mostren nivells d'expressió significativament més elevats de totes les quimiocines estudiades (exceptuant CXCL11) que les VSMCs que provenen de biòpsies de pacients sans, reflectint la seva exposició *in vivo* a l'IFN-γ.
8. En l'assaig de migració, els sobrenadants de VSMCs estimulades amb concentracions creixents de IFN-γ, provoquen un increment de la migració gràcies a l'estimulació de les quimiocines. Afegint un inhibidor químic del receptor de les quimiocines CXCL9, 10 i 11 (CXCR3), aquesta estimulació de la migració s'inhibeix de manera pràcticament total.
9. L'IFN-γ sobre les VSMC, també indueix l'expressió de les molècules d'adhesió ICAM-1 i VCAM-1.
10. En l'assaig d'adhesió podem veure que les PBMCs s'adhereixen a la monocapa de cèl·lules musculars llises d'una manera dosi depenent a l'estimulació amb IFN-γ. Les concentracions més altes de IFN-γ provoquen major estimulació de ICAM-1 i VCAM-1, i augmenten l'adhesió de les PBMCs, resultat que es pot observar en imatges amb l'experiment d'adhesió fluorescent amb el microscopi confocal.
11. Mitjançant immunohistoquímica hem vist que l'expressió de CD68 es troba disminuïda en les seccions d'artèria que tenen bloquejat l'IFN-γ amb el Clone A6, i també aquelles tractades amb corticoides. Aquest resultat es repeteix en la immunofluorescència d'una altra artèria cultivada també tractada amb l'anticòs bloquejant i estimulada amb IFN-γ recombinant.
12. En les artèries negatives cultivades de pacients sans, l'IFN-γ mostra una clara estimulació de l'expressió de CXCL9, 10 i 11, però no té cap efecte sobre altres molècules.

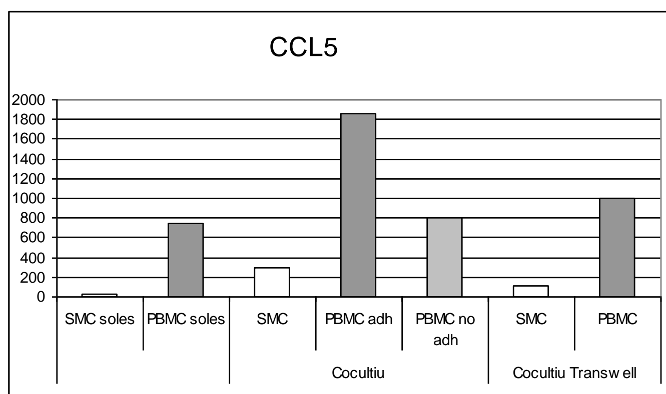
13. Tractant una artèria amb IFN-γ a dosis altes, i co-cultivant l'artèria amb l'addició de PBMCs, s'observa infiltració per part de les cèl·lules inflamatòries (CD68+) a la paret arterial, i la formació d'uns agregats multicel·lulars (també CD68+) a la secció d'artèria estimulada amb IFN-γ, que recorden a les cèl·lules gegants.

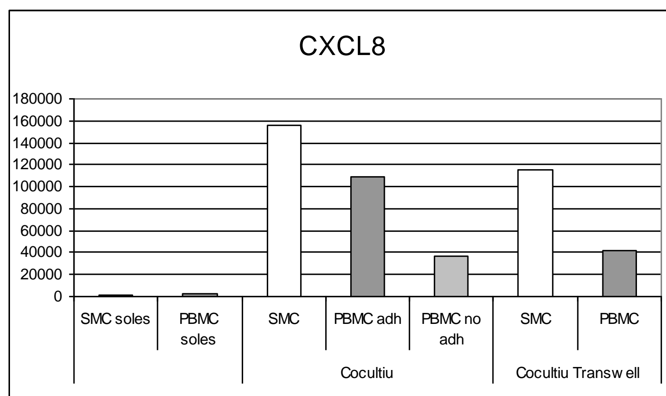
Resultats addicionals 1

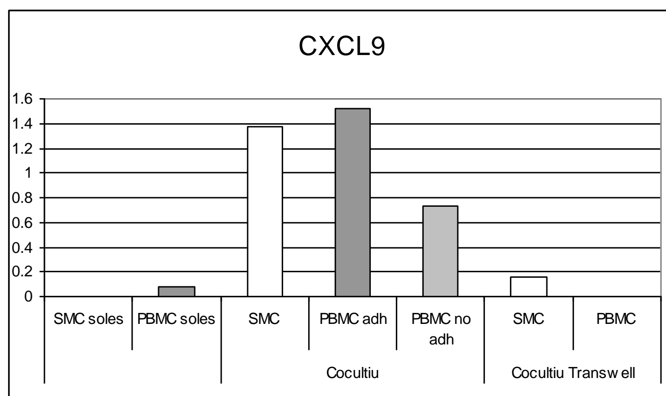


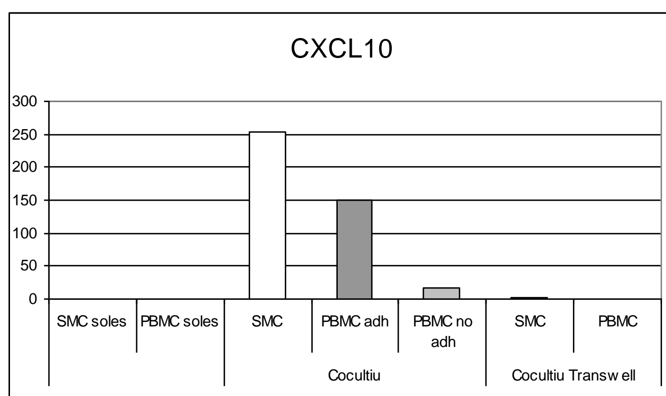


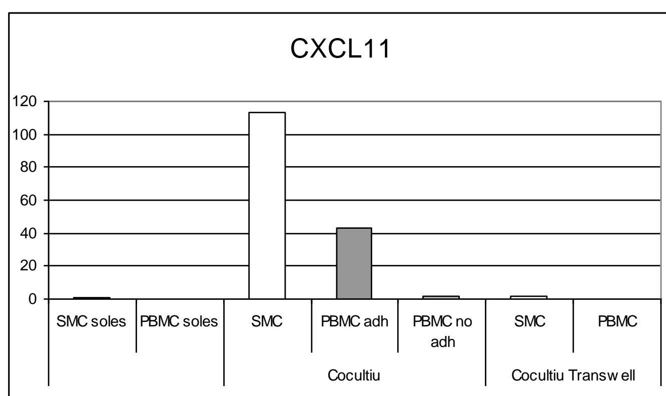






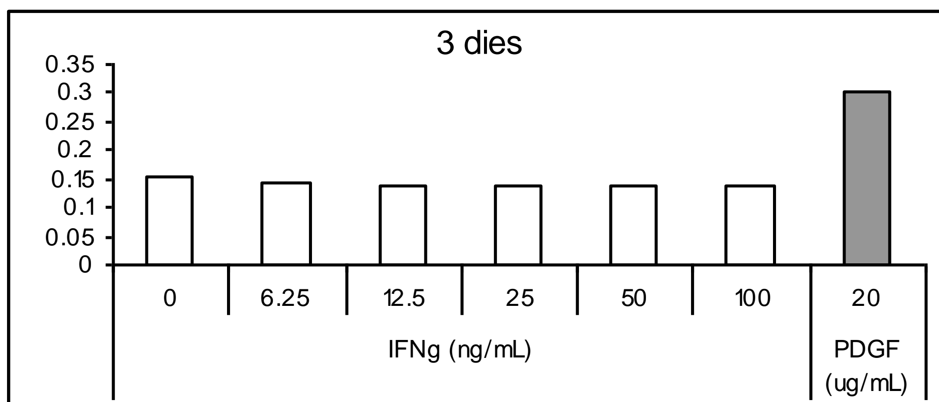


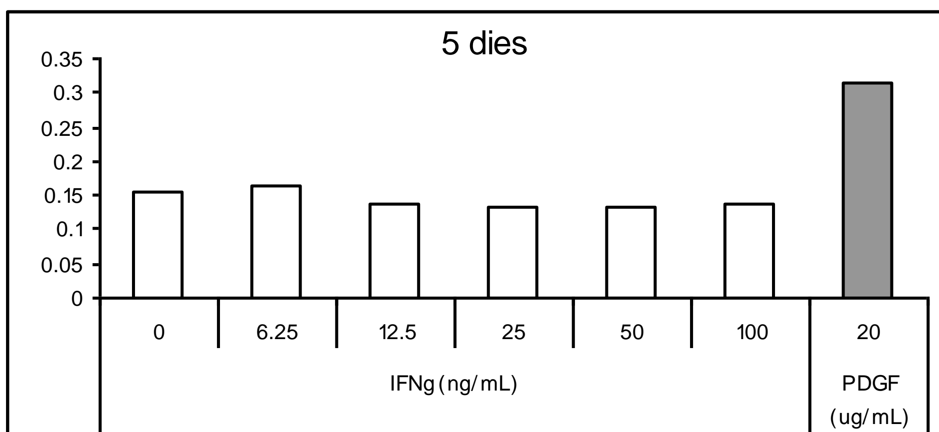


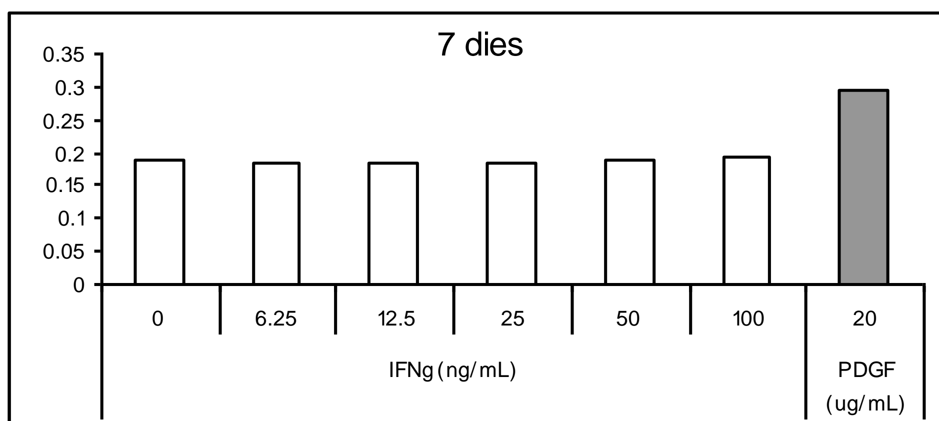


Expressió de mRNA de quimiocines en co-cultiu amb i sense *transwell*. VSMC pre-confluents són co-cultivades amb $0{,}5 \cdot 10^6$ PBMCs/pou durant 24 hores, permetent el contacte cel·lular i mitjançant l'ús de transwells. L'mRNA de cada una de les poblacions s'obté separant-les mitjançant la utilització de Versene (EDTA) per separar les PBMCs que s'han adherit a les VSMC, i tripsina per recuperar les pròpies VSMC. Les PBMCs no adherides es recuperen del sobrenedant. L'expressió de les molècules es dóna en unitats relatives.

Resultats addicionals 2

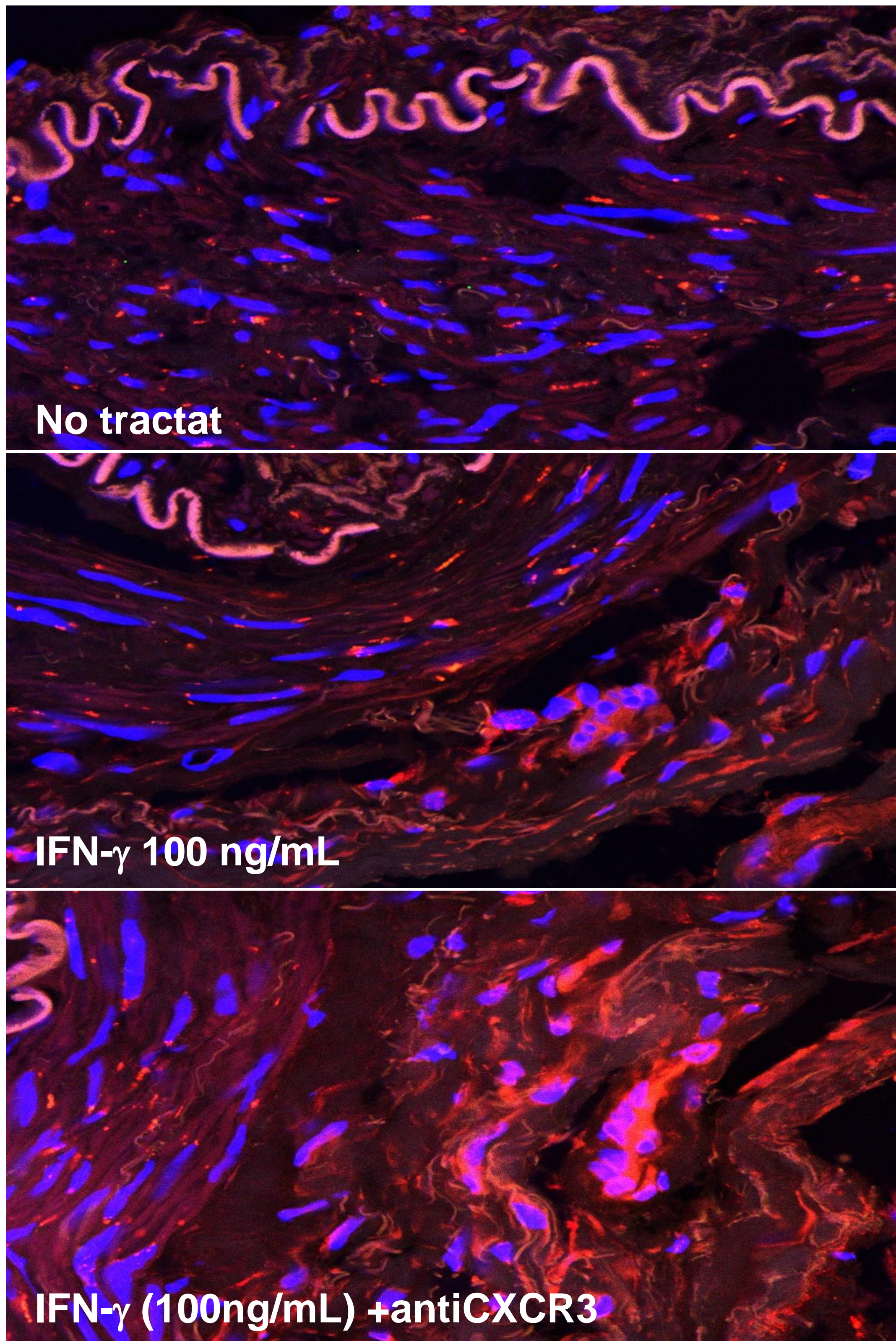






Proliferació de les VSMC amb concentracions creixents d'IFN-γ. VSMC provinents de controls són exposades a concentracions creixents de IFN-γ (ng/mL), i a una concentració de 20μg/mL de PDGF com a control positiu, en experiments de 3, 5 i 7 dies de duració. Posteriorment les cèl·lules són fixades i tenyides amb cristall violeta, i solubilitzades amb SDS (10%), per ésser quantificades en un lector de plaques. Les unitats de quantificació s'expressen en lectures de densitat òptica (a 620nm).

Resultats addicionals 3



Co-cultiu de PBMCs amb artèria temporal normal en presència o absència d'IFN-γ. Artèries provinents de controls són pre-cultivades en presència o absència d'IFN-γ (a 100ng/mL) durant 3 dies, i posteriorment co-cultivades amb PBMCs provinents de controls sans, que han estat o no pre-tractades amb un inhibidor químic de CXCR3 (2μg/mL). Les biòpsies són fixades amb paraformaldehid (4%), rentades en concentracions creixents de sacarosa (15-30%) i incloses en OCT. Finalment són tallades a un gruix de 20μm. En la immunofluorescència valorem la infiltració dels macròfags amb la tinció vermella, que correspon a marcatge de CD68, mentre que els nuclis queden tenyits en blau.

Discussió

DISCUSSIÓ

Tradicionalment descrita com una malaltia lligada a la diferenciació Th1, treballs recents han demostrat que la via Th17 juga un paper igualment significatiu en l'ACG (111). Malgrat això, donada la forta expressió d'IFN-γ en els pacients, en aquesta tesi ens hem centrat en explicar el paper d'aquesta important citoquina, lligada a la primera via de diferenciació limfocitària, en el procés fisiopatològic en l'ACG.

Durant aquest procés, s'ha descrit la presència de formacions granulomatoses, i la desestructuració progressiva de la làmina mitja de la paret arterial per l'acció de l'infiltrat inflamatori sobre les cèl·lules musculars que la conformen.

No obstant, malgrat aquests processos semblen consolidats en el coneixement de la malaltia, la descripció completa i fidedigne del procés patogenètic de l'ACG, i del rol que hi desenvolupa l'IFN-γ, és una de les assignatures pendents de la investigació en aquesta malaltia.

Aquest dèficit de coneixement va íntimament lligat al dèficit de models experimentals per a la realització d'estudis funcionals que ajudin a aclarir la importància relativa de cada un dels components moleculars que participen en aquesta evolució de la inflamació a la paret arterial.

Així doncs, fins al moment, el model de ratolí quimera de Cornelia Weyand *et al* era la única aproximació que tenim en l'ACG a un model experimental. No es tracta però d'un model animal real, d'un animal que pateixi ACG, sinó de la utilització d'un animal immunodeprimit com a suport biològic per una artèria d'un pacient amb la malaltia, implantada de manera subcutània (122).

Així doncs l'ús d'aquest model ha permès determinar canvis en l'expressió de certs mediadors inflamatoris que es troben modulats pel tractament amb glucocorticoides, com és el cas de IL-1β o IL-6, o modificats pel bloqueig de la via NOTCH com la IL-17 (36, 123).

En aquest sentit, en el nostre grup hem treballat els darrers anys en la posta a punt d'un model de cultiu d'artèria temporal de pacients, que permeti la realització d'assajos amb possibles agents terapèutics, i que presenta certs avantatges respecte al model de ratolí quimera de Weyand *et al*.

En primer lloc cultivar les artèries permet la seva monitorització diària, i garanteix una acció directa de les molècules que es pretenen testar sobre el teixit, mentre que en el model del ratolí quimera no es demostra que hi hagi vascularització cap a l'artèria implantada. El fet de tallar les artèries en seccions (aleatoritzades per minimitzar l'impacte de l'heterogeneïtat inflamatòria al llarg del segment d'artèria) permet realitzar diverses situacions diferents de tractament, i diversos duplicats per cada una de les situacions. En el ratolí quimera en canvi, cada artèria admet un sol tractament per animal.

A més d'analitzar l'expressió de mRNA de les pròpies artèries, podem també recollir el sobrenedant del cultiu i analitzar-ne la secreció de mediadors inflamatoris a nivell de proteïna.

Per acabar amb els avantatges sobre el model quimera, el cultiu d'artèria és més simple i econòmicament sostenible que el mètode del ratolí i, òbviament, permet evitar l'ús d'animals d'experimentació.

Altres treballs havien abordat el cultiu d'explants d'artèria temporal anteriorment, però ho havien fet sense el suport de matriu (124) i per tant els cultius eren viables durant poc temps.

La innovació en l'ús del Matrigel (125) permet al nostre model de cultiu mantenir la viabilitat i la morfologia de la paret arterial ben preservada fins un període de temps superior a dues setmanes.

Una de les limitacions del model és que *per se*, el cultiu de les artèries provoca l'estimulació de certes molècules potser relacionades amb el remodelat vascular. El contacte directe entre el matrigel (que és ric en proteïnes de matriu i factors de creixement) i l'artèria podria engegar programes de reparació del teixit (que ha estat quirúrgicament manipulat). Així en artèries control veiem que el cultiu d'artèria estimula molècules relacionades amb el remodelatge vascular com IL-6, PDGF, MMP9, Col III, CXCL8 o CCL2, mentre que inhibeix IFN-γ i Col I en les artèries amb ACG. Malgrat aquest fet permeti que el propi cultiu d'artèria emmascari resultats en algunes molècules proinflamatòries, reforça per contra, aquelles diferències observades que han donat resultats significatius malgrat veure's afectades per la regulació negativa del cultiu.

Amb l'ús del nostre model, hem pogut veure les diferències entre biòpsies positives i negatives. Així, tal i com esperàvem, aquelles citoquines i quimiocines lligades a processos inflamatoris es troben elevades en pacients respecte controls. És el cas de: IL-1β, IFN-γ, MMP9, CCL3, CCL4, o CCL5.

Al mateix temps, hem confirmat que l'efecte dels glucocorticoides en les artèries cultivades, es fa notar també en aquestes mateixes molècules, i trobem inhibida l'expressió de IL-1β, IL-6, IFN-γ i TNFα.

Amb aquest model a punt, hem pogut realitzar gran part dels estudis funcionals amb la nostra molècula d'interès, l'IFN-γ, ja sigui neutralitzant-lo o estimulant amb ell les nostres mostres, així com analitzar el paper d'aquesta citoquina sobre el cultiu aïllat de VSMCs derivades d'artèria o el co-cultiu amb PBMCs.

En l'ACG, s'ha associat clínicament la presència de molècules relacionades amb la via Th1 a una major freqüència de relapses (15). Aquest fet, juntament amb què l'IFN-γ és pràcticament absent en artèries sanes, i persisteix més elevat que altres citoquines durant les primeres setmanes de tractament (59), suggereix la possible associació de l'IFN-γ amb la persistència de la malaltia, i per tant situa aquesta citoquina, o a les molècules de les seves vies de senyalització, en el punt de mira d'estudis encaminats a millorar el tractament de l'ACG.

A l'iniciar aquesta via investigadora no obstant, no podem oblidar el paper dual de l'IFN-γ del que hem parlat a la introducció, per una banda limitant de la destrucció vascular, però per altre banda promovent l'acció proinflamatòria amb l'activació dels macròfags i la diferenciació limfocitària.

Per tant, l'elegibilitat com a diana terapèutica anirà lligada a l'equilibri entre aquests dos processos i la seva repercussió en el manteniment de la inflamació. Processos que d'altra banda, semblen lligats a la regulació sobre la via de senyalització de STAT1.

Mitjançant l'ús d'un anticòs humà específic contra IFN-γ (Clone A6), hem obtingut la primera evidència de què aquesta citoquina s'expressa en el teixit, i hem pogut localitzar la seva expressió dins la paret arterial, a la vegada que verificàvem l'especificitat del nostre agent bloquejant.

Un cop establert el clone A6 com a eina per l'estudi, el primer pas en aquest procés investigador ha estat utilitzar aproximacions experimentals de detecció massiva de molècules, tant a nivell de mRNA (amb targetes microfluídiques) com de proteïna (amb *arrays* de proteïna) amb les que hem observat l'activitat moduladora de l'IFN-γ sobre moltes molècules, entre les quals ha destacat la regulació d'algunes quimiocines.

Així doncs, a tenor d'aquests resultats inicials, en aquest treball ens hem centrat en clarificar quin paper pot dur a terme l'IFN-γ a través de la modulació de les quimiocines en la persistència de l'infiltrat inflamatori a la paret arterial.

Tal i com s'ha introduït abans, les quimiocines són petites molècules peptídiques encarregades de la regulació del trànsit leucocitari.
Entre elles trobem dues famílies importants que inclouen algunes de les quimiocines més estudiades en la literatura, com les quimiocines β (o CC), entre les que tenim CCL-2, 3, 4 i 5, i les quimiocines α de la família CXC, entre les que trobem a CXCL8, i les tres quimiocines directament induïdes per IFN-γ, CXCL9, 10 i 11.
Totes aquestes molècules realitzen importants i descrites funcions sobre el trànsit de macròfags, limfòcits i eosinòfils a través dels endotelis cap a les zones d'inflamació (37).

D'altra banda, la nostra molècula d'estudi, l'IFN-γ, és una de les citoquines clau en multitud de processos inflamatoris, i mitjançant un complex sistema de regulació, i unes vies de senyalització ramificades, exerceix de regulador entre d'altres coses d'algunes quimiocines, en diversos tipus cel·lulars.

Així doncs, per tal de validar els resultats inicials dels assajos de detecció múltiple, introduint la variable de l'anticòs bloquejant en el nostre model de cultiu d'artèria, hem observat l'efecte de la neutralització específica de l'IFN-γ sobre l'expressió d'altres citoquines proinflamatòries, molècules relacionades amb el remodelat vascular (proteïnes de matriu, factors de creixement i metaloproteïnasses), factors de transcripció, marcadors cel·lulars i evidentment sobre quimiocines.

A nivell d'expressió de mRNA en les artèries, l'efecte de l'anticòs Clone A6 ha estat més significatiu sobre aquelles molècules directament relacionades amb la via de l'IFN-γ, és a dir, sobre les quimiocines que es troben regulades per ell de manera directa (CXCL9, 10 i 11) i sobre STAT1, que és el seu factor de transcripció principal de la via canònica de senyalització.

A nivell de secreció de proteïna, que assagem mitjançant tècniques individuals d'ELISA, els resultats coincideixen, essent les mateixes molècules les inhibides de manera significativa (amb l'excepció de CXCL11).

Malgrat això, el cultiu d'artèria bloquejant IFN-γ ens ha permès observar certes altres tendències a la inhibició, que probablement no han esdevingut significatives per una exposició insuficient al Clone A6, ja sigui en quan a temps o a concentració de l'anticòs. Així doncs, malgrat les diferències no han resultat estadísticament rellevants, si que podem concloure que l'IFN-γ té cert efecte sobre l'expressió i la secreció de TNFα o CCL2 en les artèries cultivades.

Després de veure els efectes que tenia el bloqueig de IFN-γ sobre les artèries, hem utilitzat el nostre model per sobreestimular tant artèries de pacients amb ACG, com artèries de controls sans, amb IFN-γ recombinant, esperant obtenir resultats antagònics en l'expressió de les molècules analitzades utilitzant el Clone A6.

Pel que fa l'expressió de mRNA, l'estimulació amb IFN-γ a altes dosis ha mostrat resultats molts més clars que el bloqueig, probablement en part també per la pròpia regulació negativa intrínseca del cultiu. Així doncs, tal i com esperàvem i com es descriu a la literatura, hem vist estimulació d'aquelles molècules que es trobaven inhibides pel Clone A6, de manera estadísticament significativa (CXCL9, 10 i 11, i STAT1) però també sobre els receptors de

quimiocines CCR2 i CXCR3 (receptors específics de CCL2 i CXCL9, 10 i 11 respectivament), i molècules íntimament relacionades amb els macròfags com HLA-DRA o NOS2.

Igualment també s'han vist estimulades per IFN-γ, TNFα i STAT3. Malgrat no formar part de la via canònica de l'IFN-γ, STAT3 és estimulat per aquesta citoquina, tot i que en menor mesura que STAT1.

És el balanç entre totes dues vies el que definirà el comportament cel·lular, sent la via STAT3 la que es troba activada en absència de inflamació i per tant en absència de IFN-γ. El nivell basal d'activació d'STAT3, com veurem més endavant, és molt superior al de STAT1, ja que són altres citoquines les que l'utilitzen com a factor de transcripció principal.

Avaluant els nivells de secreció de proteïna en el cultiu d'artèria estimulat amb IFN-γ, hem pogut confirmar que les quimiocines CXCL9 i 10 es troben significativament estimulades, però CXCL11 està per sota del llindar de detecció de la tècnica d'ELISA emprada, amb el que no podem determinar si no s'excreta o es queda atrapada a la matriu extracel·lular.

Mitjançant la tècnica de *Western Blot*, hem analitzat els nivells de expressió proteica i de fosforilació de tots dos STATs, i en tots dos casos observant un significatiu increment en la fosforilació de les tirosines implicades en la via de senyalització, sobretot de STAT1 però també en menor mesura d'STAT3, en aquelles seccions tractades amb IFN-γ recombinant. Usant l'anticòs bloquejant Clone A6 hem comprovat la inhibició tant de la fosforilació com de l'expressió de la proteïna total en el cas de STAT1.

Així doncs, l'IFN-γ, malgrat tenir influència sobre l'expressió i activació de STAT3, té un impacte molt més clar sobre el seu principal factor de transcripció, STAT1.

És rellevant en aquest experiment, notar que l'expressió en les artèries sanes de STAT3 és molt superior a la de STAT1, que és molt més depenent de l'expressió d'IFN-γ, i aquesta superioritat es manifesta tant a nivell d'expressió com d'activació via fosforilació.

De fet, l'expressió d'STAT1 és exclusivament depenent dels IFNs, mentre que la de STAT3 és modulada per moltes altres citoquines com IL-6, VEGF, o Angiotensina II (126), per tant atès l'absència d'expressió d'IFN-γ en les artèries sanes, és coherent no trobar expressió ni activació d'STAT1.

No obstant, malgrat STAT1 sembla el factor de transcripció exclusivament activat pels IFNs, aquestes citoquines són capaces de senyalitzar i regular gens de manera independent de STAT1. Així doncs, cèl·lules KO per STAT1 al ser estimulades per IFN-γ mantenen la regulació sobre alguns gens inflamatoris. Regulació que no es dóna en aquelles cèl·lules que no disposen de Jak1 o de IFNGR1. Per tant, per bloquejar la via de l'IFN-γ, resulta més eficaç tallar el seu receptor, que no el seu principal factor de transcripció (77).

En aquest sentit, l'anticòs que hem utilitzat al nostres grup, el clone A6, s'uneix específicament a l'IFN-γ i per tant inhibiria la via de senyalització de manera eficaç des de més amunt.

Com ja hem comentat, una de les principals funcions de l'IFN-γ és l'activació dels macròfags, i mitjançant immunohistoquímica (tant normal com per microscòpia confocal amb fluorescència) hem pogut comprovar com disminueix el marcador de macròfags CD68 en aquelles seccions d'artèria en les que utilitzem el Clone A6.

Un dels avantatges més rellevants de l'ús del model de cultiu d'artèria temporal és que permet l'extracció i el cultiu aïllat de cèl·lules vasculars del múscul llis (VSMC).

Les VSMCs són el component cel·lular majoritari de la paret arterial, i el seu rol durant el procés fisiopatològic de la malaltia no està encara ben determinat. Durant la progressió de l'infiltrat inflamatori la paret vascular es desestructura i les SMCs canvien el seu patró d'expressió (13). Quina és la funció exacte d'aquestes cèl·lules i quin és el seu rol durant la hiperplàsia de la làmina íntima són encara preguntes sense resposta clara, però sembla evident la seva participació en aquests processos i la seva implicació en el remodelat vascular, i per tant són una diana interessant per futures investigacions.

En aquest cas, en la present tesi, ens hem centrat en clarificar el paper IFN-γ sobre aquestes SMCs, i com aquesta citoquina pot modular l’expressió d'altres molècules rellevants durant el procés inflamatori.

Malgrat en la literatura s'ha descrit que l'IFNGR s'expressa en tots els tipus cel·lulars, exceptuant eritròcits (63), hem volgut verificar-ne l'expressió mitjançant PCR a temps real en el

nostre cultiu de SMC aïllades, comprovant que efectivament, aquestes cèl·lules expressen grans quantitats de receptor de IFN-γ, i per tant estan preparades per respondre al seu estímul.

Així doncs, estimulant les SMCs amb dosis creixents d'IFN-γ hem vist que aquesta citoquina no només regula l'expressió a nivell de mRNA d'aquelles quimiocines que ja esperàvem, i que en la literatura estan descrites com regulades directament per IFN (CXCL9, 10 i 11), sinó també d'altres molècules, entre elles la quimiocina CCL2, o les molècules d'adhesió ICAM-1 i VCAM-1, a part de tenir efectes estimuladors sobre l'expressió dels STATs 1 i 3.
Aquestes estimulacions són totes dependents de la dosi, i es donen ja a temps primerencs d'estimulació, a les 6 hores, mantenint-se en alguns casos fins als 5 dies.
Saber quin és el motiu pel qual CCL2 es veu activat per IFN-γ com CXCL9, 10 i 11, però no s'estimulen les altres quimiocines, ens ha portat a realitzar un estudi dels promotors dels gens de totes aquestes quimiocines. Així doncs, hem vist que CCL2, però no cap altre de les quimiocines, comparteix amb CXCL9, 10 i 11 una diana en el seu promotor per ISGF3, factor de transcripció activat per IFN-γ, malgrat no hem aconseguit trobar cap diana per GAF (l'homodímer de STAT1), factor de transcripció principal de la via, i que en la literatura més recent (83) apareix com a activador de l'expressió de certes quimiocines.
Possiblement aquesta discrepància s'expliqui per una diferència de restricció a l'hora d'aplicar un criteri de cerca en les seqüències diana dels factors de transcripció en els promotors. És a dir, una cerca més restrictiva amb seqüències més llargues o definides, pot conduir a passar per alt seqüències més curtes, que un llindar més baix de detecció pot mostrar.
En aquest cas, el programa que nosaltres hem utilitzat per buscar les seqüències diana com GAS o ISRE, *CHIP Bioinformatics Mapper.org*, estableix una seqüència més específica i astringent per GAS (TTCC/TNG/ANAA) que la que s'ha utilitzat en altres treballs (TTNCNNNAA) (83), obtenint per tant resultats més restrictius en les cerques, malgrat buscar en una regió més ample (fins a 4000 parells de bases) abans de l'inici de transcripció.

No hem d'oblidar tampoc, que CCL2 té un receptor diferent a CCL3, 4, 5 o CXCL8, que és CCR2 i això és coherent amb tenir també una via de regulació i un paper diferent. Aquest

receptor a més (igual que CXCR3), l'hem trobat estimulat en el cultiu d'artèria incubada amb IFN-γ recombinant.

D'aquesta manera, l'IFN-γ via ISGF3 regula l'expressió de CCL2 en les SMC, i també de CCR2 en les artèries. En altres tipus cel·lulars però, aquesta regulació pot ser independent de STAT1, ja que cèl·lules del moll de l'os de ratolins KO per STAT1, estimulades amb IFN-γ presenten activació de CCL2, però no de CXCL9 o CXCL10 (77), així doncs, CCL2, tindria una segona via alternativa d'activació via IFN-γ, independent de ISGF3 i STAT1, via que no comparteix amb les altres quimiocines CXCL9 i 10, cosa que lligaria amb l'expressió constitutiva en VSMC.

L'estimulació de CCL2 via IFN-γ lligaria amb el fet de trobar significativament disminuït el marcador cel·lular de macròfags CD68 en les artèries tractades amb el Clone A6, tenint en compte que, tal i com hem explicat a la introducció, CCL2 actua principalment com a quimioatraient d'aquest tipus cel·lular. Tot i que no podem oblidar que podria haver-hi altres mecanismes implicats actuant sobre la supervivència dels macròfags a part de sobre la quimiotaxi.

Seguint en aquesta línia de treball, si comparem l'expressió de diverses molècules relacionades amb els processos inflamatoris a nivell basal, de VSMCs aïllades provinents de biòpsies sanes, amb VSMCs de biòpsies d'artèries amb ACG, al primer passatge de cultiu, veiem que les VSMCs "malaltes" (exposades a un entorn inflamatori) presenten nivells d'expressió significativament més alts, no només de citoquines proinflamatòries com IL-1β o TNFα, sinó també de quimiocines com CXCL10, CCL2 o CCL5.

No obstant, aquests resultats, poden veure's adulterats per la presència de limfòcits en el cultiu de VSMCs, cosa que donaria explicació a l'expressió d'IFN-γ per part d'aquest tipus cel·lular.

En el cultiu primari de VSMC, a un passatge tan inicial del cultiu, tenim cèl·lules sanguínies infiltrades, provinents de la sang que ha quedat retinguda a la llum de la secció del vas, o fins i tot limfòcits i monòcits residents en el teixit, que migren cap a l'exterior per la presència de quimiocines i factors de creixement en el propi Matrigel.

Així doncs, per confirmar els resultats diferencials obtinguts utilitzant cèl·lules de biòpsies sanes *versus* biòpsies amb ACG, hem realitzat un co-cultiu de PBMCs amb VSMCs de controls sans, comprovant si la presència de cèl·lules inflamatòries al cultiu podia provocar canvis en l'expressió de les VSMCs, tal i com passa *in vivo* en les biòpsies inflamades.

Aquest experiment de co-cultiu, es realitza per dos mètodes, de manera normal afegint tots dos tipus cel·lulars al mateix pou, però també mitjançant la utilització de *Transwells*.

La comunicació cel·lular, ja sigui per contacte cèl·lula-cèl·lula o mitjançant l'ús de *Transwells*, activa de manera clara l'expressió de mediadors inflamatoris en la majoria dels casos, sobretot en les cèl·lules musculars, però també en les PBMCs. No obstant, els resultats són molt més clars en el co-cultiu en els que posem les cèl·lules en contacte que per mediadors solubles.

Així doncs, el co-cultiu activa les VSMCs a expressar més quimiocines (CCL2, 3, 4, 5, CXCL8, 9 10, i 11) i citoquines proinflamatòries IL-1β, TNFα, i fins i tot nivells baixos d'IFN-γ, que amb l'estudi utilitzant *transwells* confirmen que aquesta citoquina també pot ser sintetitzada per les cèl·lules musculars, tot i que parlem de concentracions molt baixes.

En el cas de les quimiocines, sembla que l'estimulació és molt més dependent del contacte cel·lular, ja que al utilitzar *transwells* perdem gran part d'aquesta estimulació, o en els casos de CCL2 o CXCL10, podem arribar a deixar de veure-la de manera pràcticament total (*Resultats addicionals 1*).

També les PBMCs tendeixen a veure incrementada l'expressió de totes les molècules inflamatòries en el co-cultiu.

L'addició de Clone A6 al medi del co-cultiu bloquejant l'IFN-γ, inhibeix de manera radical l'expressió de CXCL9, 10 i 11 en tots dos tipus cel·lulars, però no té cap efecte sobre l'expressió a nivell de mRNA d'altres quimiocines ni citoquines proinflamatòries.

Així doncs, en aquesta situació inflamatòria *in vitro*, hem vist que les VSMCs es veuen estimulades per les cèl·lules que formarien part de l'infiltrat a sintetitzar mediadors inflamatoris que participin en el manteniment de la inflamació.

Quins efectes fenotípics pot tenir l'expressió de tots aquests marcadors que hem vist en els darrers experiments, és el que hem intentat desentrellar amb els estudis funcionals.

Així doncs, en primer lloc, hem realitzat assajos de migració cel·lular, per comprovar si les diferències que afecten a la expressió i secreció de quimiocines per part de l'IFN-γ, eren capaces de traduir-se en canvis en la capacitat migratòria de les cèl·lules de l'infiltrat inflamatori.

D'aquesta manera, mitjançant la cambra de Boyden, hem vist que les PBMCs migren significativament més en aquells pous amb el sobrenedant de les VSMCs estimulades amb concentracions més grans d'IFN-γ, que no en pous amb la citoquina sola.
És a dir, l'IFN-γ aconsegueix fer migrar les PBMCs a través d'estimular les VSMCs a secretar més quimiocines, que hem vist per PCR que eren CXCL9, 10, 11 i CCL2.
Les quimiocines directament induïdes per IFN, CXCL9, 10 i 11, exerceixen la seva funció a través d'un únic receptor de membrana, CXCR3, que hem vist estimulat en el cultiu d'artèria amb IFN-γ recombinant. Si afegim un inhibidor químic d'aquest receptor, s'observa una total inhibició de l'efecte potenciador de la migració per part de l'IFN-γ, confirmant així, que aquesta estimulació es dóna via CXCL9, 10 i 11.

En segon terme, i tenint en compte que l'IFN-γ ha demostrat estimular l'expressió tant de ICAM-1 com VCAM-1, en les cèl·lules musculars, hem realitzat assajos d'adhesió entre PBMCs i VSMCs.
Aquesta major expressió de molècules d'adhesió, que ha estat àmpliament descrita en altres tipus cel·lulars (117), es tradueix en un major percentatge de cèl·lules adherides, d'una manera dependent de la dosi d'IFN-γ amb la que estimulem les VSMCs.
Resultat que hem comprovat amb assajos d'adhesió convencional, i també amb adhesió fluorescent al microscopi confocal.

Finalment, en els experiments de proliferació no hem observat que l'IFN-γ fos capaç d'inhibir ni d'estimular la capacitat proliferativa d'aquestes cèl·lules, tal i com ocasionalment s'ha demostrat a la literatura i es podria preveure a través de les funcions de STAT1 i STAT3 (127, 128) (*Resultats addicionals 2*).

Paral·lelament, mitjançant el cultiu d'artèria de biòpsies de pacients sans (negatives per ACG) incubades amb IFN-γ, hem comprovat que l'estimulació només ha estat efectiva en incrementar l'expressió de les quimiocines directament induïdes per IFN-γ, és a dir, CXCL9, 10 i 11, però no mostra cap efecte sobre les altres molècules estudiades.

Així doncs, hem cultivat una artèria temporal en presència o absència de IFN-γ a altes dosis, i aquestes seccions d'artèria han estat posteriorment cultivades amb PBMCs amb l'objectiu de comprovar si l'IFN-γ podia generar en la paret arterial un estímul quimiotàctic d'infiltració per les cèl·lules sanguínies a través de la inducció de CXCL9, 10 i 11. L'anàlisi de les seccions s'ha dut a terme mitjançant immunohistoquímica fluorescent, en presència d'anticossos per marcadors de limfòcits i macròfags. D'aquesta manera, hem pogut comprovar l'aparició de certes formacions cel·lulars que recorden a cèl·lules gegants en aquelles seccions pre-cultivades amb IFN-γ, i en general una major intensitat de marcatge per CD68 en aquestes seccions.

Contràriament a allò esperat però, si les seccions eren tractades amb IFN-γ i un bloquejant químic de CXCR3, el número de limfòcits i macròfags infiltrats segueix sent molt alt, i podria ser fins i tot superior a la secció tractada només amb IFN-γ. Això pot ser degut a que bloquejar el receptor de les quimiocines impossibilita que els macròfags i els limfòcits que s'infiltren i s'adhereixen a la paret arterial, escapin cap a l'exterior de l'artèria, on el Matrigel conté gran quantitat de quimiocines i factors de creixement que atrauen aquestes cèl·lules.

Per tant, la infiltració d'aquestes cèl·lules a la paret, seria un procés més depenent de les molècules d'adhesió, que també són estimulades per l'IFN-γ, que no per les quimiocines que realitzen la seva funció a través d'aquest receptor, o d'altres (*Resultats addicionals 3*).

Així doncs, a través de la via de senyalització de STAT1, l'IFN-γ exerceix un potent rol regulador de diferents mediadors moleculars que semblen tenir un paper rellevant en el manteniment de l'infiltrat inflamatori degut a les seves funcions descrites fins a l'actualitat. Malgrat l'IFN-γ també estimula STAT3, el nivell basal elevat d'aquest factor de transcripció, a la vegada que la potent fosforilació d'STAT1 provocada per la citoquina afegida de manera recombinant, ens indica quina és la via de senyalització a través de la qual l'IFN-γ realitza la major part del seu control. Malgrat altres autors han posat damunt la taula la possible rellevància terapèutica del bloqueig d'aquesta via de senyalització en l'ACG (129), sembla raonable pensar que el bloqueig més amunt en la cadena resultaria més eficient (77).

Utilitzant el model de cultiu d'artèria temporal (98) hem comprovat en quina mesura i sobre quines molècules actua l'IFN-γ de manera directa, i hem obert noves possibles vies de regulació alternatives o secundàries amb tendències no significatives obtingudes a partir de la utilització d'un anticòs humà anti-IFN-γ, el Clone A6.

Així mateix l'estimulació amb IFN-γ de les artèries ens ha ofert resultats diametralment contraposats al bloqueig, tant a nivell d'expressió de mRNA com de secreció de proteïnes, reforçant les conclusions que se'n poden extreure.

Les cèl·lules del múscul llis per la seva part, que conformen la totalitat de la làmina íntima i suposen el component cel·lular majoritari de la paret arterial, tenen un rol tant important com pobrament definit durant el procés fisiopatològic de la malaltia.

Aquestes cèl·lules es veuen, en condicions patològiques, exposades a concentracions rellevants d'IFN-γ, citoquina de nul·la expressió en artèries sanes. Amb els nostres experiments hem descrit en primer lloc, que les SMC tenen nivells alts d'expressió de IFNGR, i en conseqüència, que poden respondre a l'estímul de l'IFN-γ, cosa que fan augmentant la síntesi de les quimiocines CXCL9, CXCL10, CXCL11, i CCL2, però també de les molècules d'adhesió (ICAM-1 i VCAM-1), i realitzant un *feedback* positiu sobre l'expressió dels propis factors de transcripció de la via (STAT1, però també STAT3 de manera colateral).

Aquesta estimulació de les quimiocines provoca un increment de migració leucocitària, increment que hem pogut comprovar (amb un bloqueig químic) que es dóna via CXCR3, i per tant del que en són responsables les quimiocines CXCL9, 10 i 11. L'arribada de nous leucòcits a la zona de la inflamació, tindria un efecte directe sobre la producció d'IFN-γ, que es veuria

incrementada, i aquest actuaria sobre les VSMCs produint més quimiocines de nou i reforçant el *loop* d'activació i manteniment de la inflamació a la paret arterial.

L'alta expressió de molècules d'adhesió i altres citoquines proinflamatòries com TNF-α, no fa sino facilitar i mantenir l'*status* inflamatori de la paret, i permetre el flux leucocitari cap a la zona lesionada.

Amb aquests treballs per tant, hem donat a la comunitat científica una nova eina per a l'estudi i realització d'assajos funcionals en l'ACG, el model de cultiu d'artèria temporal; i hem donat explicació a alguns processos generats per l'IFN-γ, lligats a l'evolució fisiopatològica de la malaltia, i que situen aquesta citoquina com un dels possibles factors col·laboradors en el manteniment de la inflamació a la paret arterial, i per tant afegeixen un motiu més als ja existents per la possible elegibilitat de la via de senyalització de l'IFN-γ com una potencial diana terapèutica.

Tanmateix, l'elevada expressió de molècules no regulades per IFN-γ en les lesions de ACG, subratlla probablement la necessitat de bloquejos simultanis d'altres vies.

Conclusions

CONCLUSIONS

1. El model de cultiu d'artèria temporal és un model funcional vàlid per a l'estudi de canvis en mediadors inflamatoris induïts per molècules farmacològiques, citoquines recombinants o anticossos bloquejants, i per tant una eina a tenir en compte pel disseny d'estudis funcionals que cerquin noves dianes terapèutiques.
2. El mètode de cultiu d'artèries sobre Matrigel a més, permet aïllar poblacions homogènies de cèl·lules musculars llises humanes, extretes de la làmina mitja de les artèries i per tant possibilita la realització de multitud d'assajos funcionals.
3. El tractament de les artèries amb glucocorticoides provoca la inhibició de l'expressió de moltes citoquines proinflamatòries com IL-1β, IL-6, TNF-α o IFN-γ.
4. L'IFN-γ està íntimament relacionat amb l'expressió de quimiocines a la paret arterial, i més particularment amb les cèl·lules del múscul llis que en conformen la làmina mitja, estimulant així la producció de CXCL9, 10, i 11, però també de CCL2.
5. L'augment de l'expressió de quimiocines induït per l'IFN-γ, ocorre probablement mitjançant el factor de transcripció STAT1, atès que té dianes en els promotors de tots aquests gens. El factor de transcripció STAT-3, malgrat tenir funcions sovint oposades a STAT-1, també es troba estimulat per IFN-γ en les cèl·lules musculars i en les artèries.
6. Les quimiocines CXCL9, 10 i 11, mitjançant la seva unió al receptor CXCR3, són responsables de l'estimulació de la migració de les cèl·lules de l'infiltrat inflamatori cap a les cèl·lules del múscul llis.
7. En cultiu d'artèria, l'anticòs Clone A6 inhibeix, a nivell d'expressió de mRNA, les quimiocines CXCL9, 10 i 11, i també a nivell de proteïna de CXCL9 i 10, així com fa disminuir la quantitat de marcatge de macròfags (CD68+) de la paret arterial, i inhibeix l'expressió de molècules d'expressió macrofàgica com HLA-DRA i NOS2.
8. Així mateix, en el cultiu de VSMCs, l'IFN-γ també estimula de manera dosi depenent les molècules d'adhesió ICAM-1 i VCAM-1, fet que es tradueix en un augment de l'adhesió de les cèl·lules de l'infiltrat inflamatori sobre les cèl·lules musculars.
9. L'IFN-γ per tant participa de la progressió i el manteniment de l'infiltrat inflamatori a la paret arterial, mitjançant l'estimulació en les cèl·lules musculars, de les quimiocines i

les molècules d'adhesió que actuen directament sobre els leucòcits, que són les cèl·lules productores del propi IFN-γ. Esdevenint per tant un procés proinflamatori que es perpetua a si mateix, i posa de manifest arguments a favor de l'elegibilitat de la via de senyalització de l'IFN-γ com a possible diana terapèutica en l'ACG.

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Annex

ANNEX

- Lozano E, Segarra M, **Corbera-Bellalta M**, García-Martínez A, Espígol-Frigolé G, Plà-Campo A, Hernández-Rodríguez J, Cid MC. *Increased expression of the endothelin system in arterial lesions from patients with giant-cell arteritis. Association between elevated endothelin plasma levels and the development of ischemic events*. Annals of the rheumatic diseases. 2010 Feb; 69(2):434-442.

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Increased expression of the endothelin system in arterial lesions from patients with giant-cell arteritis: association between elevated plasma endothelin levels and the development of ischaemic events

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ABSTRACT

Objective: Approximately 15–20% of patients with giant-cell arteritis (GCA) develop ischaemic complications often preceded by transient ischaemia. The expression of the endothelin (ET) system in GCA lesions was investigated to assess its relationship with the development of ischaemic complications.

Methods: Plasma ET-1 was quantified by immunoassay in 61 patients with biopsy-confirmed GCA and 16 healthy donors. ET-1, endothelin-converting enzyme (ECE-1) and endothelin receptor (ET_AR and ET_BR) messenger RNA were measured by real-time quantitative reverse transcriptase–PCR in temporal arteries from 35 of these patients and 19 control arteries. Proteins were measured by immunoassay and Western blot.

Results: ET-1 concentration was increased at the protein level in temporal artery samples from GCA patients compared with controls (0.98 (SEM 0.32) vs 0.28 (SEM 0.098) fmol/mg, $p = 0.028$). ECE-1, ET_AR and ET_BR/actin ratios (Western blot) were also significantly higher in GCA patients. Intriguingly, mRNA expression of ET-1, ECE-1 and both receptors was significantly reduced in GCA lesions compared with control arteries. When investigating mechanisms underlying these results, platelet-derived growth factor and IL-1β, present in GCA lesions, were found to downregulate ET-1 mRNA in cultured human temporal artery-derived smooth muscle cells. Glucocorticoid treatment for 8 days did not result in significantly decreased endothelin tissue concentration (0.87 (SEM 0.2) vs 0.52 (SEM 0.08); $p = 0.6$). Plasma endothelin concentrations were higher in patients with ischaemic complications (1.049 (SEM 0.48) vs 1.205 (SEM 0.63) pg/ml, $p = 0.032$).

Conclusions: The endothelin system is increased at the protein level in GCA lesions creating a microenvironment prone to the development of ischaemic complications. Recovery induced by glucocorticoids is delayed, indicating persistent exposure to endothelin during initial treatment.

Giant-cell arteritis (GCA) is a chronic granulomatous vasculitis preferentially targeting large and medium-sized arteries.[1] Inflammatory involvement triggers a vascular remodelling response resulting in lumen reduction with the potential for ischaemic complications.[2–9]

Partial or complete visual loss, usually due to anterior ischaemic optic neuropathy (AION), occurs in approximately 15–20% of patients. Visual impairment is the most common ischaemic complication in GCA and has a deep impact on patients' quality of life.[10–16] Scarce necropsy studies focusing on the anatomical substrate of AION have shown that posterior ciliary and cilioretinal arteries supplying the optic nerve and retina may be involved by GCA lesions[17–19] Irreversible visual loss is often preceded by recurrent episodes of transient blindness (amaurosis fugax), a medical emergency highly predictive of subsequent permanent visual defects in most series.[10–15] The transient and often repetitive nature of amaurosis fugax suggests the contribution of inflammation-induced vasospastic phenomena in the pathogenesis of ischaemic complications.

The endothelin (ET) family of 21 amino acid peptides includes three members, ET-1, ET-2, and ET-3. ET-1 is, by far, the main isoform produced in the cardiovascular system and is one of the most powerful vasoconstrictors identified.[20–24] The ET-1 encoding gene generates a transcript, which translates into a protein, pre-pro ET-1, intracellularly processed by a furin-like endopeptidase yielding an ET-1 precursor, big-ET-1, with low biological activity.[20 24] Cleavage of big-ET-1 by the metalloprotease endothelin-converting enzyme (ECE), generates the active peptide ET-1.[24] ET-1 is constitutively produced in blood vessels by endothelial cells and vascular smooth muscle cells (VSMC) and is also expressed by activated macrophages.[25] Endothelin peptides exert their vasoactive activity on VSMC through endothelin receptors A (ET_AR) and B (ET_BR). Binding to ET_AR elicits VSMC contraction, whereas, in some instances, binding to ET_BR may trigger vasodilatation. ET_BR is also involved in clearing circulating ET-1 by endocytosis, particularly in the lung vasculature.[21 23]

Based on the characteristics of the ischaemic complications of GCA, we hypothesised that the endothelin axis might play a role in inflammation-induced vasospastic phenomena leading to amaurosis fugax and contributing to visual loss in GCA. The aim of the study was to investigate the expression and regulation of the endothelin system in GCA lesions and to assess whether increased endothelin production is associated with the development of disease-related ischaemic events. This is the first study quantitatively exploring the expression of the endothelin system (ET-1, ECE-1, ET_AR and ET_BR) at both the messenger RNA and protein level in a human inflammatory vasculopathy.

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Table 1 Clinical findings in the study cohort patients with GCA

Clinical characteristics	GCA patients (plasma study) N (%)	GCA patients (mRNA study) N (%)	GCA patients (protein study) N (%)
No of patients	61	35	24
General characteristics			
Age in years, median (range)	78 (58–91)	78 (64–91)	77 (58–88)
Sex, women/men	44/17	29/6	19/5
Cranial symptoms	54 (89)	28 (80)	19 (79)
Headache	45 (74)	29 (83)	17 (71)
Jaw claudication	15 (25)	11 (31)	9 (38)
Scalp tenderness	28 (46)	14 (40)	10 (42)
Facial pain/oedema	15 (25)	9 (26)	3 (13)
Ocular pain	11 (18)	6 (17)	2 (8)
Tongue pain	5 (8)	1 (3)	1 (4)
Earache	19 (31)	5 (14)	5 (21)
Carotidynia	5 (8)	4 (11)	1 (4)
Toothache	9 (15)	7 (20)	4 (17)
Odynophagia	13 (21)	7 (20)	2 (8)
Abnormal temporal arteries*	54 (89)	27 (77)	16 (67)
Cranial ischaemic events	26 (43)	12 (34)	9 (38)
Permanent visual loss	19 (31)	8 (23)	4 (17)
Amaurosis fugax	2 (3)	2 (6)	2 (8)
Transient diplopia	1 (1.6)	1 (3)	1 (4)
Stroke	2 (3)	1 (3)	1 (4)
Transient ischaemic attack	1 (1.6)	0 (0)	1 (4)
Tongue ischaemia	1 (1.6)	0 (0)	0 (0)
Systemic manifestations	36 (59)	18 (51)	13 (54)
Fever	18 (30)	8 (23)	6 (25)
Weight loss	30 (49)	14 (40)	11 (46)
Polymyalgia rheumatica	31 (51)	16 (46)	10 (42)

*Abnormal temporal arteries at physical examination (painful, swollen, indurated and/or with decreased or absent pulsation).
GCA, giant-cell arteritis.

PATIENTS AND METHODS

Patients

Between 1997 and 2006, 158 patients were diagnosed with biopsy-confirmed GCA in our institution (Hospital Clinic, Barcelona). Clinical data were collected at the time of diagnosis and recorded in a database. Plasma-EDTA could be obtained from 61 of these patients during active disease (before starting therapy (43 patients) or after a single prednisone dose (18 patients)) and stored at −80°C. From 35 of them, total RNA could be extracted from frozen fragments of the temporal artery biopsies excised before starting treatment (30 patients) or after a single prednisone dose (five patients). Protein extracts could be obtained from 24 of these patients. Preliminary experiments showed no significant differences in any of the components of the endothelin axis between treatment-naive patients and patients who had received a single prednisone dose.

Clinical findings of the study group (table 1) were similar to previously published series except for a higher frequency of ischaemic complications, which served well the purpose of this study.[10 12–14] Twenty-six out of these 61 patients developed GCA-related ischaemic complications. Nineteen suffered permanent visual loss (four binocular and 15 monocular) in 14 due to AION, in two due to retinal ischaemia, in one due to central retinal artery occlusion and in two fundoscopy was normal. In four out of the 19 patients, visual loss was preceded by amaurosis fugax and in two by transient diplopia. Two additional patients had stroke, two reversible amaurosis fugax, one transient diplopia, one a transient ischaemic attack and one tongue ischaemia. Patients were considered to have a weak systemic inflammatory response when they had up to two of the following: fever greater than 37°C; weight loss greater than 3 kg; haemoglobin less than 110 g/l; erythrocyte sedimentation rate of 85 mm or greater. Patients with three to four of these findings were considered to have a strong systemic inflammatory response, as reported[3 10 26 27]

For control purposes, plasma-EDTA was obtained from 16 volunteers with similar age (median 73 years, range 60–87) and gender distribution (10 women and six men). Controls had no past history of cancer, chronic inflammatory disease or recent (⩽3 months) infection. As controls for biopsy studies, we included consecutive temporal artery biopsies from 19 patients (13 women and six men) with a median of 77 years (range 64–91) in whom GCA was initially considered but were subsequently diagnosed as having other diseases. The prevalence of cardiovascular risk factors (smoking habit, hypertension, diabetes or hypercholesterolaemia) was similar between patients and controls.

To assess the effect of glucocorticoids on the endothelin system in lesions we consecutively selected temporal artery samples from 18 additional patients (labelled as treated patients) diagnosed within the same period of time with biopsy-confirmed GCA who had received prednisone (1 mg/kg per day) for an average of 8 days (range 3–20) when the temporal artery biopsy was excised.

The study was approved by the Ethics Committee of our institution (Hospital Clínic). Patients signed informed consent for the collection and storage of biological material.

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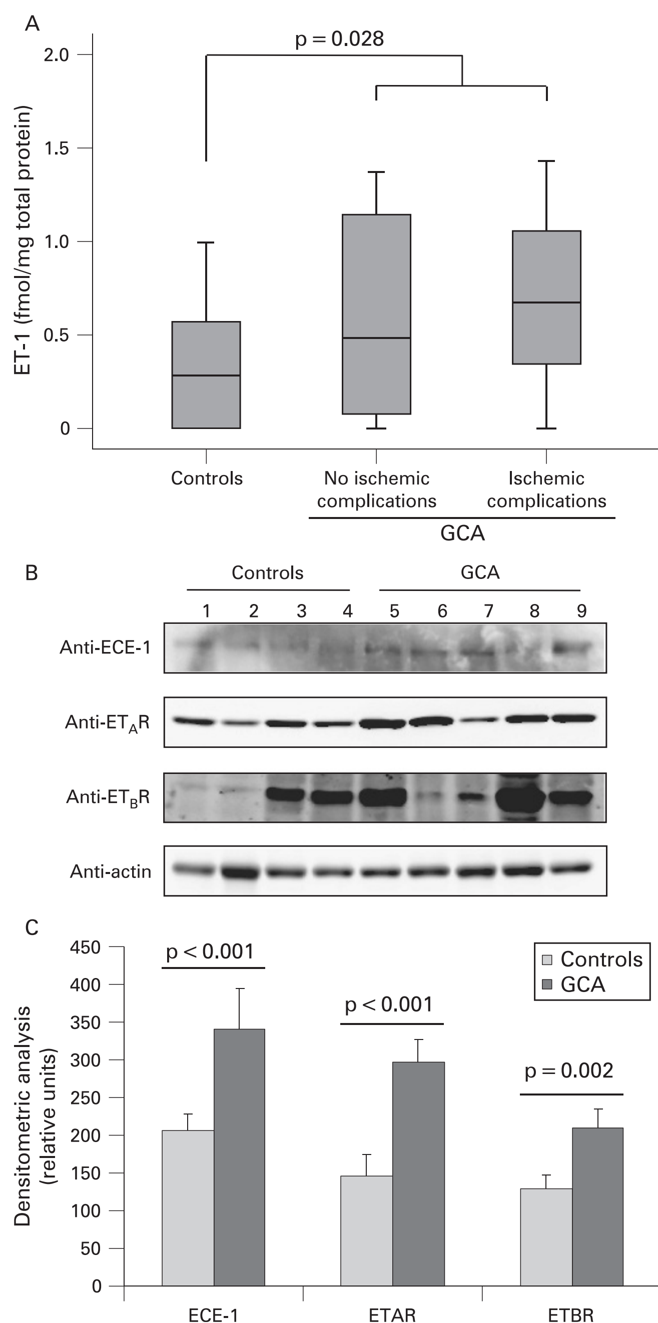

Figure 1 Endothelin (ET) system in temporal artery biopsies from giant-cell arteritis (GCA) patients. (A) ET-1 concentration in GCA arteries from 24 active patients (eight with and 16 without ischaemic complications) and controls (N = 19). (B) Representative immunoblot disclosing endothelin-converting enzyme (ECE-1) and endothelin receptors A and B (ET_AR and ET_BR) expression. Actin immunodetection was used as a control for loading. (C) ECE-1, ET_AR and ET_BR protein/actin ratio determined by densitometric analysis of Western blots of temporal artery biopsy protein extracts from 24 active patients and 19 controls.

Reagents

Human ET-1 was purchased from MP Biomedicals (Aurora, Ohio, USA). Recombinant human transforming growth factor beta (TGFβ), tumour necrosis factor alpha (TNFα), IL-1β, IL-6 and purified platelet-derived growth factor (PDGF)-AB were obtained from R&D Systems (Minneapolis, Minnesota, USA).

Measurement of circulating endothelin

Plasma was concentrated fourfold by speed-vacuum lyophilisation, and the ET-1 concentration was determined in the entire cohort of patients and controls by using the Parameter ELISA kit (R&D Systems) according to the manufacturer's protocol. This kit exhibits cross-reactivity with other endothelin peptides as follows: ET-2 45%; ET-3 14%; and big-ET less than 1%.

Measurement of the endothelin system mRNA by quantitative real-time RT–PCR

Temporal artery biopsies were embedded in optimal cutting temperature (OCT, Sakura, The Netherlands), snap-frozen in liquid nitrogen and stored at −80°C until used. Sections consecutive to those that provided the histopathological diagnosis were processed for RNA isolation using TRIzol reagent (Invitrogen, Carlsbad, California, USA).

Total RNA (1 μg) was reverse transcribed to complementary DNA using the Archive kit (Applied Biosystems, Foster City, California, USA) in a final volume of 100 μl, employing random hexamer priming. cDNA was measured by quantitative real-time PCR using specific Pre-Developed TaqMan gene expression assays (ET-1 Hs00174961_m1, ET-2 Hs00266516_m1, ECE-1 Hs00154837_m1, ET_AR Hs00609865_m1 and ET_BR Hs00240747_m1) from Applied Biosystems, as previously described.[9]

Endothelin system protein detection in tissue

Immunoassay

Temporal artery protein extracts were obtained from the phenol phase during RNA isolation. The ET-1 concentration was determined by immunoassay (Biomedica Medizinprodukte GmbH, Vienna, Austria) and normalised for total protein content. This kit exhibits cross-reactivity with other endothelin peptides as follows: ET-2 100%; ET-3 less than 5%; and big-ET less than 1%.

Western blot analysis

Twenty-five micrograms of protein per condition were resolved on 10% reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis gels and blotted onto nitrocellulose membranes (Invitrogen). Immunodetection was performed with the following antibodies: goat anti-human ECE-1 (R&D Systems), mouse anti-human ET_AR (BD Biosciences Pharmingen, Franklin Lakes, New Jersey, USA) and rabbit polyclonal anti-human ET_BR (Abcam, Cambridge, UK) at 1 : 1000 dilution. Chemiluminescence signals were measured with the LAS-3000 imaging system (Fujifilm Corporation, Tokyo, Japan).

Cell culture

Primary culture of HTASMC

Human temporal artery-derived vascular smooth muscle cells (HTASMC) were isolated from fresh remnant segments of temporal artery biopsies from patients with GCA, as previously described.[9] Cells obtained by this method had a myointimal phenotype as confirmed by the expression of α-smooth muscle actin by flow cytometry and type I collagen expression by reverse transcriptase (RT)–PCR, as reported.[9]

HUVEC isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from freshly delivered cords as reported.[28] The growth medium consisted of M199 (Invitrogen) supplemented with 20% iron-fortified bovine calf serum (Hyclone Laboratories, Logan, Utah,

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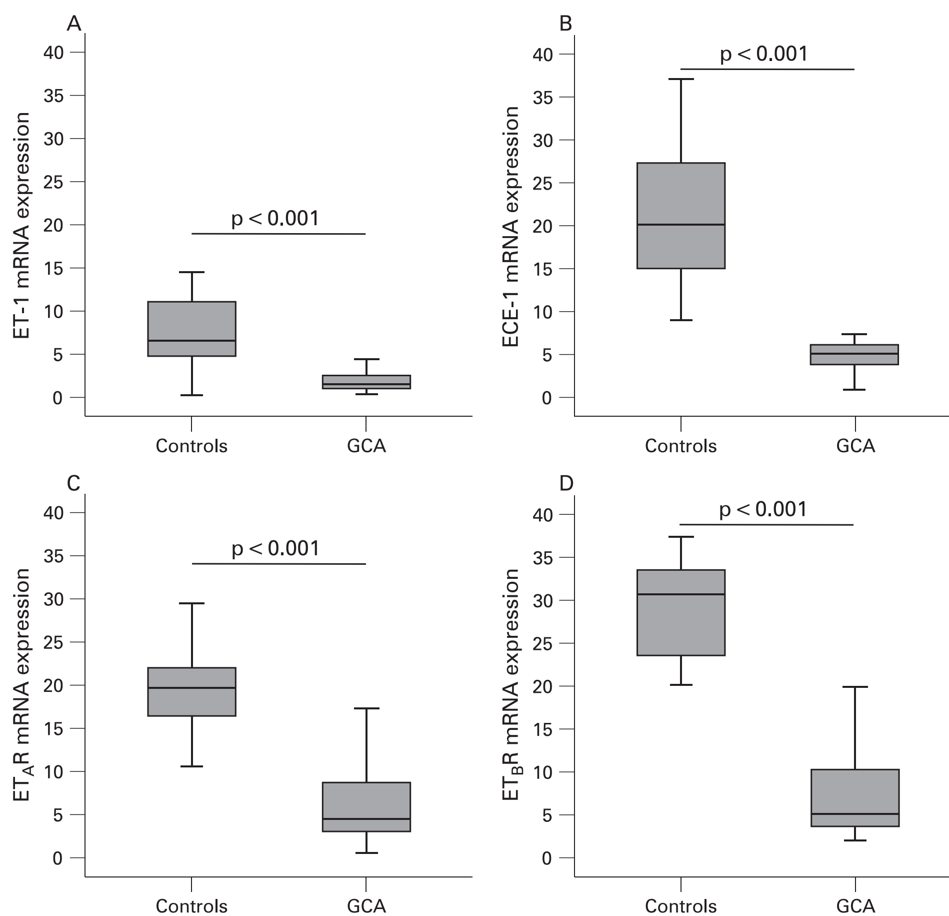

Figure 2 Endothelin (ET) system mRNA expression in temporal artery biopsies from giant-cell arteritis (GCA) patients. (A) ET-1; (B) endothelin-converting enzyme (ECE-1); (C) endothelin receptor A (ET_AR) and (D) endothelin receptor B (ET_BR) mRNA expression in temporal arteries from 35 active GCA patients and 19 controls.

USA), 200 μg/ml endothelial cell growth supplement (BD Biosciences), 100 U/ml penicillin/streptomycin, 50 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 2 mM glutamine and 50 U/ml sodium heparin.

Statistics

The Mann–Whitney test and Spearman's rho correlation coefficient were used for statistical analysis.

RESULTS

ET-1, ECE-1 and ET_AR and ET_BR are overexpressed in GCA lesions

As displayed in fig 1A, temporal artery biopsies from patients with GCA contained significantly higher concentrations of ET-1 than normal arteries (0.979 (SEM 0.315) vs 0.280 (SEM 0.098) fmol/mg total protein; $p = 0.028$). ECE-1, ET_AR and ET_BR expression was also significantly higher in GCA lesions (fig 1B,C). No differences were found in temporal artery concentrations of the endothelin system components between patients with or without ischaemic complications.

Endothelin system mRNA expression is downregulated in GCA lesions

Unexpectedly, a substantial decrease in ET-1, ECE-1, ET_AR and ET_BR mRNA was observed in GCA lesions compared with temporal arteries from controls (fig 2A–D). Consequently, ET-1 protein in lesions negatively correlated with its own mRNA ($r = -0.612$, $p = 0.015$). To exclude the possibility that cross-reactive ET-2 may account for the increase in ET-1 peptide found in lesions, ET-2 mRNA was subsequently measured by real-time RT–PCR. ET-2 mRNA concentrations were virtually negligible, 1000 times less abundant than ET-1 mRNA with no differences between patients and controls (0.001866 vs 0.001291 relative units, $p = 0.926$). A positive correlation was found between the expression of ET-1 and ECE-1 mRNA ($r = 0.673$, $p<0.001$), between ET-1 and ET_BR mRNA ($r = 0.675$, $p<0.001$) and, to a lesser extent, between ET-1 and ET_AR mRNA ($r = 0.322$, $p = 0.052$), suggesting coordinated regulation.

PDGF and IL-1β downregulate in vitro ET-1 mRNA expression by VSMC from GCA arteries (HTASMC)

Dissociation between ET-1 mRNA and protein may suggest a negative regulation by ET-1 itself or by other mediators present in GCA lesions. As VSMC are quantitatively the major source of endothelin in medium-sized arteries, we chose this model to investigate the regulation of ET-1 production by ET-1 itself or by other factors produced in the GCA inflammatory micro-environment, including TGFβ, PDGF, IL-1β, IL-6 and TNFα.[2–4 8 9] ET-1 regulation in response to inflammatory mediators was also investigated in endothelial cells, using HUVEC as a model.

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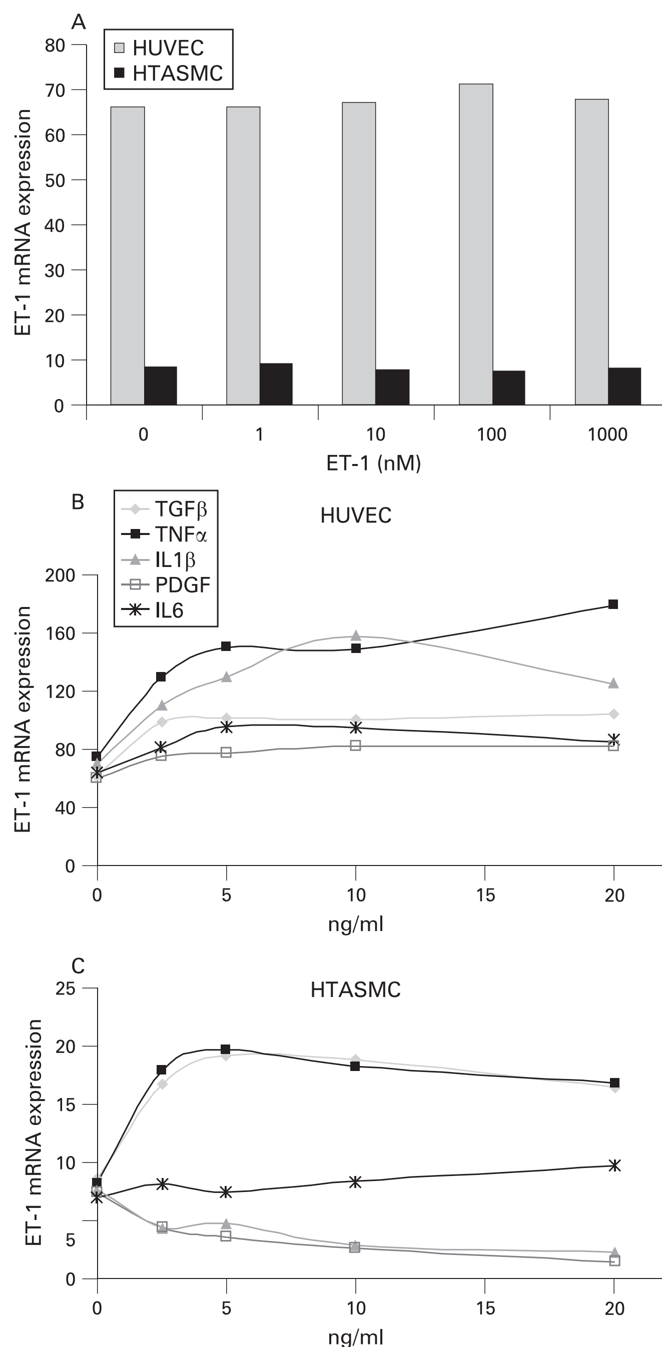

Figure 3 Regulation of the endothelin (ET) system in cultured human umbilical vein endothelial cells (HUVEC) and human temporal artery-derived vascular smooth muscle cells (HTASMC). Subconfluent cells were incubated with increasing concentrations of depicted factors for 24 h. ET-1 mRNA expression was measured by real-time PCR as described in the Methods section. Experiments were repeated three times with consistent results and a representative experiment is shown. (A) Effect of ET-1 on its own expression. (B) Effect of cytokines and growth factors on ET-1 expression by HUVEC and (C) by HTASMC. PDGF, platelet-derived growth factor; TGFβ, transforming growth factor beta; TNFα, tumour necrosis factor alpha.

Overall, HUVEC produced significantly more ET-1 mRNA than HTASMC (fig 3). ET-1 did not downregulate its own expression either in HUVEC or in HTASMC (fig 3A). Among other factors tested, TGFβ and TNFα significantly increased ET-1 expression by HTASMC (fig 3B,C) and TNFα and IL-1β increased ET-1 expression by HUVEC. Interestingly, PDGF and IL-1β remarkably reduced ET-1 expression by HTASMC only, in a dose-dependent manner (fig 3C). At the range of concentrations tested, IL-6 did not elicit significant changes in ET-1 expression by HUVEC nor by HTASMC (fig 3B,C). These results indicate that some inflammatory mediators present in GCA lesions are able to downregulate ET-1 mRNA expression markedly and that ET-1 expression is differently regulated in endothelial cells compared with HTASMC.

Glucocorticoid therapy partly modulates endothelin system expression

To analyse the effect of glucocorticoid therapy on the endothelin system, we cross-sectionally compared the protein concentration in arteries from active versus treated GCA patients. We did not observe significant differences in the ET-1 concentration between temporal arteries from active patients and temporal arteries from patients treated for a median of 8 days; in both groups ET-1 remained elevated compared with control arteries (fig 4A). However, ECE-1 and ET_AR concentrations were lower in treated compared with active patients, reaching values found in normal temporal arteries. The decrease in ET_BR levels in treated patients was not significant (fig 4B).

These findings suggest that glucocorticoid treatment for a median of 8 days decreases some components of the endothelin system, but ET-1 levels persist elevated. Three of our patients agreed to a second biopsy, which was performed within 46–50 weeks after the initiation of glucocorticoid treatment. As shown in fig 4(C–D), ET-1 and ECE-1 levels decreased remarkably in paired biopsies obtained before and after prolonged treatment. Changes in endothelin receptors were less conclusive.

We found again dissociation between mRNA and protein with regard to the effects of glucocorticoid treatment on ET-1 expression (fig 5). Temporal arteries from treated patients contained significantly higher concentrations of ET-1, ECE-1 and ET_BR mRNA than temporal arteries from active patients, although they did not reach values found in normal temporal arteries. ET_AR expression was similar in active versus treated patients but, in both groups, remained inferior to controls (fig 5A–D).

Circulating plasma ET-1 concentrations are elevated in GCA patients with ischaemic complications

Circulating ET-1 levels were similar in GCA patients as in healthy donors (1.112 (SEM 0.04) pg/ml vs 1.119 (SEM 0.06) pg/ml, $p = 0.642$). However, among GCA patients, circulating ET-1 concentrations were significantly higher in patients with ischaemic complications (1.205 (SEM 0.63) pg/ml) compared with patients without ischaemic events (1.048 (SEM 0.48) pg/ml, $p = 0.032$; fig 6A). ET-1 was also higher in patients with a weak systemic inflammatory response than in those with a strong systemic inflammatory reaction (1.120 (SEM 0.06) vs 0.990 (SEM 0.05) pg/ml, $p = 0.002$) who, as previously published, are at lower risk of ischaemic events[10 12 13] (fig 6B).

DISCUSSION

Increased endothelin expression and function contributes to the pathogenesis of a number of diseases including primary pulmonary hypertension, portal hypertension and systemic sclerosis.[29 30] Recognition of the role of the endothelin system in these disorders has led to efficient new therapeutic approaches.[23]

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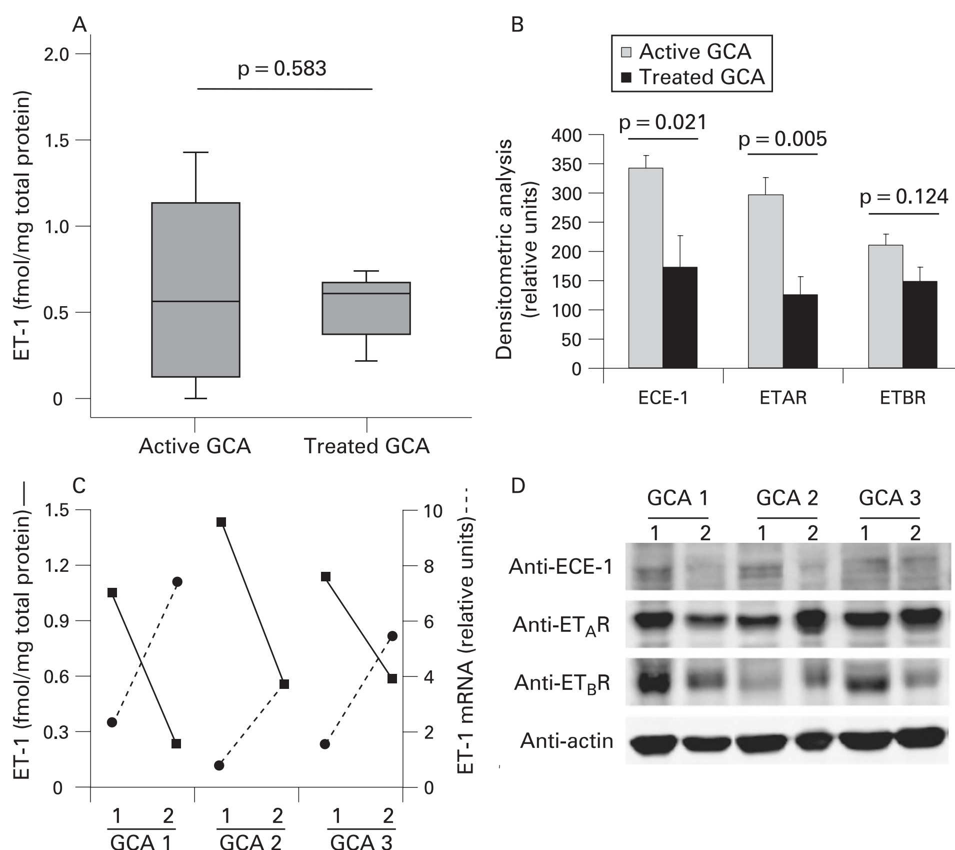

Figure 4 Effect of glucocorticoid therapy on the endothelin (ET) system. (A) Temporal artery biopsy ET-1 concentrations in active versus treated patients as described in the Methods section. (B) Densitometric analysis of endothelin-converting enzyme (ECE-1), endothelin receptor A (ET_AR) and endothelin receptor B (ET_BR) protein expression in temporal arteries from active versus treated patients assessed by Western blot. (C) ET-1 mRNA (– – –) and protein (——) concentration in paired temporal artery biopsies obtained from three giant-cell arteritis (GCA) patients before treatment (1) and 46–50 weeks after glucocorticoid treatment (2). (D) ECE-1, ET_AR and ET_BR protein expression in the paired temporal artery biopsies from the same patients as in C. Number 1 refers to the first biopsy and number 2 refers to the second biopsy.

In this study we found that ET-1, ECE-1 and endothelin receptors are increased in vascular lesions from patients with GCA. Although our data do not definitely demonstrate a direct participation of the endothelin system in the generation of vascular occlusion in GCA, these findings certainly configure a scenario prone to endothelin-induced vasospastic and vasocclusive responses.

Plasma concentrations of ET-1 were similar between patients and controls. Elevated plasma ET-1 was previously reported in four patients with GCA compared with reference values obtained from the general population.[31] The discrepancy may be due to the fact that we studied a much larger cohort of patients and used control individuals within a similar age range. As with other mediators involved in vascular remodelling,[5] ET-1 may increase with age. However, the plasma ET-1 concentration was significantly higher in patients with ischaemic events, suggesting that elevated ET-1 may render patients more prone to develop vasospasm in small arteries supplying the optic nerve. Conversely, ischaemia itself may contribute to elevated ET-1 because ET-1 expression and release are under control by hypoxia inducible factor.[32] Plasma ET-1 concentrations have been found, indeed, to correlate with disease severity in other conditions such as congestive heart failure,[33] pulmonary artery hypertension[29 34] and atherosclerotic disease.[35]

The measurement of ET-1 in tissue extracts disclosed increased ET-1 concentrations in temporal arteries from GCA patients. However, contrary to what was observed for circulating endothelin, no differences were found between patients with or without ischaemic complications. Several possibilities may account for this apparent discrepancy. Tissue endothelin measurement was performed in a smaller cohort and in a vascular tissue distant from the vascular beds where ischaemic complications usually occur. In addition, as with other potent mediators, ET-1 bioavailability is not only transcriptionally regulated, but it is regulated at multiple levels including proteolytic cleavage by ECE-1, receptor expression and control of ET-1 secretion. Secretion at a given time point may be more relevant than the overall ET-1 content in cells and tissues.[36 37] The observation that endothelin receptors are remarkably upregulated in target vascular lesions increases the potential biological relevance of increased circulating ET-1 found in patients with ischaemic complications.[37]

Unexpectedly, and further supporting the complexity of endothelin regulation, all the components of the endothelin system were downregulated at the mRNA level. Destruction or dysfunction of the main cellular component of the artery wall, VSMC, which constitutively express ET-1, might account for reduced active transcription of the endothelin system at a given

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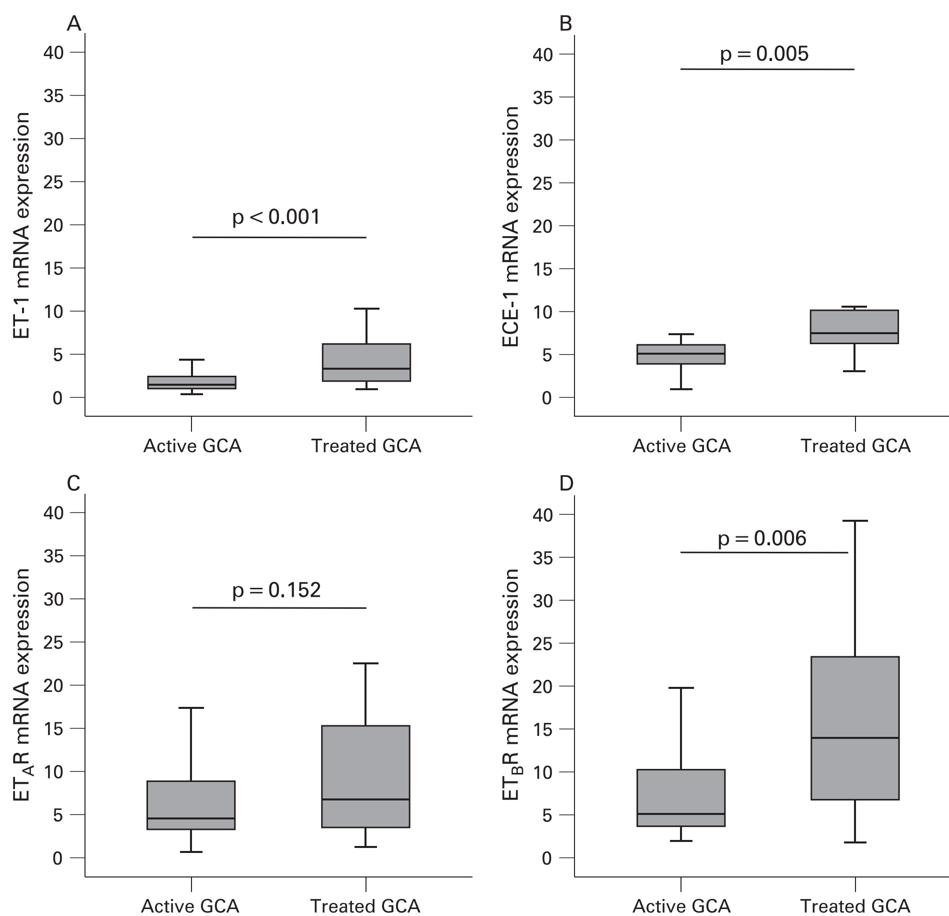

Figure 5 Changes on endothelin (ET) system mRNA expression in temporal artery biopsies according to glucorticosteroid treatment. (A) ET-1; (B) endothelin-converting enzyme (ECE-1); (C) endothelin receptor A (ET_AR) and (D) endothelin receptor B (ET_BR). GCA, giant-cell arteritis.

time point, while ET-1 peptide produced by infiltrating macrophages may still remain.[25] In addition, counterregulatory mechanisms may also operate and may influence mRNA expression or stability. Contrary to other systems in which accumulation of the final product exerts a negative feedback loop on its own de novo expression, we could not demonstrate ET-1 downregulation by ET-1 itself, either in endothelial cells or in HTASMC. Among several factors expressed in temporal artery lesions, IL-1β and, in particular, PDGF substantially decreased ET-1 mRNA in HTASMC but not in endothelial cells. Interestingly, PDGF is a potent stimulator of the proliferating, migrating and secretory myointimal phenoptype of HTASMC. Myointimal cells may no longer be responsive to the vasoconstrictor effects of ET-1, which, indeed, may be inefficient in advanced lesions with intimal hyperplasia. Therefore, it is not surprising that PDGF downregulates ET-1 production by HTASMC.

Approximately 10–17% of GCA patients presenting with visual symptoms continue to have deteriorating vision during the first 1–2 weeks after the initiation of glucocorticoid treatment, indicating that glucocorticoids do not immediately/completely prevent visual loss.[38–40] Treatment for a median of 8 days did not efficiently result in decreased ET-1 concentration in tissue, although some of the components of the system such as ECE-1 and ET_AR were reduced. Although after long-term glucocorticoid treatment ET-1 concentration eventually decreased in paired biopsies, our findings indicate that the endothelin system may persist increased in GCA lesions for at least one week after the start of therapy.

Based on these results it is attractive to hypothesise that the endothelin system may play a role in the development of transient or irreversible visual loss in patients with GCA. Incomplete regulation of the endothelin system with glucocorticoid treatment may at least partly explain why some patients continue to lose sight during the first days after glucocorticoid therapy.[38–40] Interference with the endothelin system may, then, be a therapeutic option for patients with GCA, particularly those who continue to present with visual symptoms after the beginning of glucocorticoids and for whom no additional options exist. The number of patients who continue to lose vision after glucocorticoid therapy is fortunately small, but this situation, for which there are no established alternatives, is one of the unsolved issues in the management of GCA.[11 38–40] Endothelin receptor antagonists have clinical efficacy and are approved for the treatment of patients with pulmonary hypertension[41] and for patients with digital ulcers secondary to systemic sclerosis.[42] However, the potential efficacy of endothelin receptor blockade in reducing the risk of visual loss in patients with visual symptoms remains hypothetical but would be worth considering in multicentre clinical trials.

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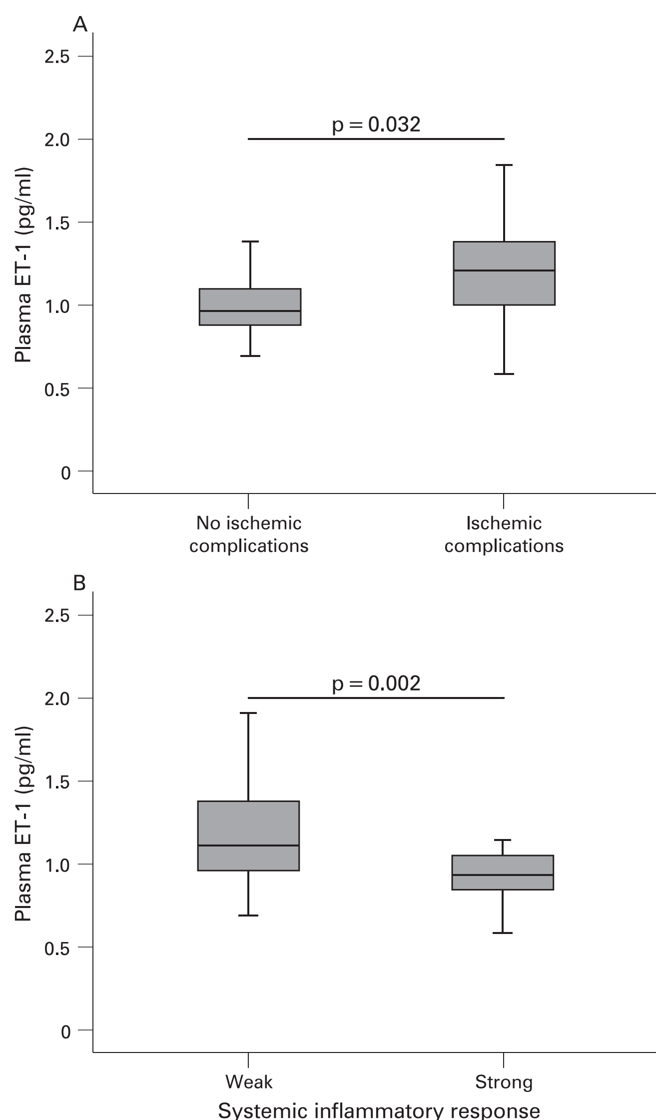

Figure 6 Circulating endothelin (ET-1) concentration in patients with giant-cell arteritis. (A) ET-1 concentration in plasma from patients with ischaemic complications (N = 25) compared with patients without ischaemic events (N = 36). (B) ET-1 concentration in plasma from patients with a weak systemic inflammatory response (N = 39) compared with those with a strong systemic inflammatory reaction (N = 22).

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Patient consent: Obtained.

The results were partly presented at the 71st Annual Meeting of the American College of Rheumatology. Boston, USA, November 2007.

Provenance and peer review: Not commissioned; externally peer reviewed.

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Increased expression of the endothelin system in arterial lesions from patients with giant-cell arteritis: association between elevated plasma endothelin levels and the development of ischaemic events

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Thalidomide decreases gelatinase production by malignant B lymphoid cell lines through disruption of multiple integrin-mediated signaling pathways

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ABSTRACT

Background
Thalidomide and its analogs are effective agents in the treatment of multiple myeloma. Since gelatinases (matrix metalloproteinases-2 and -9) play a crucial role in tumor progression, we explored the effect of thalidomide on gelatinase production by malignant B lymphoid cell lines.

Design and Methods
We investigated the effect of therapeutic doses of thalidomide on integrin-mediated production of gelatinases by malignant B lymphoid cell lines by gelatin zymography, western-blot, reverse transcriptase polymerase chain reaction and invasive capacity through Matrigel-coated Boyden chambers. We also explored the effect of thalidomide on the activation status of the main signaling pathways involved in this process.

Results
Thalidomide strongly inhibited gelatinase production by B-cell lines and primary myeloma cells in response to fibronectin, the most efficient gelatinase inducer identified in lymphoid cells. Thalidomide disrupted integrin-mediated signaling pathways involved in gelatinase induction and release, such as Src and MAP-kinase ERK activation, resulting in decreased cell motility and invasiveness. Unexpectedly, treatment with thalidomide elicited an increase in fibronectin-induced Akt phosphorylation through phosphoinositide 3-kinase-independent pathways since thalidomide decreased fibronectin-induced phosphoinositide 3-kinase phosphorylation and reversed the inhibition of Akt phosphorylation achieved by the phosphoinositide 3-kinase inhibitors wortmannin and LY294002.

Conclusions
Disruption of integrin-mediated signaling may be an important mechanism through which thalidomide and its analogs impair tumor cell interactions with the microenvironment. The unexpected effects of thalidomide on Akt activation indicate the need for further studies to elucidate whether the interference with Akt downstream effects would synergize with the antitumor activity of thalidomide.

Key words: matrix metalloproteinases, thalidomide, B-cell malignancies, integrins.

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The online version of this article has a Supplementary Appendix.

Introduction

Thalidomide has received a great deal of attention in recent years due to its remarkable therapeutic efficacy in the treatment of multiple myeloma (MM).[1,2] Thalidomide and its analogs are also being investigated in other hematologic malignancies and hematologic disorders for which there is no effective treatment such as mantle cell lymphoma, chronic lymphocytic leukemia, and myelodysplastic syndromes.[3] Thalidomide is not directly cytotoxic and the mechanisms through which it exerts its therapeutic benefits are not well defined and appear to be highly complex. Understanding the molecular basis of the effects of thalidomide is crucial in order to be able to design more efficient and less harmful analogs.[4]

Survival and progression of myeloma cells are highly dependent on stimuli from the bone marrow microenvironment.[5,6] These include soluble factors, contact-dependent interactions with surrounding cells, and angiogenesis. Interleukin-6, tumor necrosis factor-α and insulin-like growth factor are pivotal in promoting proliferation and survival of myeloma cells.[6] Contact-dependent interactions with bone marrow stromal cells, endothelial cells, and matrix proteins, including fibronectin, transduce survival signals, which may confer resistance to apoptosis induced by radiation, dexamethasone, or traditional chemotherapy.[5] Angiogenesis is a crucial requirement for myeloma progression and the extent of angiogenesis is inversely correlated with survival in patients with disseminated MM[7] and also in patients with solitary plasmacytomas.[8]

Thalidomide was tried in MM on the basis of its ability to inhibit angiogenesis.[9] Thalidomide inhibits proliferation of endothelial cells and decreases fibroblast growth factor-2 and vascular endothelium growth factor-induced angiogenesis in several models.[10] In addition, it reduces the production of angiogenic factors vascular endothelial growth factor, hepatic growth factor and fibroblast growth factor-2 by endothelial cells obtained from bone marrow biopsies of MM patients.[11] However, a correlation between the extent of bone marrow angiogenesis and response to thalidomide has not been consistently demonstrated[12] and extramedullary plasmacytomas are resistant to thalidomide in spite of being highly vascularized.[13] These observations suggest that, besides inhibiting angiogenesis, thalidomide has additional therapeutic effects probably targeting interactions between tumor cells and the bone marrow microenvironment.[1,13,14]

Gelatinases (the matrix metalloproteinases MMP2 and MMP9) have a crucial function in the progression of lymphoproliferative disorders.[15-18] These proteinases facilitate tumor progression, not only by breaking natural barriers such as basement membranes or interstitial matrix but through many additional mechanisms including activation of cytokines and growth factors by proteolytic cleavage, release of matrix-bound growth factors and exposure of cryptic sites or release of active fragments from large matrix proteins.[19] It has been recently shown that integrin-mediated cell interaction with matrix molecules, particularly fibronectin, is the strongest inducer of gelatinase production and activation in cells of lymphoid origin.[20,21] Integrin engagement not only induces gelatinase production but also gelatinase activation through coordinated induction of the MMP2 activator MMP14, and down-regulation of tissue inhibitor of metalloproteinases (TIMP)-2. Moreover, integrin-mediated signaling drives a rapid post-transcriptional release of gelatinases through pathways related to cell migration.[21]

Given the relevance of gelatinases in cell interactions with the microenvironment and ultimately in tumor progression,[19] we investigated the effects of thalidomide on matrix-induced MMP production by malignant lymphoid B-cell lines, in search for direct effects on malignant cells with relevant impact on their relationship with the surrounding milieu. We found that thalidomide decreases gelatinase production in response to fibronectin by interfering with multiple integrin-mediated signaling pathways which are also involved in the regulation of cell motility and survival. Thalidomide-mediated disruption of integrin signaling may then impair multiple contact-dependent tumor cell interactions with the bone marrow microenvironment which are though to be crucial for malignant cell survival.[5]

Design and Methods

Reagents

Thalidomide (Chemie Grünenthal, Germany) was dissolved in dimethylsulfoxide to give a stock solution of 10 mg/mL, and stored at -20° C. Wortmannin was purchased from Sigma-Aldrich (St Louis, MO, USA). LY294002 was obtained from Calbiochem (EMD Chemicals, Inc., Gibbstown, NJ, USA). Lenalidomide was a generous gift from Dr Dolors Colomer (Hematopathology Unit, Dept. of Hematology, Hospital Clínic, Barcelona, Spain).

Cells and cell culture

Human B lymphoblastoid cell lines Raji (derived from an Epstein-Barr virus-positive Burkitt's lymphoma) and IM9 (derived from a plasma cell leukemia) were obtained from the European Collection of Cell Cultures (Salisbury, UK). RPMI 8226 and KMM1 myeloma cell lines, Hbl-2 (diffuse B-cell lymphoma) and MEC-1 (B-chronic lymphocytic leukemia) cell lines were kindly provided by Dr Dolors Colomer. Cells were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Biological Industries, Israel), 2mM L-glutamine and 50 μg/mL gentamycin at 37°C in 5% CO_2.

Primary MM cells were obtained from remaining bone marrow aspirates obtained for diagnostic purposes from patients with MM. The use of this tissue was approved by the Ethics Committee and patients signed informed consent. Mononuclear cells were isolated by Ficoll-Histopaque density centrifugation. Adherent cells were removed by allowing cells to adhere to a 0.1% gelatin-coated dish at 37°C in 5% CO_2 for 24 h. Non-adherent cells were collected, cultured in RPMI 1640 with 10% fetal calf serum and used for experiments. Unless otherwise indicated, cells were exposed to chemicals for 4 h and cell viability was confirmed by trypan blue exclusion.

Gelatin zymography

Cells were resuspended in serum-free RPMI 1640 medium at 0.5×10^6 cells/mL. For each condition, 5×10^6 cells were pre-incubated with chemicals at the indicated concentrations for 30 min at 37°C before the addition of fibronectin at 10 μg/mL. The supernatant fluid was collected 4 h later and concentrated 200-fold with Urifil-10 concentrator devices (Millipore, Molsheim, France).

Concentrated samples were subjected to gelatin zymography as previously described.[20,21]

Reverse transcriptase polymerase chain reaction

RNA was extracted from 5×10^6 cells using TRIzol® Reagent (GIBCO). One microgram of RNA was used for cDNA synthesis with the Superscript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), using oligo d(T)s priming. MMP2, MMP9 and MMP14 cDNA was amplified as previously described.[20,21] Thirty-five reaction cycles were run, each consisting of three steps of 45 s at 94°C, 57 °C (for MMP2 and MMP9) or 60°C (MMP14), and 72°C respectively, followed by an elongation period of 10 min at 72°C. Polymerase chain reaction products were analyzed in 1.2% agarose gels (Invitrogen). Multiplex amplification of β2-microglobulin was used as an internal control.

Adhesion assay

Ninety-six-well plates (Nalgene Nunc International, Denmark) were coated with fibronectin (50 μg/well) overnight at 4°C. Cells were suspended in serum-free medium, plated on fibronectin-coated wells at a density of 0.15×10^6 cells/well and incubated at 37°C for 1 h. Non-adherent cells were aspirated and the remaining cells were fixed and stained with 0.2% crystal violet (Sigma) in 20% methanol, washed with distilled H_2O and air-dried. The dye was solubilized with 1% sodium dodecyl sulfate (SDS) and optical density was read with a spectrophotometer at 600 nm wavelength. Conditions were tested in quadruplicate wells.

Matrigel invasion assay

Ten micrometer-pore polycarbonate filters (Nucleopore, Toronto, Canada) were coated with Matrigel (kindly provided by Dr Hynda K Kleinman, NIH, Bethesda, MD, USA) diluted in RPMI 1640 at 1.25 mg/mL, and placed between the lower and upper compartments of 48-well Boyden chambers (Neuro Probe Inc, Gaithersburg, MD, USA). The lower compartments were filled with 25 μL RPMI 1640 with 10% fetal calf serum and 0.1×10^6 cells in serum-free medium with the indicated concentrations of thalidomide loaded onto the upper chambers. After incubation for 6 h at 37°C, the filter was removed, fixed with methanol and stained with hematoxylin. Cells on the upper side were swept and the number of cells/field on the lower side was counted under an inverted microscope in six randomly selected fields/well. Experiments were performed in quadruplicate wells.

Flow cytometry analysis

For each condition 0.5×10^6 cells were incubated for 30 min at 4°C with 2 μg of the following monoclonal antibodies diluted in 100 μL of RPMI-1% bovine serum albumin: anti-integrin α4 chain (clone HP2/1) (Immunotech, Marseille, France), anti-integrin α5 chain (clone SAM1) (Immunotech), anti-αvβ3 integrin (clone LM609) (Chemicon International, Inc., Temecula, CA, USA), anti-integrin β1 chain (clone K20) (Immunotech) or HUTS-21 (β1 activated epitope), a generous gift from Dr F Sánchez-Madrid (Hospital La Princesa, Madrid, Spain). Cells were subsequently were washed with cold phosphate-buffered saline-1% bovine serum albumin and incubated for 30 min at 4°C with goat anti-mouse IgG Alexa Fluor® 488 secondary antibody (Molecular Probes, Leiden, The Netherlands) at a 1:200 dilution. After two washes with cold phosphate-buffered saline-1% bovine serum albumin, cells were fixed in 200 μL of 5% formaldehyde in phosphate-buffered saline and fluorescence measured using a FACScan® flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The expression of HUTS-21 was analyzed in the absence and in the presence of 200μM of Mn^{2+} divalent cations.[22]

Western blot

For each condition, 5×10^6 cells were incubated with the indicated concentrations of chemicals and then exposed or not to fibronectin at 10 μg/mL in serum-free RPMI 1640 medium for 4 h. Cells were lysed in 0.5 mL of modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton-X-100, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS) supplemented with freshly added protease inhibitors (Complete™, Boehringer Mannheim, Germany) and Na_3VO_4 at 200 μM. The protein content of the lysates was measured with the BCA protein assay (Pierce, Rockford, IL, USA).

For each condition, 20 μg of lysate were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose. Membranes were incubated overnight at 4°C with the appropriate primary antibody: ERK MAP kinase activation state was evaluated using anti-phospho-p44/42 MAPK. The nitrocellulose membrane was then stripped and re-probed with p44/42 MAPK rabbit polyclonal IgG antibodies (Cell Signaling, Beverly, MA, USA) at 1:1000 dilution. Src activation was evaluated with anti-phospho-Src at Y416 (activated form), then re-probed with anti-phospho-Src at Y527 (inactivated form) and finally re-probed with anti-Src rabbit polyclonal IgG antibodies (Cell Signaling) at 1:1000 dilution. To assess Akt activation state, a combination of two anti-phospho-Akt (Ser473 and Thr308) was used and the membrane re-probed with Akt rabbit polyclonal IgG antibodies (Cell Signaling) at a dilution of 1:1000. The activation state of phosphatase and tensin homolog (PTEN) was evaluated with anti-phospho-PTEN rabbit polyclonal IgG antibody (Cell Signaling) at a dilution of 1:1000. Anti-phospho-mammalian target of rapamycin (mTOR) and anti-mTOR antibodies were also obtained from Cell Signaling. Monoclonal mouse anti-human β-actin antibody (clone AC-15) (Sigma) was used at a 1:2000 dilution. MMP14 was detected with a polyclonal rabbit anti-human MMP14 (Chemicon) at a 1:1000 dilution.

Immunodetection was performed by incubating membranes with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit HRP-conjugated, Cell Signaling or anti-mouse HRP-conjugated, Transduction Laboratories, Lexington, KY, USA) at a 1:2000 dilution. Chemiluminescence signals were detected with LUMIGLO® reagent (Cell Signaling). Membranes were exposed to a LAS3000 chemiluminiscence detector (Fuji Photo Film, Düsseldorf, Germany) and the data collected analyzed with Image Gauge V4.0 software.

Results

Thalidomide strongly decreases fibronectin-induced matrix metalloproteinase production by malignant B lymphoid cells

Fibronectin is a matrix protein present in the bone marrow microenvironment which is crucial in promoting tumorigenesis through several mechanisms including stimulation of cell migration, invasiveness and survival.[23] These responses are mainly mediated by integrin engagement and signaling. We used two B lymphoid cells, IM9 and Raji, to study gelatinase production induced by fibronectin. As we have previously demonstrated in T-cell lines,[20,21] exposure to fibronectin strongly induced metalloproteinase (MMP9, MMP2 and MMP14) expression and

release by both IM9 and Raji cells (Figures 1 and 2). Interestingly, thalidomide decreased fibronectin-induced gelatinase production in a dose-dependent manner (Figures 1 and 2A and 2B). Production of the MMP2 activator, MMP14, which is also up-regulated by fibronectin in lymphoid cells,[20,21] was strongly inhibited by thalidomide, particularly in its activated form (Figures 1 and 2C). Thalidomide, therefore, not only reduced fibronectin-induced MMP expression, but also restrained gelatinase activation and release.

The Raji and IM9 cells used in this study come from different lymphoid malignancies, suggesting that the effects of thalidomide on integrin signaling are not restricted to a particular cell line. Accordingly, thalidomide's impairment of gelatinase production in response to fibronectin was also confirmed in primary MM cells obtained from bone marrow aspirates of two patients (Figure 2D). Patient # 1 had 100% plasma cell infiltration whereas patient # 2 had 50% infiltration. The increased gelatinolytic signal observed in cells from patient # 2 may indicate a contribution of additional cells (i.e. from the myelomonocytic lineage) which have greater ability to produce gelatinases than cells of lymphoid origin and suggest that thalidomide may also decrease MMP production by other cell types present in the MM microenvironment.

Thalidomide decreases Raji cell adhesion and invasiveness

We have previously shown that fibronectin-induced gelatinase production by lymphoid cells is mediated by integrins α4, α5 and αv.[20] Given that thalidomide decreased integrin-mediated induction and release of gelatinases, we assessed its effect on additional integrin-mediated functions, such as cell adhesion to matrix proteins. Preliminary experiments showed that Raji cells significantly adhered to fibronectin whereas adhesion to other matrix proteins such as collagen I, collagen IV or laminin was poor (*data not shown*). Thalidomide significantly decreased Raji cell adhesion and spreading onto fibronectin in a dose-dependent manner (Figure 3A). Moreover, as shown in Figure 3B, thalidomide significantly reduced Raji cell invasion through the reconstituted basement membrane Matrigel. As reported by others,[24] IM9 cells were not naturally adherent to fibronectin. Although IM9 cells interacted with fibronectin and produced gelatinases efficiently in response to fibronectin engagement, these cells were unable to complete additional steps required for cell attachment and migration and were not, therefore, tested in these systems. To confirm that thalidomide analogs elicit similar responses, and to verify activity in other cell lines, we explored the effects of lenalidomide in other cell types including a MM-derived cell line, RPMI8226. It was found that lenalidomide was highly effective in reducing cell adhesion, and gelatinase production in response to fibronectin (*Online Supplementary Figure S1*).

We next investigated whether the inhibitory effects of thalidomide on integrin-mediated responses were due to a decrease in integrin expression. As illustrated in Figure 3C, both cell lines significantly expressed fibronectin receptors αvβ3, α4 and α5. According to previously published data, these integrins mediate fibronectin-induction of gelatinases.[20] In these experimental conditions, thalidomide did not significantly down-regulate integrin surface expression, as detected by flow cytometry (Figure 3C), nor did it modify the expression of β1 integrin chain-activation related epitope recognized by the monoclonal antibody HUTS-21[22]

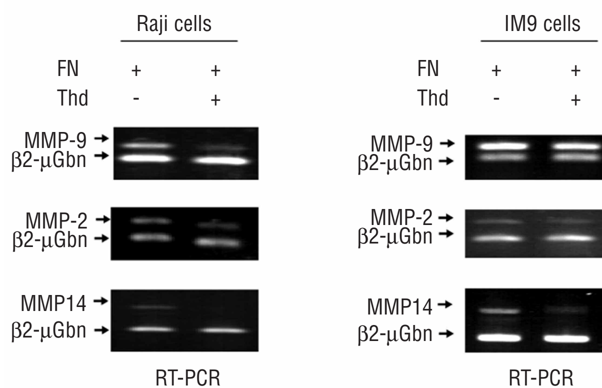

Figure 1. Thalidomide (Thd) inhibits gelatinase (MMP2 and MMP9) and MMP14 gene expression in response to fibronectin (FN). Reverse transcriptase polymerase chain reaction amplification of MMP2, MMP9 and MMP14 in Raji (A) and IM9 (B) cells cultured in the presence of fibronectin at 10 μg/mL and exposed to thalidomide at 20 μg/mL for 4 h.

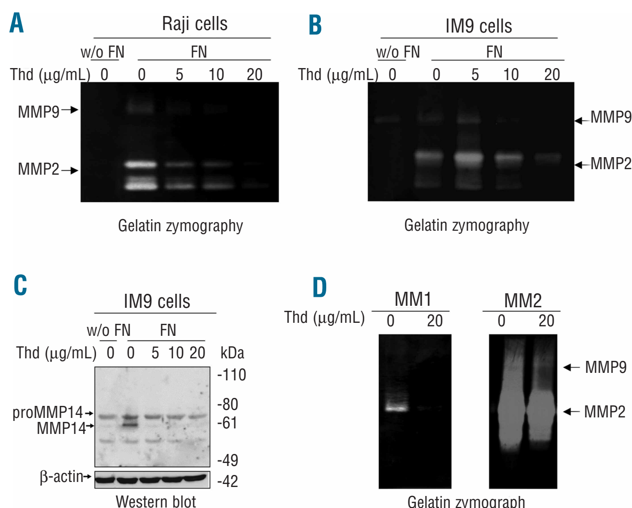

Figure 2. Thalidomide (Thd) inhibits gelatinase (MMP2 and MMP9) and MMP14 production in response to fibronectin (FN). Gelatin zymography of the supernatants of Raji (A) and IM9 (B) cells in the absence or in the presence of fibronectin at 10 μg/mL and exposed to thalidomide at the indicated concentrations for 4 h. (C) Western blot detection of MMP14 in IM9 cell lysates cultured in the same conditions. Similar results were obtained with Raji cells. (D) Gelatin zymography of supernates obtained from primary myeloma cells obtained from bone-marrow aspirates of two different patients and cultured with fibronectin (10 μg/mL) for 4 h in the absence or presence of thalidomide 20 μg/mL.

which was weakly expressed at baseline but strongly induced by Mn^{2+}. Taken together, these findings indicate that the effects of thalidomide on fibronectin-induced adhesion and gelatinase production do not primarily depend on changes in integrin expression and suggest that thalidomide may influence integrin-mediated signaling pathways.

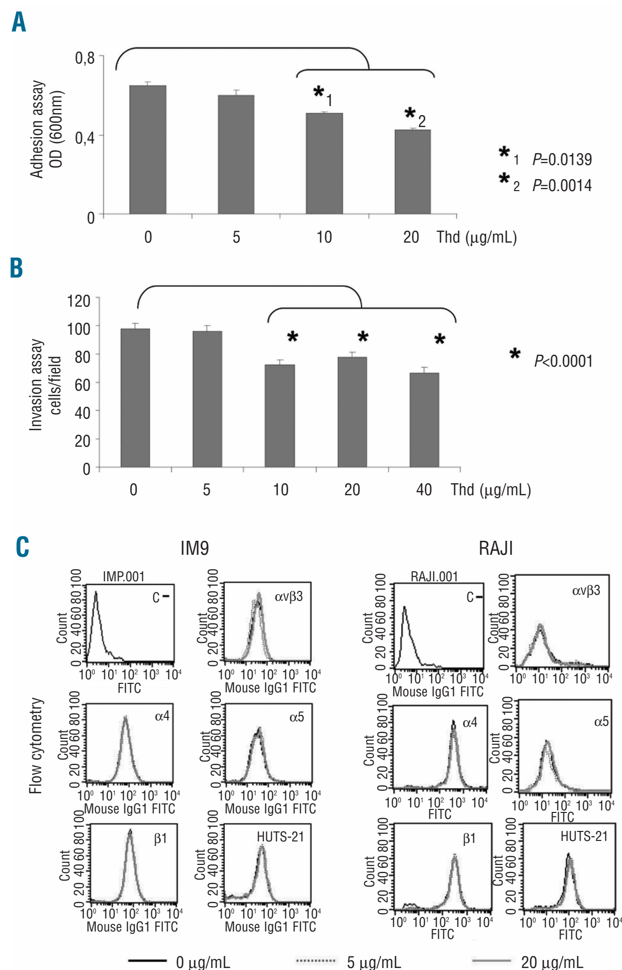

Figure 3. Thalidomide (Thd) decreases cell attachment to fibronectin and invasiveness through Matrigel-coated membranes without modifying integrin surface expression. (A) Raji cells treated with the indicated concentrations of thalidomide were incubated on fibronectin-coated wells for 1 h. Bars represent mean in optical density ± SEM. Statistical significance of differences observed was calculated with the Mann-Whitney U test. (B) Invasion of Raji cells treated with various doses of thalidomide through Matrigel-coated polycarbonate membranes over a 6 h period. Bars indicate mean cell number ± SEM on the lower side of the membrane. Statistical significance of differences observed was calculated with the Mann-Whitney U test. (C) Flow cytometry analysis of surface expression of αvβ3 integrin, α4, α5 chain and β1 integrin chains, and β1 activation related epitope recognized by the antibody HUTS21 in IM9 and Raji cells exposed to the indicated concentrations of thalidomide for 1-h. Identical results were obtained after 6-h exposure to thalidomide. Since baseline expression of HUTS21 was weak (*data not shown*) results represented in the figure were obtained after incubation with Mn^{2+}. Negative control (C-) represents cells incubated with the secondary antibody only.

Treatment with thalidomide results in decreased phosphorylation of integrin-activated kinases involved in the regulation of gelatinase production in response to fibronectin

In previous studies, we showed that fibronectin-induced gelatinase expression and release by lymphoid cells is mediated by Src, PI3-kinase and MAP kinases ERK1/2.[20,21] However, while inhibition of Src by PP2 also results in decreased gelatinase synthesis and secretion, inhibition of ERK phosphorylation by PD98059 and of PI3-kinase by wortmannin leads to increased fibronectin-induced rapid gelatinase release into the medium followed by a reduction in gelatinase mRNA.[20,21] Similar effects were observed with the B-cell lines used in this study (*data not shown*). We next investigated the effect of thalidomide on these pathways. Fibronectin-induced Src activation was decreased by thalidomide as illustrated by the decrease in Src phosphorylation at Y416 elicited by thalidomide treatment (Figure 4A).[25] In contrast, Src phos-

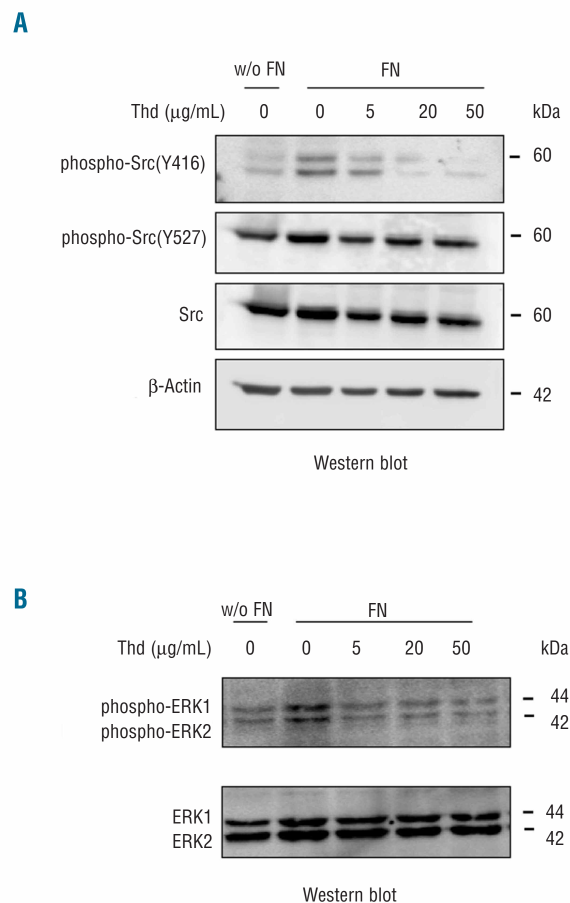

Figure 4. Thalidomide (Thd) inhibits phosphorylation of Src and ERK kinases in response to fibronectin (FN). (A) Western blot detection of Src phosphorylated at Y416 and at Y527 in Raji cells exposed or not to fibronectin (10 μg/mL) and incubated with the indicated concentrations of thalidomide. The membrane was stripped and re-probed with anti p60 Src and with anti β-actin antibody. (B) Western blot detection of phosphorylated p42 and p44 ERK in Raji cells cultured in the same conditions as in A. The blot was subsequently stripped and re-probed with anti-ERK antibody. Similar results were obtained from IM9 cells.

phorylation at Y527, leading to Src inactivation, remained unmodified (Figure 4A). Thalidomide also resulted in strongly decreased ERK phosphorylation induced by fibronectin (Figure 4B).

Thalidomide increases integrin-induced Akt phosphorylation in a PI3-kinase-independent pathway

Previous studies have shown that PI3-kinase participates in fibronectin-induced release of MMP.[20] Given that thalidomide reduced phosphorylation of several kinases crucial to integrin-signaling, we next explored the effect of thalidomide on PI3-kinase activation and ensuing downstream effects. When activated by tyrosine phosphorylation of its p85 subunit, PI3-kinase catalyzes phosphatidylinositol trisphosphate (PIP3) formation. PIP3 then recruits Akt to the cell membrane where it can be phosphorylated by active phosphoinositide-dependent kinase 1 (PDK1).[26,27] Akt activation promotes cell survival by stimulating anti-apoptotic pathways and this mechanism is considered to be a pivotal downstream effect of PI3-kinase function.[26,27] Exposure to thalidomide decreased PI3-kinase activation in both cell lines (Figure 5A).

We also explored the effects of thalidomide on PTEN activation. PTEN is a lipid phosphatase that dephosphorylates PIP3, decreasing Akt activation.[28-30] *PTEN* is considered to be a tumor suppressor gene given that loss of PTEN activity, as a result of deletion or mutation of the gene, is commonly found in solid tumors. However, although *PTEN* mutations can be found in some cases of MM,[28] loss of PTEN function is an uncommon pathway of malignant transformation in lymphoproliferative disorders.[6,31] We found that thalidomide reduced fibronectin-induced PTEN phosphorylation in IM9 cells (Figure 5B). Raji cells lacked detectable PTEN protein (*data not shown*). Since PTEN is inhibited by phosphorylation, this finding indicates that thalidomide increases

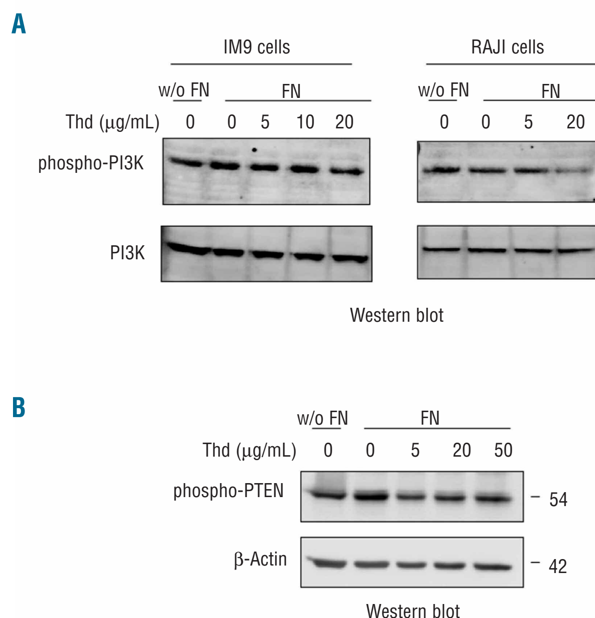

Figure 5. Thalidomide (Thd) decreases PI3-kinase phosphorylation and increases PTEN activation. (A) Western-blot detection of phosphorylated p85 subunit of PI3-kinase in IM9 and Raji cell lysates after incubation with thalidomide in the presence or absence of fibronectin (FN) (10 μg/mL). (B) Western-blot detection of phosphorylated PTEN and corresponding β-actin in cell lysates obtained from IM9 cells exposed to the indicated concentrations of thalidomide in the absence or presence of fibronectin (10 μg/mL).

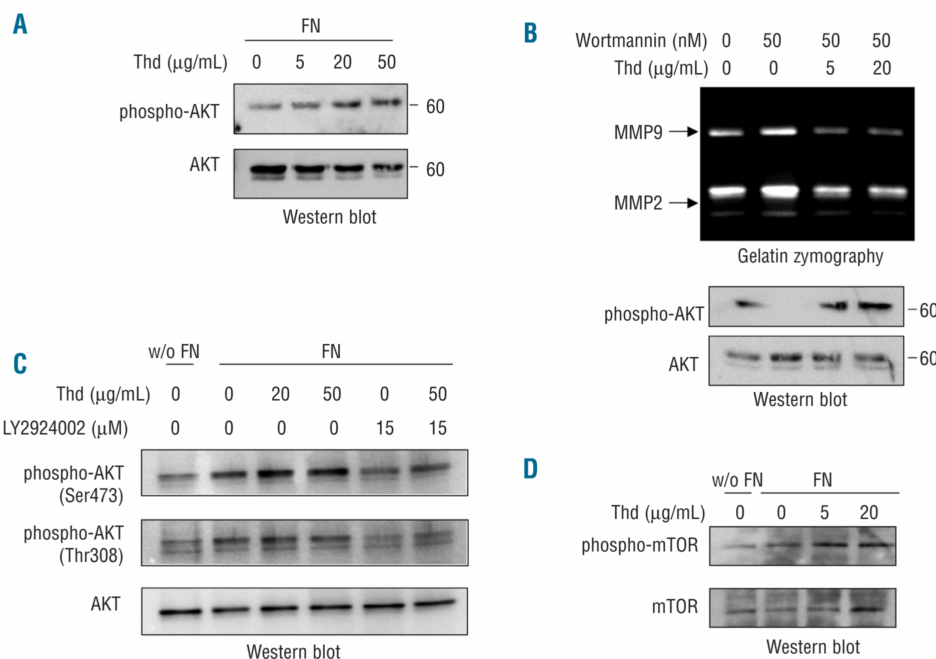

Figure 6. Thalidomide (Thd) increases fibronectin (FN)-induced Akt phosphorylation through a PI3-kinase independent pathway. (A) Western-blot detection of phosphorylated Akt of IM9 cell lysates after exposure to the indicated doses of thalidomide for 4 h in the presence of fibronectin (10 μg/mL). (B) Gelatin zymography and western-blot detection of phosphorylated Akt in Raji cell supernates and lysates, respectively, after exposure to thalidomide at the depicted concentrations, in the absence or presence of wortmannin at 50 nM. Similar results were obtained with IM9 cells. (C) Western-blot detection of phosphorylated Akt (Ser473 and Thr308) in Raji cells exposed or not to fibronectin (10 μg/mL) and incubated with the indicated concentrations of thalidomide and PI3-kinase specific inhibitor LY294002 (15 μM). The blot was subsequently stripped and re-probed with anti-Akt antibody. (D) Western-blot detection of mTOR phosphorylation in Raji cell lysates exposed to thalidomide in the presence or absence of fibronectin. Similar results were obtained in IM9 cells.

PTEN activation, which may reduce even more downstream effects of PI3-kinase in IM9 and cells expressing functional PTEN. The increase in PTEN activity could also contribute to the anti-tumor effects of thalidomide since PTEN inhibits cell proliferation through Akt-dependent and independent pathways.[32]

Akt phosphorylation is a crucial downstream effect of PI3-kinase activation. We hypothesized that reduced PI3-kinase activation and increased PTEN activity induced by thalidomide might, therefore, result in decreased Akt activation, in accordance with thalidomide's anti-tumor effects. Unexpectedly, and in spite of reduced PI3-kinase phosphorylation and increased activity of PTEN in IM9 cells, thalidomide increased fibronectin-induced phosphorylation of Akt in both cell lines (Figure 6A) and was able to overcome the inhibitory effect of the PI3-kinase inhibitor wortmannin on Akt phosphorylation (Figure 6B). This finding was confirmed with the more specific PI3-kinase inhibitor LY294002 (Figure 6C). These findings indicate that thalidomide increases Akt phosphorylation through a PI3-kinase-independent pathway. In support of the functional relevance of the increase in Akt phosphorylation, exposure to thalidomide resulted in increased phosphorylation of the Akt substrate mTOR in both cell lines (Figure 6C).

The increase in Akt phosphorylation induced by thalidomide was confirmed in myeloma cell lines RPMI 8226 and KMM1 (*Online Supplementary Figure S2*). In these cell lines total Akt content and Akt phosphorylation in response to fibronectin and to thalidomide were less intense than in Raji and IM9 cells, which are derived from more aggressive malignancies.

Discussion

Our data indicate that thalidomide has strong direct effects on tumor cells, leading to disruption of integrin-mediated interactions with the surrounding microenvironment. Interference with integrin-mediated signaling may also contribute to previously identified effects of thalidomide, such as inhibition of angiogenesis and T-cell co-stimulation, since cell-cell and cell-matrix interactions mediated by integrins are crucial in these processes. The signaling pathways disrupted by thalidomide regulate crucial cell functions for tumor progression such as cell motility, cell-cell and cell-matrix interactions, gelatinase production, and cell growth. Src kinases and ERK are key enzymes in promoting tumor cell growth and invasiveness. Increased Src activity by v-Src was one of the first recognized mechanisms of malignant transformation.[25,33] Src also plays a crucial role in cell motility and we and others have shown that Src-family kinases are key regulators of integrin-mediated gelatinase production and rapid release through multiple interactions with focal adhesion kinase (FAK) and FAK-associated signaling molecules[21,34] Src not only mediates gelatinase production but also post-transcriptional rapid release of gelatinases in response to fibronectin. Src and ERK inhibition may, therefore, both be important mechanisms through which thalidomide decreases cell motility and MMP production in response to matrix proteins. Changes in protein phosphorylation elicited by thalidomide suggest that interference with these and other protein kinase and/or phosphatase activities may lead to additional alterations in cell function which could contribute to the drug's anti-angiogenic, anti-inflammatory and anti-tumor effects.

However, the increase in Akt activity may seem inconsistent with the anti-tumor activity of thalidomide, given that activated Akt transduces powerful anti-apoptotic signals[26,35] and an increase in Akt activation is crucial for the survival of many tumors including MM.[35] Recent trials have shown that while thalidomide delays progression in MM, advance is accelerated in some patients when progression occurs, indicating that thalidomide may, indeed, activate some survival pathways.[36] Thalidomide-mediated survival signals through Akt activation may be partially overcome by interference with other important pathways stimulating cell growth and migration such as Src or MAP kinases in response to extracellular signals. Interestingly, increased Akt activity renders cells more sensitive to the suppressive effects of mTOR inhibitors on cell survival and on angiogenesis.[37] The effect of thalidomide on Akt and mTOR activation suggests that inhibitors of mTOR may potentiate the anti-tumor effects of thalidomide, as indicated by experimental animal models.[38,39]

In conclusion, functional testing of new thalidomide analogs is currently based on their ability to elicit known effects of thalidomide, such as T-cell co-stimulatory function or inhibition of cytokine production and angiogenesis.[4,40] The newly recognized effects of thalidomide reported here may help in the screening of new therapeutic agents.

Authorship and Disclosures

MS, EL, MC-B, CV, M-TC and JE performed the laboratory work. MS, NI, JB and MCC participated in the design of the research. MS and MCC analyzed the data and wrote the paper.

The authors reported no potential conflicts of interest.

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Selective Up-Regulation of the Soluble Pattern-Recognition Receptor Pentraxin 3 and of Vascular Endothelial Growth Factor in Giant Cell Arteritis

Relevance for Recent Optic Nerve Ischemia

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Objective. **To assess local expression and plasma levels of pentraxin 3 (PTX3) in patients with giant cell arteritis (GCA).**

Methods. **Plasma and serum samples were obtained from 75 patients with GCA (20 of whom had experienced optic nerve ischemia in the previous 3 weeks and 24 of whom had experienced symptom onset in the previous 6 months and had no history of optic nerve ischemia) and 63 controls (35 age-matched healthy subjects, 15 patients with rheumatoid arthritis, and 13 patients with chronic stable angina). In 9 patients in whom GCA was recently diagnosed, circulating levels of interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, CCL2/monocyte chemotactic protein 1, CCL3/macrophage inflammatory protein 1α (MIP-1α), CCL4/MIP-1β, CCL11/eotaxin, CXCL9/monokine induced by interferon-γ, CXCL10/interferon-γ–inducible 10-kd protein, tumor necrosis factor α (TNFα), interferon-γ, vascular endothelial growth factor (VEGF), granulocyte–macrophage colony-stimulating factor, and FasL were measured via a multiplexed cytometric assay. PTX3 and VEGF concentrations were assessed by enzyme-linked immunosorbent assay. PTX3 and CD68 expression were determined by immunohistochemistry and immunofluorescence on temporal artery samples.**

Results. **GCA patients with very recent optic nerve ischemia had significantly higher PTX3 and VEGF levels compared to other GCA patients and controls. GCA patients with a disease duration of <6 months had significantly higher PTX3 levels compared to other GCA patients and controls. Immunohistochemistry revealed selective PTX3 expression in the wall of inflamed arteries.**

Conclusion. **Our findings indicate that local expression of PTX3 is a feature of vascular inflammation in GCA; elevated circulating levels of PTX3 identify patients with very recent optic nerve ischemia or a recent diagnosis. Optic nerve ischemia is also associated with increased circulating VEGF levels.**

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Pentraxins (PTX) are a superfamily of acute-phase reactants characterized by a cyclic multimeric structure. C-reactive protein (CRP) and serum amyloid P (SAP) are well-characterized short PTXs. Inflammatory molecules, most prominently interleukin-6 (IL-6), prompt CRP synthesis by hepatocytes. PTX3 is the prototypical long PTX and, unlike the classic short PTX, whose sequence and regulation diverge between mouse and human, has a high degree of conservation (1). Several stimuli, in particular IL-1β, tumor necrosis factor α (TNFα), and Toll-like receptor agonists, promote its synthesis both in tissue cells, including vascular endothelial cells and smooth muscle cells (2,3), and in peripheral blood leukocytes (4,5). IL-10 and glucocorticoid hormones also enhance PTX3 production elicited by inflammatory signals (6,7). Conversely, interferon-γ (IFNγ) inhibits PTX3 expression elicited by IL-1β, TNFα, or lipopolysaccharide (8). IFNγ, IL-6, monocyte chemotactic protein 1 (MCP-1)/CCL2 and granulocyte–macrophage colony-stimulating factor (GM-CSF) per se have negligible effects on PTX3 expression (5,8).

PTX3 contributes to protection against bacteria, fungi, and viruses (1). Moreover, it is involved in the modulation of the innate response associated with ongoing cell death (9,10) and interacts with the extracellular matrix, contributing to the assembly of the cumulus oophorus (11). PTX3 synthesis is a hallmark of vascular injury (12). It occurs in atherosclerotic lesions (13–16), as a response to acute mechanical damage of the vessel wall, such as that associated with coronary stenting (17), and during acute myocardial infarction (18,19). PTX3 inhibits neointimal thickening after balloon injury of rat carotid arteries via interference with fibroblast growth factor 2 (20,21). PTX3 levels are elevated in preeclampsia, a common vascular complication of pregnancy (22,23), and in small-vessel vasculitis. In the latter case, PTX3 expression reflects local synthesis and is modulated by disease activity and immunosuppressive treatment (24,25).

Giant cell arteritis (GCA) is characterized by the association of systemic inflammatory symptoms, prompted by macrophage-derived inflammatory cytokines (i.e., IL-1β, TNFα, and IL-6) (26), with large and/or medium-sized artery involvement (27,28). Among ischemic complications, optic nerve ischemia is one of the most feared (29–32). Arterial wall thickening with involvement of the intima, which is often hyperplastic, leads to critical narrowing and occlusion of arteries that supplement the optic nerve. Intimal hyperplasia is the consequence of many steps, including the interaction of dendritic cells with T cells, IFNγ-mediated macrophage activation, and growth factor synthesis. Little is known about the role of microenvironmental signals in shaping vascular involvement, although there is evidence that they regulate recruitment and local activation of T cells (33,34). Intimal hyperplasia and neovessel formation may depend on in situ vascular endothelial growth factor (VEGF) expression (35). Some studies suggest that patients who experience ischemic events tend to have a weaker systemic acute-phase reaction (36,37).

In this study, we investigated whether GCA is associated with local PTX3 expression and increased plasma levels. We also assessed whether plasma PTX3 levels are correlated with the circulating levels of some relevant cytokines and growth factors.

PATIENTS AND METHODS

Patients and controls. Two groups of GCA patients were studied. Group 1 (patients 1–45 in Table 1) comprised 45 consecutive GCA patients (32 women and 13 men) with a mean age of 73 years (range 59–92 years) and a mean disease duration of 31.21 $\pm$ 5.11 months (range 0.25–125 months) evaluated at the San Raffaele University Hospital in Milan. Group 2 (patients 46–75 in Table 1) comprised 30 GCA patients (24 women and 6 men) with a mean age of 81 years (range 65–94 years) evaluated at the Hospital Clinic in Barcelona at the time of diagnosis, when symptoms had been present for a mean of 1.90 $\pm$ 0.35 months (range 0.12–8 months). A detailed medical history was obtained from all 75 patients, and all patients underwent a complete physical examination and routine hematologic and biochemical analyses, comprising blood cell counts, tests of kidney function, and assessments of erythrocyte sedimentation rate (ESR) and CRP concentrations. All patients were diagnosed as having GCA according to the American College of Rheumatology (ACR) 1990 criteria (38) (see Table 1 for criteria met). After clinical assessment, written informed consent was obtained and venous blood samples were collected. The study was approved by the local ethics committees.

At the time of blood sample collection, 27 patients (13 from group 1 and 14 from group 2) (Table 1) had experienced ocular ischemic complications (ischemic optic neuropathy or amaurosis fugax); the timing of ischemic event occurrence ranged from $<$24 hours to 5 years before sample collection. In particular, 20 of these patients (6 patients from group 1 and all 14 patients from group 2) had experienced optic nerve ischemia very recently (i.e., in the previous 3 weeks) (Table 1). One patient from group 1 (patient 42 in Table 1) had developed optic nerve ischemia recently (i.e., 3 months before blood sample collection). Among the patients without a history of very recent or recent optic nerve ischemia, 24 patients (10 from group 1 and 14 from group 2) had experienced symptom onset in the previous 6 months, while 30 patients (28 from group 1 and 2 from group 2) had a disease duration of $>$6 months. In 9 consecutive patients from group 1, in whom GCA was newly diagnosed (patients 28, 32, 34, 35, 37, 39, 40, 41, and 42 in Table 1), blood was collected again after a 4-week interval.

Control groups included 35 age-matched healthy subjects, 15 age-matched patients with rheumatoid arthritis (RA) who satisfied both the ACR 1987 criteria (39) and the 2010

Table 1. Serologic and clinical characteristics of the GCA patients*

Patient	Sex	Temporal artery alterations†	New headache†	Abnormal artery biopsy findings†	ESR >50 mm/hour†	Disease duration, months‡§	ESR, mm/hour‡	CRP, mg/liter‡	Prednisone, mg/day‡	Optic nerve ischemia¶
1	M	+	+	NA	+	16	5	0.2	12.5	Past
2	F	+	+	+	−	28	13	1.1	0	−
3	F	+	+	+	+	43	11	0.1	2.5	−
4	F	−	+	−	+	42	28	0.2	7.5	−
5	F	−	+	−	+	30	59	0.9	5	−
6	F	+	+	+	+	61	44	0.7	0	Past
7	F	−	+	+	+	31	14	1.2	0	−
8	F	−	−	+	+	36	32	1.7	5	Past
9	F	+	+	−	+	106	28	0.2	2.5	−
10	F	+	−	+	+	28	20	0.1	0	−
11	F	−	+	−	+	16	34	2.0	7.5	−
12	F	+	−	−	+	94	20	1.9	0	Past
13	F	−	+	−	+	114	24	0.4	5	−
14	F	+	+	+	+	45	15	0.6	7.5	Past
15	F	−	+	+	−	83	25	0.1	5	−
16	F	+	+	−	+	78	8	0.1	5	−
17	M	+	+	−	−	3	8	0.4	37.5	−
18	F	+	+	−	+	15	10	0.3	5	−
19	M	+	+	−	+	20	14	0.3	10	−
20	F	+	+	−	+	63	4	0.9	5	−
21	F	+	+	ND	+	4	53	12.6	0	−
22	M	+	−	ND	+	14	14	0.2	12.5	−
23	F	+	−	NA	+	88	20	1.9	0	−
24	M	+	+	−	+	34	2	0.4	0	−
25	F	+	+	−	+	27	31	0.8	5	Past
26	F	+	+	+	+	35	26	1.1	12.5	−
27	M	+	−	+	+	13	83	5.2	IVMP	Very recent
28	M	+	+	−	−	2	7	13.9	0	−
29	F	+	−	−	+	51	17	9.1	5	−
30	M	−	+	+	+	125	55	9.6	0	−
31	F	+	+	+	+	3	100	9.1	0	−
32	M	+	−	+	+	10	72	2.3	12.5	−
33	F	−	+	−	+	0.67	65	11.2	IVMP	Very recent
34	F	+	+	NA	+	0.5	57	0.7	37.5	−
35	M	−	+	−	+	1.25	31	0.2	75	−
36	F	+	+	−	+	2.5	78	ND	0	−
37	M	+	−	−	+	4	65	2.1	0	−
38	F	−	+	−	+	2	64	ND	37.5	−
39	F	+	+	ND	+	1	20	ND	IVMP	Very recent
40	M	+	+	−	+	1	58	9.0	50	Very recent
41	F	+	+	+	+	0.5	61	8.3	0	Very recent
42	M	+	+	−	+	3	63	4.5	0	Recent
43	F	+	+	+	−	24	46	1.9	25	−
44	F	−	+	ND	+	5	27	1.2	12.5	−
45	F	+	+	+	+	0.25	56	32.2	IVMP	Very recent
46	F	+	−	+	+	2	130	12	0	−
47	F	+	+	+	+	1	106	6.3	60	Very recent
48	F	+	+	+	+	1.25	103	16.3	IVMP	Very recent
49	F	+	+	+	+	1	78	18.4	0	Very recent
50	F	−	+	+	+	3	117	11.5	IVMP	Very recent
51	F	+	+	+	+	1	125	15.2	IVMP	Very recent
52	F	+	+	+	+	1.5	90	5.4	0	Very recent
53	F	−	+	+	+	0.25	100	0.8	0	Very recent
54	F	+	+	+	+	1.25	80	0.1	60	Very recent
55	M	+	+	+	+	0.75	115	19.4	0	Very recent
56	F	−	−	+	+	1.5	124	13.2	0	Very recent
57	F	−	−	+	+	0.75	90	6.0	60	Very recent
58	F	+	−	+	+	6	110	7.9	IVMP	Very recent
59	F	+	−	+	+	0.125	105	12.0	60	Very recent
60	F	+	−	+	+	0.25	71	1.5	0	Very recent

Table 1. *(Cont'd)*

Patient	Sex	Temporal artery alterations†	New headache†	Abnormal artery biopsy findings†	ESR >50 mm/hour†	Disease duration, months‡§	ESR, mm/hour‡	CRP, mg/liter‡	Prednisone, mg/day‡	Optic nerve ischemia¶
61	F	+	−	+	+	5	102	10.2	0	−
62	M	−	−	+	+	2.5	113	19.8	0	−
63	F	+	−	+	+	8	127	6.3	0	−
64	M	−	−	+	+	7	122	6.5	0	−
65	F	+	+	+	+	0.5	130	18.2	0	−
66	F	+	+	+	+	1	92	6.2	0	−
67	F	+	+	+	+	0.5	100	3.3	0	−
68	F	+	+	+	+	1	77	4.5	0	−
69	M	+	−	+	+	4	120	8.5	0	−
70	M	+	+	+	+	1	110	12.5	0	−
71	M	+	−	+	+	0.5	105	16.2	0	−
72	F	+	+	+	+	1	121	20.3	0	−
73	F	−	−	+	+	3	72	4.1	0	−
74	F	−	+	+	+	1	65	8.2	80	−
75	F	+	+	+	+	3.5	104	15.8	0	−

* All patients were older than 50 years at the time of diagnosis of giant cell arteritis (GCA). ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; NA = not adequate; ND = not determined; IVMP = intravenous methylprednisolone.
† Classification criteria satisfied at the time of the diagnosis of GCA.
‡ At the time of blood sample collection.
§ Time between disease onset and blood sample collection.
¶ Optic nerve ischemia was classified as past (occurring >6 months before sample collection), recent (occurring 3 months before sample collection), or very recent (occurring <3 weeks before sample collection).

ACR/European League Against Rheumatism criteria (40), and 13 age-matched patients with chronic stable angina, defined as effort angina (lasting >3 months) with angiographic evidence of coronary artery stenosis (stenosis >50% in diameter) in the absence of a previous history of unstable angina or myocardial infarction. A detailed medical history was obtained from each control subject. At the time of blood collection, RA patients had a mean disease duration of 147 months (range 12–480 months). The mean ESR was 24.5 mm/hour (range 12–74), the mean CRP concentration was 0.62 mg/liter (range 0.20–1.48), and the mean prednisone dosage was 5 mg/day (0–12.5 mg/day). Two of the patients with RA were untreated; the rest were receiving disease-modifying antirheumatic drugs and prednisone (n = 7) or receiving anti-TNF agents (n = 6). The same control subjects (healthy subjects, patients with RA, and patients with chronic stable angina) were used for both sets of GCA patients (group 1 and group 2).

Assessment of patient protein and serum samples. PTX3 levels in plasma samples were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (41). This assay is highly sensitive and specific (41,42). No cross-reactions were observed with other PTXs, including CRP and SAP protein. The samples were analyzed in batches in a random order in a blinded manner. The Flex Set cytometric bead array technology (Becton Dickinson) permits multiplexed analysis of several analytes in one sample (43). We obtained sera from 9 consecutive patients in whom GCA had recently been diagnosed (within the previous 7 days) (patients 28, 32, 34, 35, 37, 39, 40, 41, and 42 in Table 1), and assessed the circulating concentrations of IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12 p70, CCL2/MCP-1, CCL3/macrophage inflammatory protein 1 (MIP-1α), CCL4/MIP-1β, CCL11/eotaxin, CXCL9/monokine induced by interferon-γ (MIG), CXCL10/IFN-γ–inducible 10-kd protein (IP-10), TNFα, IFNγ, VEGF, CSF2/GM-CSF, and CD178/FasL. Assays were performed according to the recommendations of the manufacturers; flow cytometry was carried out on an LSRII flow cytometer (BD Biosciences). VEGF levels in serum samples were assessed using a commercially available ELISA kit (Quantikine Human VEGF Immunoassay) according to the instructions of the manufacturer (R&D Systems). Absorbance was determined using an automated microplate reader (Bio-Rad Model 680).

Immunohistochemistry and immunofluorescence. Four-micrometer–thick sections of 5 temporal artery biopsy samples used to diagnose GCA and 5 samples with no sign of vasculitis were analyzed in parallel. For immunohistochemical analysis, after 30 minutes in xylol, sections were rehydrated using alcohol solutions (99%, 95%, 70%, and 50%). After microwave treatment (3 cycles at 650W for 5 minutes each), sections were incubated for 60 minutes at room temperature in a 0.3% H_2O_2 solution to block endogenous peroxidases. After rinsing in phosphate buffered saline, sections were incubated for 20 minutes at room temperature in normal serum diluted 1:20. After washing, sections were incubated with the biotinylated PTX3-specific rat monoclonal antibody MNB4 for 2 hours at room temperature (final concentration 40 μg/ml), as previously described (42,44). The MNB4 binding was then revealed using peroxidase-conjugated streptavidin (Vectastain Elite ABC kit; Vector). Peroxidase was revealed by 3,3′-diaminobenzidine. In a similar manner, sections were stained with commercially available anti-CD68 mouse monoclonal antibodies (Novocastra) to identify macrophages and anti-CD31 (platelet endothelial cell adhesion molecule 1) to identify endothelial cells. Sections were counterstained with hematoxylin. For immunofluorescence analysis, tissue sections were

subjected to Pronase antigen retrieval, then incubated with MNB4 and anti-CD68, and revealed with fluorescein-labeled antibodies.

Statistical analysis. One-way analysis of variance and Bonferroni post hoc analysis were used to compare the concentrations of circulating molecules in different groups of patients and controls. Correlations between quantitative continuous variables were evaluated using Spearman's correlation analysis; data are shown as the correlation index rho and significance level *P*. Statistical analyses were performed with IBM SPSS statistics software version 18 and GraphPad Prism for Windows version 5 (StataCorp). Values are expressed as the mean ± SEM.

RESULTS

Elevated PTX3 levels in GCA patients with very recent optic nerve ischemia and in those with a disease duration of <6 months. The mean ± SEM PTX3 concentration was significantly higher in GCA patients (23.31 ± 4.06 ng/ml) than in healthy age-matched controls (3.97 ± 0.28 ng/ml; $P \leq 0.003$) and in patients with RA (3.13 ± 0.37 ng/ml; $P \leq 0.043$) (Figures 1A and 2A). In particular, patients in whom optic nerve ischemia had occurred very recently had a significantly higher mean PTX3 concentration compared to other GCA patients and controls in both group 1 and group 2 (Figure 2A). When the 2 groups were considered together, the mean ± SEM PTX3 concentration was 63.82 ± 10.29 ng/ml in patients with very recent optic nerve ischemia and 8.58 ± 1.46 ng/ml in the other GCA patients ($P \leq 0.001$) (Figures 1B and 2A). In the group with very recent optic nerve ischemia, no correlation was found between the level of PTX3 and the CRP level, ESR, or daily prednisone dosage (data not shown).

Patients with past optic nerve ischemia, defined as an event that occurred >6 months before blood samples were obtained (n = 6) (patients 1, 6, 8, 12, 14, and 25 in Table 1), had a mean ± SEM blood PTX3 level of 2.76 ± 0.35 ng/ml, which was not significantly different from the mean ± SEM blood PTX3 level in GCA patients who had symptoms of long-standing duration (>6 months) but no optic nerve ischemia (mean ± SEM 4.57 ± 1.06 ng/ml; n =24) and in control groups. In the group of patients with a history of optic nerve ischemia (very recent, recent, or past) (Table 1), a significant correlation was found between circulating PTX3 levels and ESR and CRP but not between PTX3 levels and daily prednisone dosage (Figure 3A).

Among patients without very recent optic nerve ischemia, those with GCA onset in the previous 6 months had significantly higher PTX3 levels (mean ± SEM PTX3 level 14.12 ± 2.82 ng/ml; n = 24) compared to those with a disease duration of >6 months (mean ±

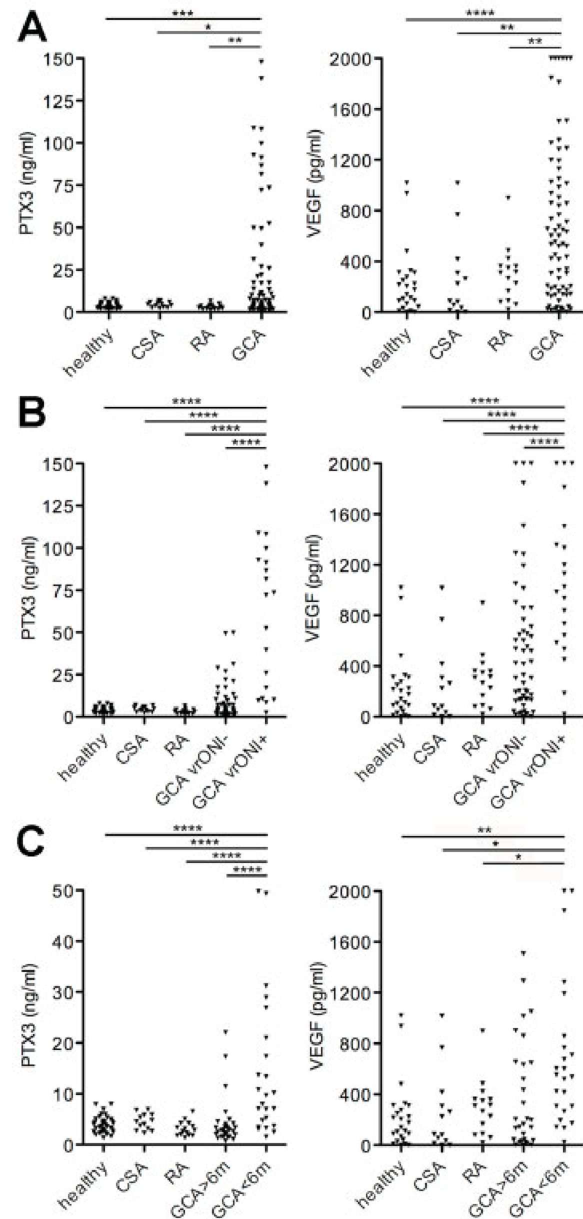
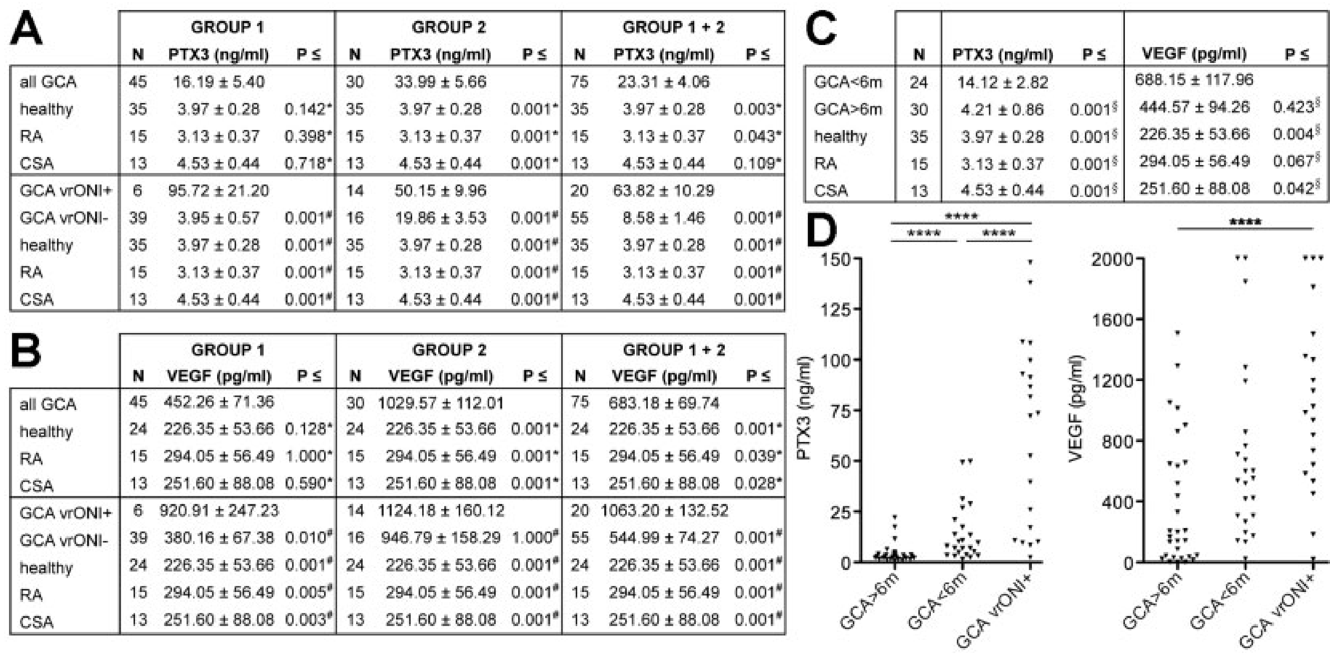


Figure 1. Pentraxin 3 (PTX3) and vascular endothelial growth factor (VEGF) concentrations in patients with giant cell arteritis (GCA) and control groups. **A,** PTX3 and VEGF concentrations in age-matched healthy controls, patients with chronic stable angina (CSA), patients with rheumatoid arthritis (RA), and patients with GCA. **B,** PTX3 and VEGF concentrations in age-matched healthy controls, patients with chronic stable angina, patients with RA, patients with GCA without very recent optic nerve ischemia (GCA vrONI−), and patients with GCA with very recent optic nerve ischemia. **C,** PTX3 and VEGF concentrations in age-matched healthy controls, patients with chronic stable angina, patients with RA, patients with GCA with no history of optic nerve ischemia and a disease duration of >6 months, and patients with GCA with no history of optic nerve ischemia and a disease duration of <6 months. Symbols represent individual subjects. * = $P \leq 0.1$; ** = $P \leq 0.05$; *** = $P \leq 0.005$; **** = $P \leq 0.001$.



A

	GROUP 1			GROUP 2			GROUP 1 + 2		
	N	PTX3 (ng/ml)	P ≤	N	PTX3 (ng/ml)	P ≤	N	PTX3 (ng/ml)	P ≤
all GCA	45	16.19 ± 5.40		30	33.99 ± 5.66		75	23.31 ± 4.06	
healthy	35	3.97 ± 0.28	0.142*	35	3.97 ± 0.28	0.001*	35	3.97 ± 0.28	0.003*
RA	15	3.13 ± 0.37	0.398*	15	3.13 ± 0.37	0.001*	15	3.13 ± 0.37	0.043*
CSA	13	4.53 ± 0.44	0.718*	13	4.53 ± 0.44	0.001*	13	4.53 ± 0.44	0.109*
GCA vrONI+	6	95.72 ± 21.20		14	50.15 ± 9.96		20	63.82 ± 10.29	
GCA vrONI-	39	3.95 ± 0.57	0.001#	16	19.86 ± 3.53	0.001#	55	8.58 ± 1.46	0.001#
healthy	35	3.97 ± 0.28	0.001#	35	3.97 ± 0.28	0.001#	35	3.97 ± 0.28	0.001#
RA	15	3.13 ± 0.37	0.001#	15	3.13 ± 0.37	0.001#	15	3.13 ± 0.37	0.001#
CSA	13	4.53 ± 0.44	0.001#	13	4.53 ± 0.44	0.001#	13	4.53 ± 0.44	0.001#

B

	GROUP 1			GROUP 2			GROUP 1 + 2		
	N	VEGF (pg/ml)	P ≤	N	VEGF (pg/ml)	P ≤	N	VEGF (pg/ml)	P ≤
all GCA	45	452.26 ± 71.36		30	1029.57 ± 112.01		75	683.18 ± 69.74	
healthy	24	226.35 ± 53.66	0.128*	24	226.35 ± 53.66	0.001*	24	226.35 ± 53.66	0.001*
RA	15	294.05 ± 56.49	1.000*	15	294.05 ± 56.49	0.001*	15	294.05 ± 56.49	0.039*
CSA	13	251.60 ± 88.08	0.590*	13	251.60 ± 88.08	0.001*	13	251.60 ± 88.08	0.028*
GCA vrONI+	6	920.91 ± 247.23		14	1124.18 ± 160.12		20	1063.20 ± 132.52	
GCA vrONI-	39	380.16 ± 67.38	0.010#	16	946.79 ± 158.29	1.000#	55	544.99 ± 74.27	0.001#
healthy	24	226.35 ± 53.66	0.001#	24	226.35 ± 53.66	0.001#	24	226.35 ± 53.66	0.001#
RA	15	294.05 ± 56.49	0.005#	15	294.05 ± 56.49	0.001#	15	294.05 ± 56.49	0.001#
CSA	13	251.60 ± 88.08	0.003#	13	251.60 ± 88.08	0.001#	13	251.60 ± 88.08	0.001#

C

	N	PTX3 (ng/ml)	P ≤	VEGF (pg/ml)	P ≤
GCA<6m	24	14.12 ± 2.82		688.15 ± 117.96	
GCA>6m	30	4.21 ± 0.86	0.001§	444.57 ± 94.26	0.423§
healthy	35	3.97 ± 0.28	0.001§	226.35 ± 53.66	0.004§
RA	15	3.13 ± 0.37	0.001§	294.05 ± 56.49	0.067§
CSA	13	4.53 ± 0.44	0.001§	251.60 ± 88.08	0.042§

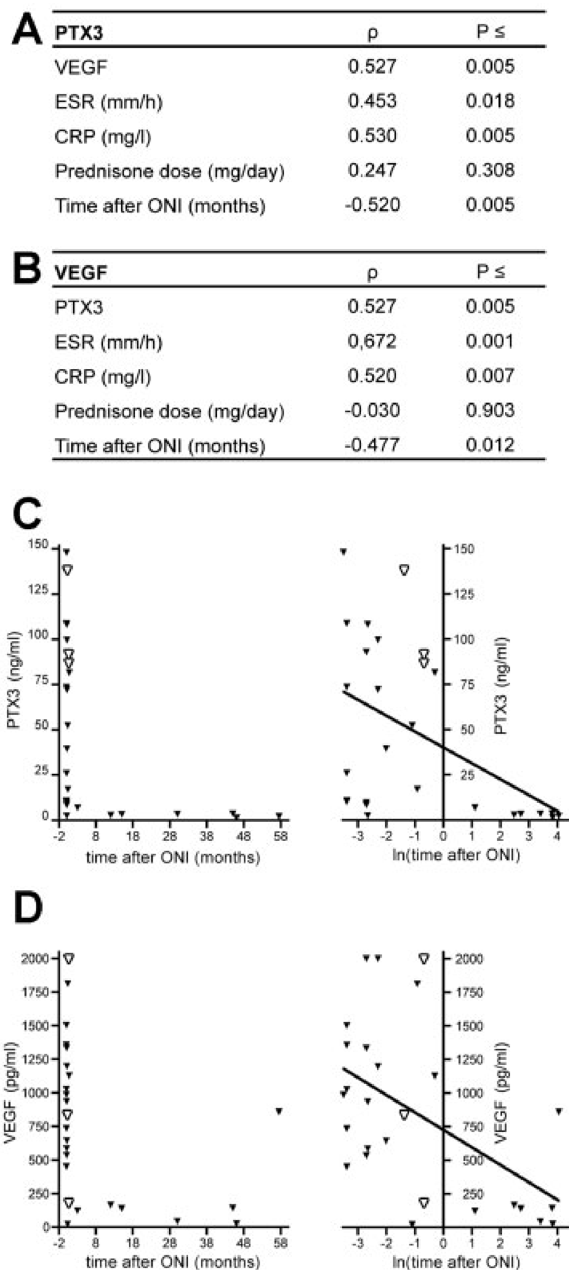
Figure 2. PTX3 and VEGF concentrations in all patients with GCA, patients with GCA divided into subgroups, and control groups (healthy controls, patients with RA, and patients with chronic stable angina). Group 1 consisted of patients with GCA evaluated at San Raffaele University Hospital in Milan, and group 2 consisted of patients with GCA evaluated at the Hospital Clinic in Barcelona. The same control subjects were used for each subset of patients with GCA. **A** and **B,** Comparison of PTX3 (**A**) and VEGF (**B**) concentrations in all patients with GCA, control groups, patients with GCA with very recent optic nerve ischemia (occurring within the 3 weeks prior to sample collection), and patients with GCA without very recent optic nerve ischemia. * = *P* versus all patients with GCA; # = *P* versus patients with GCA with very recent optic nerve ischemia. **C,** Comparison of PTX3 and VEGF concentrations in patients with GCA with a disease duration of <6 months, patients with GCA with a disease duration of >6 months, and control groups. § = *P* versus patients with GCA with a disease duration of <6 months. **D,** Comparison of PTX3 and VEGF concentrations in patients with GCA with a disease duration of >6 months, patients with GCA with a disease duration of <6 months, and patients with GCA with very recent optic nerve ischemia. Symbols represent individual subjects. **** = $P \leq 0.001$. See Figure 1 for definitions.

SEM PTX3 level 4.21 ± 0.86 ng/ml; n = 30) ($P \leq 0.001$) and control groups ($P \leq 0.001$ for each) (Figures 1C and 2C). GCA patients in whom symptoms had been present for <6 months also had significantly higher CRP and ESR levels ($P \leq 0.001$). In the group of GCA patients without very recent optic nerve ischemia, PTX3 levels correlated inversely with the disease duration ($\rho = -0.665$, $P \leq 0.001$) and directly with the ESR ($\rho = 0.598$, $P \leq 0.001$) and CRP level ($\rho = 0.617$, $P \leq 0.001$). No correlation was found with the daily prednisone dosage.

Notably, patients with very recent optic nerve ischemia had significantly higher PTX3 levels (mean ± SEM 63.82 ± 10.29 ng/ml) than patients with no history of optic nerve ischemia in whom symptoms had been present for <6 months (mean ± SEM 14.12 ± 2.82 ng/ml; $P \leq 0.001$) and patients with no history of optic nerve ischemia in whom symptoms had been present for >6 months (mean ± SEM 4.21 ± 0.86 ng/ml; $P \leq 0.001$) (Figure 2D).

No significant difference was found in PTX3 concentrations between treated and untreated GCA patients, despite significantly different ESRs (mean ± SEM 48.64 ± 5.93 mm/hour in treated patients versus 77.61 ± 6.50 mm/hour in untreated patients; $P \leq 0.002$) and CRP levels (mean ± SEM 4.51 ± 1.09 mg/liter in treated patients versus 8.30 ± 1.09 mg/liter in untreated patients; $P \leq 0.017$).

Elevated VEGF levels in GCA patients versus controls and in GCA patients with very recent optic nerve ischemia. In order to assess whether differences in the levels of circulating cytokines and growth factors could account for the observed discrepancy in the PTX3 levels, a multiple simultaneous cytometric bead array analysis was performed in 3 patients with very recent optic nerve ischemia (patients 39, 40, and 41 in Table 1) and in 6 consecutive patients in whom GCA was recently diagnosed (within the previous 7 days) without very recent optic nerve ischemia (patients 28, 32, 34, 35, 37, and 42 in Table 1). The 2 groups were not significantly different with regard to disease duration, ESR, CRP level, hemoglobin concentration, or daily prednisone



A

PTX3	ρ	P ≤
VEGF	0.527	0.005
ESR (mm/h)	0.453	0.018
CRP (mg/l)	0.530	0.005
Prednisone dose (mg/day)	0.247	0.308
Time after ONI (months)	-0.520	0.005

B

VEGF	ρ	P ≤
PTX3	0.527	0.005
ESR (mm/h)	0,672	0.001
CRP (mg/l)	0.520	0.007
Prednisone dose (mg/day)	-0.030	0.903
Time after ONI (months)	-0.477	0.012

Figure 3. Correlation analysis in patients with GCA with a history of optic nerve ischemia (ONI). **A** and **B,** Correlations of PTX3 concentration (**A**) and VEGF concentration (**B**) with each other and with erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, daily prednisone dosage, and time after optic nerve ischemia. **C** and **D,** Correlations of PTX3 concentration (**C**) and VEGF concentration (**D**) with time or logarithm of time from occurrence of optic nerve ischemia to blood sample collection. Open symbols indicate patients in whom multiplexed cytometric bead assay was performed. See Figure 1 for other definitions.

dosage (Table 2). Patients with very recent optic nerve ischemia had significantly higher circulating VEGF levels (mean ± SEM 496.81 ± 230.99 pg/ml versus 86.85 ± 29.71 pg/ml in patients without very recent optic nerve ischemia; $P \leq 0.039$). No significant differences were found in the concentrations of the other analytes, including cytokines known to induce PTX3 expression (IL-1β and TNFα) and those known to inhibit PTX3 expression (IFNγ). The mean circulating level of IL-6 was lower in patients with very recent optic nerve ischemia, but the difference was not statistically significant (Table 2).

Circulating VEGF levels were then assessed by conventional ELISA in sera from all GCA patients (n = 75), age-matched healthy controls (n = 24), RA patients (n = 15), and patients with chronic stable angina (n = 13). GCA patients had significantly higher VEGF concentrations (mean ± SEM 683.18 ± 69.74 pg/ml) compared to healthy controls (226.35 ± 53.66 pg/ml; $P \leq 0.001$), RA patients (294.05 ± 56.49 pg/ml; $P \leq 0.039$), and patients with chronic stable angina (251.60 ± 88.08 pg/ml; $P \leq 0.028$) (Figures 1A and 2B). GCA patients with very recent optic nerve ischemia had significantly higher VEGF levels (mean ± SEM 1,063.20 ± 132.52 pg/ml) than other GCA patients (544.99 ± 74.27 pg/ml; $P \leq 0.001$) and control groups ($P \leq 0.001$ for each) (Figures 1B and 2B), thus confirming the results obtained via multicytokine assay. When groups 1 and 2 were considered separately, the difference was significant in group 2 but not in group 1 (Figure 2B).

In the group of GCA patients with very recent optic nerve ischemia, VEGF levels correlated with the ESR ($\rho = 0.505$, $P \leq 0.023$). We found no correlation between VEGF levels and PTX3 levels, CRP levels, or daily prednisone dosage in this subset of GCA patients (data not shown). Patients with past optic nerve ischemia (Table 1) had a mean ± SEM VEGF concentration of 229.11 ± 128.11 pg/ml, which was not significantly different from that in GCA patients with long-standing symptoms and no optic nerve ischemia (mean ± SEM 498.43 ± 111.72 pg/ml; n = 24). In the group of patients with a history of optic nerve ischemia (very recent, recent, or past), a significant correlation was found between VEGF concentration and PTX3 level, ESR, and CRP level but not between VEGF concentration and the daily prednisone dosage (Figure 3B).

VEGF concentrations in GCA patients with a disease duration of <6 months and no history of optic nerve ischemia (mean ± SEM 668.15 ± 117.96 pg/ml; n = 24) were not significantly higher than those in GCA patients in whom symptoms had been present for >6 months (mean ± SEM 444.57 ± 94.26 pg/ml; n = 30) (Figures 1C and 2C). In the subset of GCA patients without very recent optic nerve ischemia, VEGF level correlated directly with PTX3 level ($\rho = 0.342$, $P \leq$

Table 2. Multicytokine analysis in selected patients with GCA*

	Patients with very recent optic nerve ischemia (n = 3)	Patients without optic nerve ischemia (n = 6)
PTX3, ng/ml	105.27 ± 16.43	5.26 ± 1.05†
Disease duration, months	0.83 ± 0.17	3.46 ± 1.40
ESR, mm/hour	46.33 ± 13.19	49.17 ± 10.22
CRP, mg/liter	8.65 ± 0.35	3.96 ± 2.08
Hemoglobin, gm/dl	11.27 ± 0.34	12.33 ± 0.74
Prednisone, mg/day	20.83 ± 12.36	20.40 ± 15.15
Cytokines, pg/ml		
IL-1β	<2.30	<2.30
IL-2	0.95 ± 0.95	1.60 ± 1.09
IL-4	<1.40	<1.40
IL-6	2.94 ± 1.68	10.11 ± 4.44
IL-7	5.88 ± 2.36	3.06 ± 1.56
IL-8	7.39 ± 1.15	12.96 ± 2.36
IL-10	2.75 ± 0.23	2.35 ± 0.41
IL-12p70	<0.60	0.67 ± 0.45
CCL2/MCP-1	21.37 ± 7.77	57.26 ± 18.78
CCL3/MIP-1α	3.76 ± 1.06	3.84 ± 0.24
CCL4/MIP-1β	21.79 ± 3.57	41.89 ± 27.83
CCL11/eotaxin	54.31 ± 26.38	93.58 ± 14.55
CXCL9/MIG	446.82 ± 340.20	9,000.93 ± 6,603.38
CXCL10/IP-10	82.12 ± 37.92	77.99 ± 19.05
TNFα	0.35 ± 0.35	0.20 ± 0.19
IFNγ	<1.80	2.07 ± 1.08
VEGF	496.81 ± 230.99	86.85 ± 29.71‡
CSF2/GM-CSF	1.47 ± 0.12	1.34 ± 0.27
CD178/FasL	11.25 ± 1.39	15.07 ± 1.75

* Flex Set cytometric bead array technology was used to measure cytokine levels. Values are the mean ± SEM. GCA = giant cell arteritis; PTX = pentraxin 3; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IL-1β = interleukin-1β; MCP-1 = monocyte chemotactic protein 1; MIP-1α = macrophage inflammatory protein 1α; MIG = monokine induced by interferon-γ; IP-10 = interferon-γ–inducible 10-kd protein; TNFα = tumor necrosis factor α; IFNγ = interferon-γ; VEGF = vascular endothelial growth factor; GM-CSF = granulocyte–macrophage colony-stimulating factor.
† $P < 0.001$ versus patients with very recent optic nerve ischemia.
‡ $P < 0.039$ versus patients with very recent optic nerve ischemia.

0.011), ESR ($\rho = 0.475$, $P \leq 0.001$), and CRP level ($\rho = 0.334$, $P \leq 0.014$) and inversely with the daily prednisone dosage ($\rho = -0.342$, $P \leq 0.011$). No significant correlation was found between VEGF level and disease duration ($\rho = -0.175$, $P \leq 0.205$). Moreover, no significant correlation was found between PTX3 and VEGF levels in any of the control groups (patients with RA, patients with chronic stable angina, and healthy controls).

Patients with very recent optic nerve ischemia had a significantly higher mean ± SEM VEGF concentration (1,063.20 ± 132.52 pg/ml) than patients with a disease duration of >6 months (444.57 ± 94.26 pg/ml; $P \leq 0.001$) (Figure 2D). VEGF levels were not significantly higher in patients with very recent optic nerve ischemia than in GCA patients who had no history of optic nerve ischemia and in whom symptoms had been present for <6 months (mean ± SEM 668.15 ± 117.96 pg/ml; $P \leq 0.089$) (Figure 2D). No significant difference was found between treated and untreated GCA patients with regard to VEGF levels, despite significantly different ESRs (48.64 ± 5.93 mm/hour in treated patients versus 77.61 ± 6.50 mm/hour in untreated patients; $P \leq 0.002$) and CRP levels (4.51 ± 1.09 mg/liter in treated patients versus 8.30 ± 1.09 mg/liter in untreated patients; $P \leq 0.017$).

Transience of the increase in PTX3 levels in patients with optic nerve ischemia. Laboratory investigations, including multiplexed cytometric bead array analysis, were repeated using blood samples obtained from 3 patients with very recent optic nerve ischemia (patients 39, 40, and 41 in Table 1) and from 4 patients in whom GCA was recently diagnosed who did not have optic nerve ischemia (patients 32, 35, 37, and 42 in Table 1) 4 weeks after the first sample was obtained. In the first group, the mean ± SEM PTX3 concentration was significantly lower 1 month after the onset of ischemic symptoms (13.01 ± 5.84 ng/ml) than at the time of diagnosis (105.27 ± 16.43 ng/ml; $P \leq 0.001$), while the difference between VEGF concentrations at the 2 time points was not significant (496.81 ± 230.99 pg/ml at

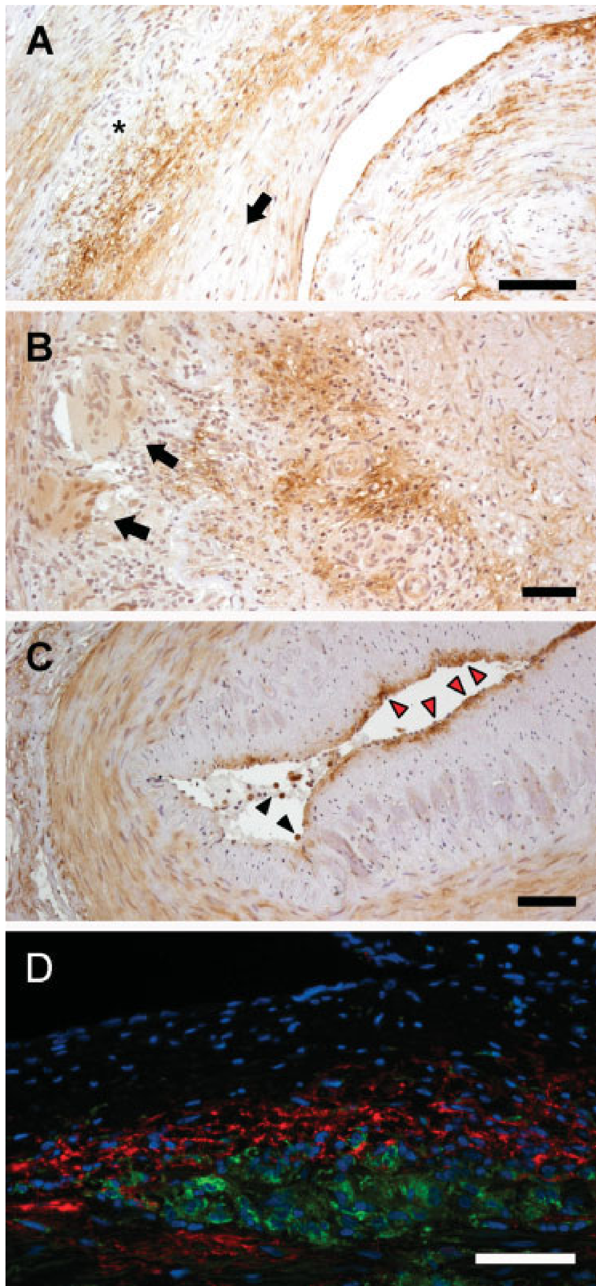


Figure 4. PTX3 expression in temporal artery specimens from patients with GCA. **A,** PTX3 expression in the extracellular matrix of the hyperplastic intima, located mainly at the intima–media junction. **Asterisk** indicates disrupted internal elastic lamina; **arrow** indicates hyperplastic intima. **B,** PTX3 expression in the extracellular matrix of the hyperplastic intima. Giant cells along the internal elastic lamina (**arrows**) are only faintly positive. **C,** Endothelial and subendothelial PTX3 expression (**red arrowheads**). Intraluminal leukocytes strongly express PTX3 (**black arrowheads**). **D,** High levels of PTX3 expression in the extracellular matrix surrounding CD68-positive macrophages and giant cells. Red staining shows PTX3, green staining shows CD68, and blue staining shows nuclei. Bars = 100 μm. See Figure 1 for definitions.

time 0 and 206.91 ± 42.29 pg/ml after 4 weeks). In the group of patients in whom GCA was newly diagnosed and no ischemic event had occurred, no significant difference was found between circulating levels of PTX3 and VEGF at the time of diagnosis and circulating levels of PTX3 and VEGF 1 month later. VEGF concentrations at the time of the second sample collection were higher in GCA patients with very recent optic nerve ischemia (206.91 ± 42.29 pg/ml) than in GCA patients with low PTX3 levels (89.49 ± 37.68 pg/ml), although the difference was not significant. No significant difference in the circulating levels of the other cytokines and growth factors measured via multiplexed cytokine assay was demonstrated between the 2 different time points of sample collection (data not shown).

In the subset of all GCA patients with a history of optic nerve ischemia (very recent, recent, or past) (Table 1), a significant inverse correlation was found between time after optic nerve ischemia (months between onset of optic nerve ischemia and sample collection) and the circulating levels of both PTX3 ($\rho = -0.520$, $P \leq 0.005$) and VEGF ($\rho = -0.477$, $P \leq 0.012$) (Figure 3).

Expression of PTX3 in vasculitic lesions. PTX3 was expressed in all temporal artery specimens with signs of active disease (n = 5). Temporal artery specimens with no sign of vasculitis (n = 5) stained negative. (Results are available from the author upon request.) PTX3 expression in specimens with active vasculitis was mainly located in the media, the adventitia, and the extracellular matrix (Figures 4A and B). Occasionally, the endothelium and subendothelial matrix expressed PTX3 (Figure 4C). Immunofluorescence analysis demonstrated that PTX3 was highly expressed in the extracellular matrix surrounding CD68-positive activated macrophages and giant cells (Figure 4D).

DISCUSSION

PTX3 generation occurs under conditions associated with vascular injury. It may help to protect the tissue against the deleterious effects of Noxa and to limit the harmful action of inflammatory leukocytes (2,16,19). Extensive and productive inflammation, although segmental, characterizes large vessels in GCA. In this study, we demonstrated that PTX3 is locally produced in the inflamed vessel domains of patients with GCA. Potential stimuli for PTX3 generation, including IL-1β and TNFα, have been detected in temporal artery specimens from GCA patients (26,45). However, local expression of IFNγ, which is known to inhibit PTX3 synthesis, has also been demonstrated (45). We have no direct evidence regarding the nature of the signal responsible for local

production of the molecule, even if the relative balance among the various cytokines is likely to finely tune the extent of PTX3 generation.

Circulating levels of PTX3 are increased in GCA patients. We observed a well-defined cluster of patients with notably elevated plasma expression of PTX3. These patients were clinically homogeneous, since they all had experienced optic nerve ischemia in the 3 weeks before blood sampling. The event was transient, since PTX3 levels were lower (even if still elevated) in 3 patients from whom we obtained a second sample 4 weeks later. Moreover, PTX3 levels in patients with a history of past optic nerve ischemia (occurring >6 months before blood samples were obtained) were similar to those in controls.

A transient elevation of PTX3 has previously been demonstrated in patients with ongoing acute myocardial infarction. In those patients, kinetics were significantly faster than in the patients with GCA in the present study, since PTX3 levels peaked 7.5 hours after coronary care unit admission, were high at 24 hours, but were in the normal range in the following days (42). Forty-eight hours after acute myocardial infarction, PTX3 elevation is no longer detectable (18). Different sources may contribute to PTX3 synthesis in patients with GCA and acute myocardial infarction. In this study, we observed that activated macrophages and giant cells at the intima–media junction and endothelial cells express PTX3 in inflamed temporal arteries. These cells are not likely to be an important source of PTX3 during myocardial infarction (42). Interestingly, endothelial cells are the main source of PTX3 in antineutrophil cytoplasmic antibody–associated vasculitis lesions (24); in these samples, PTX3-expressing macrophages are less common, indicating relevant pathophysiologic differences between large- and small-vessel vasculitis.

In the subset of GCA patients without very recent optic nerve ischemia in the present study, PTX3 levels correlated with CRP levels and ESR, and patients with recent disease onset (within the past 6 months) had significantly higher PTX3 levels compared to GCA patients with a longer disease duration and compared to control groups. This reflects the strong vascular inflammation in the early phases of the disease, for which a high initial steroid dose and a very slow tapering are required. It has been demonstrated that despite early symptom relief after beginning therapy, arteritis is detectable in temporal arteries after >2 weeks (32), and ischemic complications often occur within the first 4 weeks after steroids have been started (30).

Polymyalgia rheumatica (PMR) shares several features, including important systemic inflammation, with GCA, but actual vessel involvement is in general elusive, since it can be demonstrated only in a minority of PMR patients (32). In a previous study, no significant increase in circulating PTX3 levels was observed in PMR patients when compared to healthy controls (46). The extent of vascular inflammation during the early phases of the disease and the presence of very recent optic nerve ischemia may thus account for the higher plasma PTX3 concentration we have observed in GCA patients. PTX3 has a peculiar characteristic among inflammatory molecules, since it is locally produced at sites of inflammation, and specifically in the midst of large vessel walls. Further studies are warranted to address whether local production of PTX3 directly contributes to the local remodeling of the inflamed vessel (2,34), an event which has been shown to be regulated by environmental factors, including the activation of the endothelin system (47).

Moreover, we plan to evaluate whether PTX3 levels may contribute to the early identification of patients with GCA among those presenting with PMR features. In fact, classification criteria for GCA (38) set the cutoff point for ESR at a higher level compared to PMR criteria (32). Nonetheless, ESR levels rapidly decrease, and temporal artery biopsy findings may be negative after the initiation of steroid therapy. Differential diagnosis may therefore be difficult in patients initially classified and treated as having PMR who report a "new headache." The finding of increased PTX3 levels may support a diagnosis of GCA in this subset of patients, even if steroids have been already started and ESR is low, since we did not find a significant effect of the treatment or a correlation between PTX3 levels and daily prednisone dosage, despite the experimental evidence that corticosteroids regulate PTX3 expression (7). Further studies are warranted to explore this issue.

The finding of notably high circulating PTX3 levels in GCA patients with optic nerve ischemia suggests that this parameter could be useful for differentiating GCA-associated optic nerve ischemia from nonarteritis optic nerve ischemia in the elderly (48). A prospective study is required to evaluate this possibility.

Among the cytokines and growth factors tested in this study, only VEGF levels were higher in patients with very recent optic nerve ischemia. Four weeks after the first determination, VEGF levels in this subset of GCA patients were still higher than those in the subset of GCA patients with low PTX3 levels. Interestingly, no significant difference was found in circulating VEGF levels between patients with recent disease onset (within the past 6 months) and patients with past disease onset (before the past 6 months) nor between treated patients and untreated patients. In previous studies, VEGF syn-

thesis has been detected in the inflamed arterial wall (35), and this molecule is believed to be involved in neoangiogenesis and intimal hyperplasia, which are critical events in determining critical narrowing or occlusion of the arterial lumen, and thus ischemia (34). Single studies have suggested VEGF gene polymorphisms to be implicated in susceptibility to develop GCA (49) or GCA-related ischemic events (50). To the best of our knowledge, this is the first study to demonstrate increased systemic VEGF levels in GCA patients. Taken together, our data suggest a link between PTX3 and ongoing vascular inflammation and ischemic events in GCA patients. VEGF may be involved as well.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Manfredi had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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EXTENDED REPORT

Increased IL-17A expression in temporal artery lesions is a predictor of sustained response to glucocorticoid treatment in patients with giant-cell arteritis

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ABSTRACT

Background Interleukin 17A (IL-17A) exerts pivotal proinflammatory functions in chronic inflammatory and autoimmune diseases.

Objective To investigate IL-17A expression in temporal artery lesions from patients with giant-cell arteritis (GCA), and its relationship with disease outcome.

Methods Fifty-seven patients with biopsy-proven GCA were prospectively evaluated, treated and followed for 4.5 years (52–464 weeks). Relapses, time (weeks) required to achieve a maintenance prednisone dose <10 mg/day, and time (weeks) to complete prednisone withdrawal were prospectively recorded. IL-17A mRNA was measured by real-time quantitative RT-PCR in temporal arteries from all patients and 19 controls. IL-17 protein expression was assessed by immunohistochemistry/immunofluorescence.

Results IL-17A expression was significantly increased in temporal artery samples from GCA patients compared with controls (6.22±8.61 vs 2.50±3.9 relative units, p=0.016). Surprisingly, patients with strong IL-17A expression tended to experience less relapses, and required significantly shorter treatment periods (median 25 vs 44 weeks to achieve <10 mg prednisone/day, p=0.0079). There was no correlation between IL-17A and RORc or RORα expression suggesting that these transcription factors may not exclusively reflect Th17 differentiation, and that cells other than Th17 cells might contribute to IL-17 expression in active patients. Accordingly, $FoxP3^+IL\text{-}17A^+$ cells were identified in lesions by confocal microscopy and were dramatically reduced in specimens from treated patients.

Conclusions IL-17A expression is increased in GCA lesions, and is a predictor of response to glucocorticoid treatment. The contribution of FoxP3+ cells to IL-17A production in untreated patients suggests that induced-Tregs may facilitate disease remission when proinflammatory cytokine production is downregulated by glucocorticosteroids.

INTRODUCTION

Giant-cell arteritis (GCA) is a large and medium-sized vessel vasculitis considered to be a Th1-mediated disease on the basis of its granulomatous appearance and the strong expression of IFNγ and IFNγ-induced products in lesions.[1–5]

Patients with GCA experience a dramatic improvement with high-dose glucocorticoids (GC). However, response is not sustained, and about 40%–50% of patients relapse when GC are tapered.[6 7] Persistent activity results in prolonged GC treatment, and more than 80% of patients experience GC-related complications.[8] Mechanisms involved in disease persistence are unknown. Increased expression of TNFα and CCL2 in vascular lesions and elevated serum concentrations TNFα and IL-6 are associated with relapsing disease and prolonged GC-requirements.[5 9 10] However, blocking TNFα with infliximab did not result in reduced relapse rate or cumulated GC doses when compared with placebo in a randomised clinical trial, indicating that blocking TNFα is not sufficient to abrogate disease activity.[11] In the context of this trial, comparison of gene expression in second temporal artery biopsies performed in four patients after 1 year of treatment disclosed that IFNγ and IL12/23p40, but not IL12p35, were upregulated in relapsing patients.[3] Discrepancy between expression of the two IL-12 subunits, p35 and p40, suggested that upregulated IL-12/23p40 might be part of IL-23. This led to the search for IL-23 and its related cytokine IL-17 in GCA lesions.[3 12 13]

IL-17A production characterises a distinct T cell phenotype, Th17, with pivotal proinflammatory functions in several autoimmune and chronic inflammatory disorders previously thought to be Th1-mediated.[14 15] Several cytokines participate in Th17 differentiation in humans, including IL-1β, IL-6 and TGFβ. IL-21 also contributes, particularly in the absence or paucity of IL-6, and IL-23 participates in the expansion/maintenance of the Th17 phenotype.[15] All these cytokines are abundantly expressed in GCA lesions.[3 9 16 17]

The aim of this study was to expand these initial observations by investigating the expression of IL-17A in temporal artery biopsies from a sizable series of patients with GCA to investigate the relationship between IL-17A expression and clinically relevant findings, such as the intensity of the systemic inflammatory response, relapses and response to therapy.

PATIENTS AND METHODS

Patients

The study group consisted of 57 patients with biopsy-proven GCA diagnosed between 1997 and 2006 at our institution (Hospital Clinic, Barcelona)

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(see online supplementary data S1 and figure S1 for selection criteria). All patients were prospectively evaluated and treated by the authors (GEF, JHR, SPG and MCC) with a predefined homogeneous glucocorticoid-tapering schedule.[6 10] Clinical data recorded at the time of diagnosis included disease symptoms, number of relapses, time to achieve a prednisone dose <10 mg/day, and time to complete prednisone discontinuation with no relapse within the following 6 months. The following baseline blood tests were recorded: erythrocyte sedimentation rate (ESR), haemoglobin, C-reactive protein (CRP) and haptoglobin concentrations, and platelet counts. Relapse was defined as reappearance of cranial symptoms, polymyalgia rheumatica, or systemic symptoms that could not be attributed to other conditions.[3 11] Isolated fluctuations on ESR or CRP were not considered relapses. Symptoms of relapse had to resolve by an increase of 10 mg above the previous effective dose.

Clinical data of the patients are displayed in online supplementary table S1. In 38 patients, the temporal artery was removed before starting treatment, and the remaining 19 had been treated with prednisone (60 mg/day) for a median of 7 days (range 2–12). In four patients, a contralateral temporal artery biopsy was performed after 1 year of treatment.[3]

Uninvolved temporal arteries from 19 patients (13 women and 6 men) with a median of 77 years (range 64–91) in whom GCA was considered but not confirmed, served as controls. All of them were subsequently diagnosed with other diseases (see online supplementary methods).

The study was approved by the Ethics Committee of Hospital Clínic (Barcelona), and patients signed an informed consent.

RNA Isolation and cDNA synthesis

Temporal artery biopsies were embedded in optimal cutting temperature (OCT, Sakura, The Netherlands), snap-frozen in liquid nitrogen and stored at −80°C until used. Sections consecutive to those that provided the histopathologic diagnosis were processed for RNA isolation using TRIzol Reagent (Invitrogen, Carlsbad, California, USA).

Total RNA (1 μg) was reverse-transcribed to cDNA using the Archive kit (Applied Biosystems, Foster City, California, USA), employing random hexamer priming.

Real-time quantitative PCR

cDNA was measured by quantitative real-time PCR using specific Pre-Developed TaqMan gene expression assays (see online supplementary methods) from Applied Biosystems as previously described.[9 16] All samples were normalised to the expression of the housekeeping gene, GUSb. The comparative CT method was used to assess relative gene expression.

Immunohistochemistry

To determine the topography of IL-17 expression, serial 4–6 μm sections were obtained from frozen temporal arteries of two of the patients and two controls. Sections were air-dried, fixed with cold acetone and permeabilised with 0.1% saponin. Endogenous peroxidase was blocked with H_2O_2, and the slides were incubated with the primary polyclonal antibody goat anti-human IL-17A (R&D Systems, Minneapolis, Minnesota, USA). Optimal dilutions were tested on frozen sections of human tonsils (positive control). Immunoglobulins obtained from the same species served as negative controls. Immunodetection was performed with a HRP-labeled polymer conjugated to a secondary antibody (EnVision Visualisation method, Dako, Glostrup, Denmark) using 3,3′-diaminobenzidine as a chromogen.

Enzyme-linked immunosorbent assay (ELISA) detection of IL-17A in tissue

Temporal artery protein extracts could be obtained from seven of the patients (five responders and two relapsers) from the phenolic phase during RNA extraction. IL-17A concentration was measured by immunoassay using Quantikine Human IL-17A from R&D Systems, according to the manufacturer's protocol.

Immunofluorescence staining and confocal microscopy

For qualitative assessment of cytokine distribution at the cellular level, immunofluorescence staining was performed in three additional temporal artery biopsies obtained from an active patient and from two patients who had received treatment with prednisone at 60 mg/day for 8 days at the time of the artery excision. A fragment of these biopsies was fixed in 4% paraformaldehyde with increasing concentrations of sucrose, frozen with OCT and stored at −80°C. Cryostat 10 μm sections were fixed with 4% paraformaldehyde, permeabilised with Triton 0.1% and immunostained with the primary and secondary antibodies detailed in online supplementary methods. Nuclei were stained with Hoechst dye (Molecular Probes, Life Technologies Ltd, Paisley, UK) at 1 : 1000. Slides were mounted in Mowiol 4-88 Reagent (Merck4Biosciences, Nottingham, UK) and examined using a laser scanning confocal Leica TCS *SP5* microscope (Leica Microsystems, Heildelberg, Germany). Images were processed with Leica Confocal software and Image J software (Wayne Rasband, Bethesda, Massachusetts, USA). The number of IL-17 positive and IL-17/FoxP3 double positive cells per field was counted in 10 fields per specimen at 200× magnification.

STATISTICAL ANALYSIS

Mann–Whitney test, Spearman's rho correlation coefficient, and Kaplan–Meier survival curves analysed with log-rank test were used for statistical analysis. With the exception of comparison between untreated (38) and treated (19) patients, statistical analysis was restricted to the cohort of 38 treatment-naïve patients.

RESULTS

IL-17A is upregulated in GCA lesions

IL-17A mRNA was significantly more abundant in temporal arteries from untreated patients than in control arteries (6.22±8.61 vs 2.50±3.90 relative units; p=0.016) (figure 1A). By contrast, plasma IL-17A was not detectable, or was slightly above the detection threshold in the majority of patients and controls (figure 1B).

As shown in figure 2, immunostaining revealed intense IL-17A protein expression by inflammatory cells in GCA lesions. Giant-cells immunostained negative for IL-17A. Interestingly, although control arteries had some constitutive IL-17A mRNA expression, control arteries immunostained negative for IL-17A.

Lack of correlation between IL-17A expression and systemic inflammatory findings

Given the known proinflammatory functions of IL-17A, we explored whether IL-17A mRNA expression correlated with the intensity of the systemic inflammatory response. No differences in IL-17A mRNA expression were observed between patients with strong versus weak systemic inflammatory response at diagnosis (see online supplementary table S2). No significant correlation was found between IL-17A mRNA expression and acute-phase reactants including ESR (r=0.0886, p=0.60), haemoglobin (r=−0.03563, p=0.82) or CRP concentrations (r=0.1495, p=0.45).

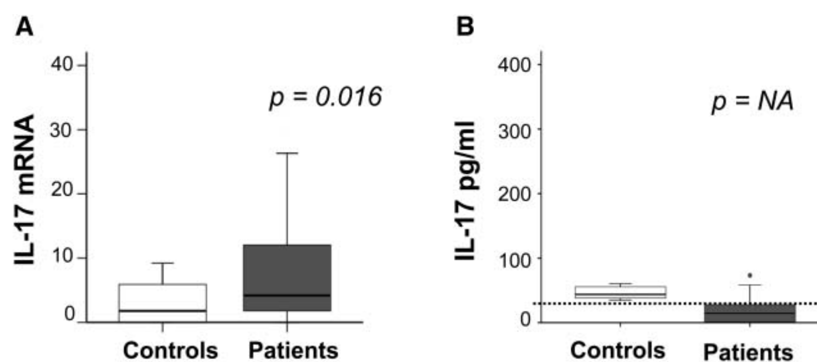

Figure 1 IL-17A concentrations in temporal artery biopsies and serum from patients with giant-cell arteritis (GCA). (A) IL-17A mRNA expression (relative units) in temporal arteries from 38 treatment-naïve patients with GCA and 19 controls. (B) IL-17A concentrations in sera from 33 GCA treatment-naïve patients and seven controls. Dotted line indicates detection threshold. NA, not applicable.

Correlation between IL-17A and other proinflammatory cytokines in GCA lesions

We next tested the potential correlation between IL-17A expression in treatment-naïve samples and the expression of cytokines known to participate in Th17 differentiation (IL-6, TGFβ) or in the maintenance and expansion of the Th17 phenotype (IL23p19).[15] IL-17A mRNA concentrations in untreated GCA arteries significantly correlated with IL-6 (r=0.363, p=0.025) and with IL23p19 mRNA (r=0.397, p=0.008). These findings suggest coordinated regulation in accordance with the role for

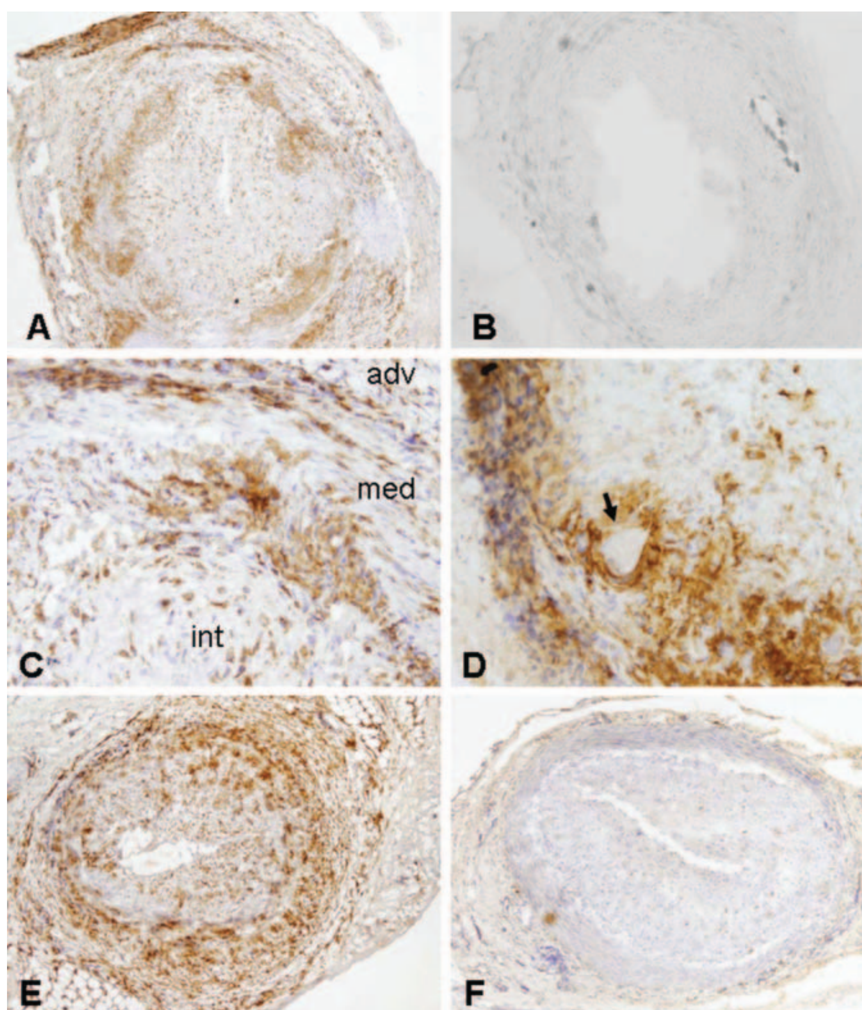

Figure 2 Immunohistochemical detection of IL-17A expression in temporal artery lesions. (A) IL-17 expression in a giant-cell arteritis (GCA)-involved temporal artery section. (B) Lack of IL-17A immunostaining in a temporal artery from a control individual. (C) Closeup view of a GCA-involved artery where distinct IL-17A+cells can be observed in all arterial layers: adventitia (adv), media (med) and intima (int). (D) Giant cells (arrow) do not express IL-17A. (E) IL-17A expression in the temporal artery from a treatment-naive patient compared with IL-17A expression in the contralateral biopsy obtained after being treated with prednisone for 1 year (F).

IL-6 and IL-23 in inducing and maintaining the Th17 phenotype, respectively. No correlation was found between IL-17A and TGFβ (r=−0.037, p=0.848).

IL-17A concentration is decreased in temporal arteries from treated GCA patients

IL-17A mRNA concentrations in temporal artery biopsies from the 19 treated patients were significantly lower than those found in the 38 treatment-naïve patients (1.74±2.48 relative units vs 6.22±8.61; p=0.017) (figure 3A). In four patients who underwent a second temporal artery biopsy after being treated for 1 year, immunohistochemical detection of IL-17 protein, which was intense at the time of diagnosis (figure 2E), was limited to scattered remaining inflammatory cells in the second specimen (figure 2F).

IL-17A expression and long-term response to glucocorticoid treatment

Surprisingly, IL-17A mRNA concentrations in lesions tended to be higher in patients who achieved sustained remission or experienced just one disease flare compared with those who experienced multiple relapses (7.46±9.73 vs 3.19±3.70 relative units; p=0.058) (figure 3B). The same trend was observed in IL-17A protein concentration which tended to be higher in non-relapsing patients (19.30±29.96 vs 0.68±0.63 pg/ml) (figure 3C). Accordingly, IL-17A mRNA levels tended to be higher in patients able to completely discontinue prednisone at 3 years (5.17±8.11 vs 0.29±0.46, p=0.06) (figure 3D).

Similarly, untreated patients with high IL-17A mRNA content (above 75% percentile) in their arteries achieved a maintenance prednisone dose <10 mg/day significantly sooner than patients with lower IL-17A mRNA values (median 25 vs 44 weeks, p=0.0079) (figure 4A). Likewise, patients with elevated IL-17A mRNA concentrations were able to completely withdraw prednisone earlier than patients with lower IL-17 mRNA levels (p=0.028) (figure 4B).

Plasticity between regulatory T lymphocytes (Tregs) and Th17 lineages may contribute to the association between increased IL-17A expression and response to therapy

We next investigated the expression of transcription factors RORα and RORc, master regulators of Th17 lineage. RORα and RORc were expressed at similar levels in GCA and control arteries; their expression tended to correlate (r=0.33, p=0.053), and were not influenced by treatment (figure 5). No correlation was found between IL-17A and RORα (r=−0.20, p=0.36) or RORc expression (r=−0.11, p=0.596). Taken together, these findings suggest that RORα and RORc may be expressed by cells other than Th17 lymphocytes, and that Th17 cells may not be the only producers of IL-17A in GCA. By contrast,

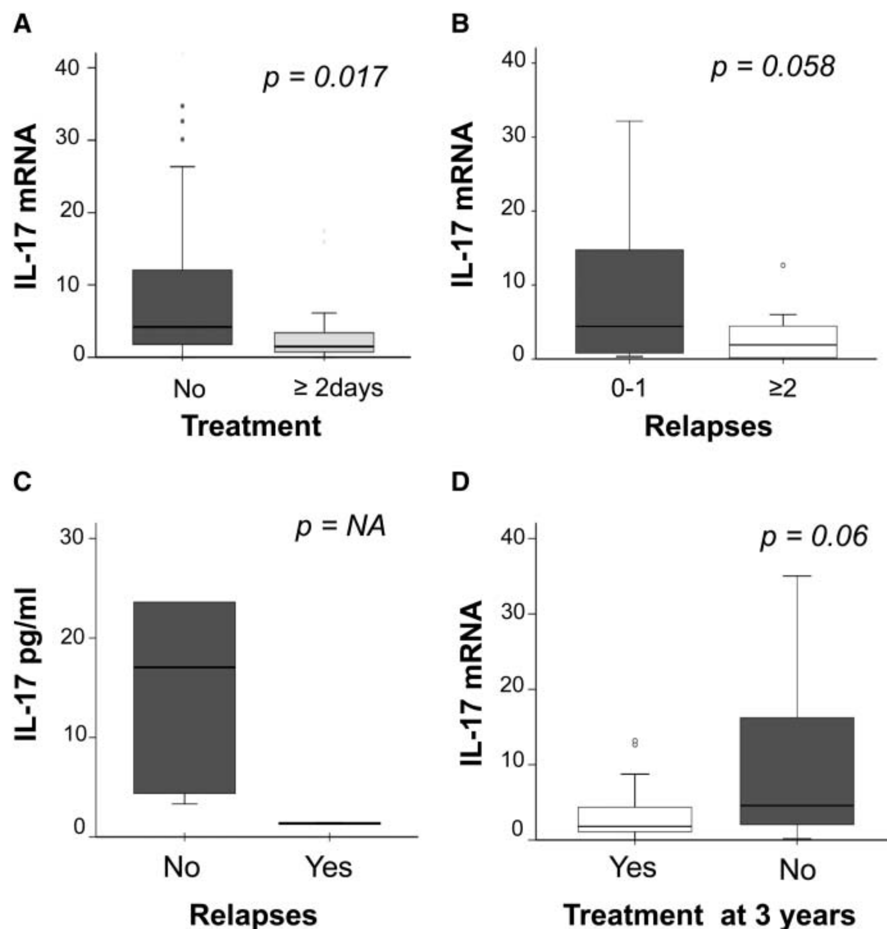

Figure 3 Relationship between IL-17A and response to therapy. (A) IL-17A mRNA expression (relative units) in temporal arteries from 38 treatment-naïve and 19 prednisone treated giant-cell arteritis (GCA) patients. (B) IL-17A mRNA content in temporal arteries from 38 treatment-naïve GCA patients according to relapses. (C) IL-17 protein content in temporal arteries from seven GCA patients according to relapses (patient numbers are too small for reliable statistics). (D) IL-17A mRNA concentration in initial temporal artery biopsies from patients still requiring prednisone compared with that obtained from patients in sustained treatment-free remission, 3 years after diagnosis. NA: not applicable

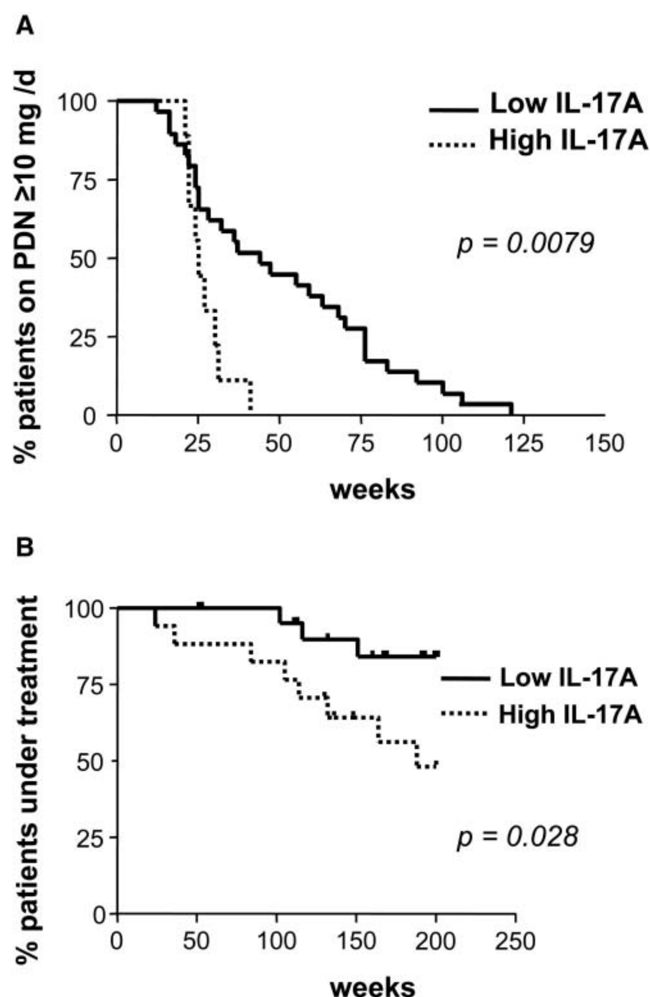

Figure 4 Association between IL-17A expression in giant-cell arteritis (GCA) lesions and treatment requirements. (A) Percentage of patients requiring >10 mg prednisone/day to maintain remission, according to the intensity of IL-17A expression in vascular lesions. High IL-17A refers to mRNA concentration within the 75th percentile, and low IL-17A below the 75th percentile. (B) Percentage of patients requiring prednisone treatment over time, according to the intensity of IL-17A expression in lesions as in A.

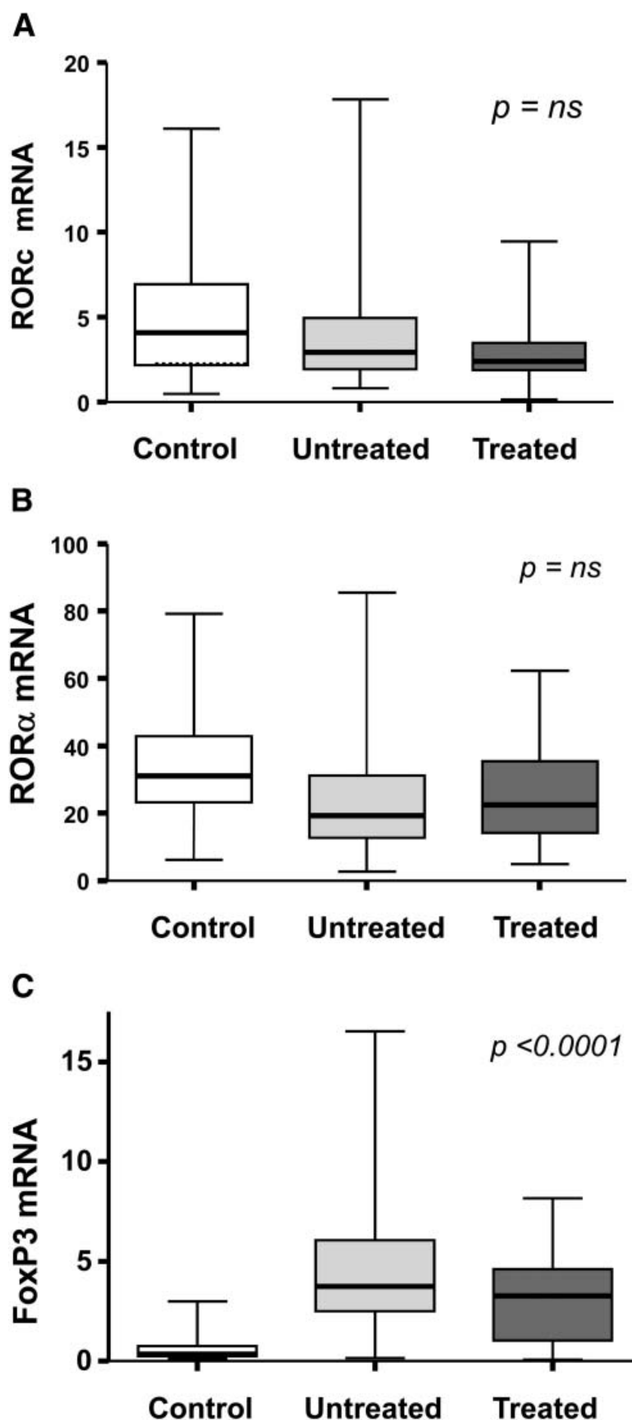

Figure 5 Expression of master regulators of Th17 (RORc and RORα) and Treg differentiation (FoxP3) in temporal artery lesions. (A) Expression of RORc (relative units) in 19 control arteries, 38 treatment-naïve patients and 19 prednisone-treated patients. Expression of RORα (B) and FoxP3 (C) in the same patient subsets.

FoxP3 was significantly upregulated in GCA lesions and significantly decreased with treatment (figure 5).

It has been recently shown that induced regulatory Tregs may transiently lose their suppressive capacity and produce IL-17A in an inflammatory microenvironment.[18–21] Confocal microscopy of temporal artery sections showed that a subset of Tregs (identified as FoxP3-positive cells) contributed, indeed, to IL-17A production in active GCA lesions, and that these cells may be reduced in specimens from treated patients (figure 6). Initial IL-17A expression may then reflect the functional activity of Th17 cells and also the contribution of IL-17A-producing FoxP3 cells with their potential to recover suppressive activity when inflammatory stimuli decrease in the microenvironment.

DISCUSSION

We found that IL-17A expression is prominent in GCA lesions. Consistently, IL-17A expression correlated with expression of IL-6, a cytokine involved in Th17 differentiation, and particularly, with expression of the IL-23 subunit IL-23p19, a cytokine involved in the maintenance and expansion of the Th17 phenotype.[15 21] The lack of correlation with TGFβ, also required for Th17 differentiation, as well as the significant correlation with IL-23, may be related to the fact that temporal artery biopsies are rarely performed during early events and are usually obtained when the inflammatory process is fully developed. At the stages when temporal arteries are obtained, TGFβ may play additional roles in vascular remodelling.[3 22] Our findings, obtained in a sizable series of patients with prospectively recorded

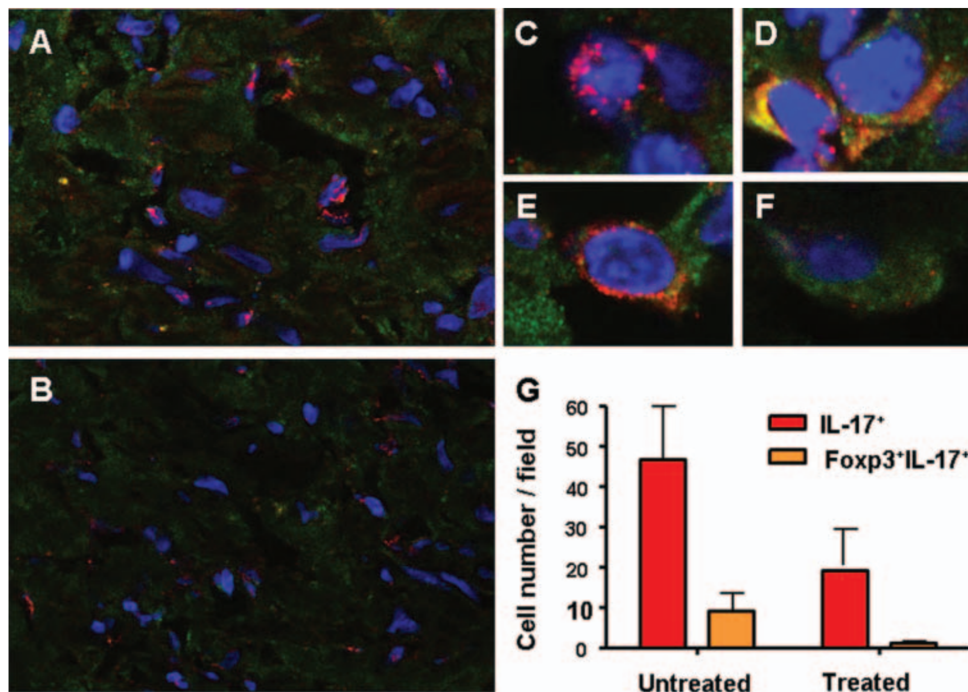

Figure 6 Expression of IL-17A and Foxp3 cells in temporal artery lesions from patients with giant-cell arteritis. (A) IL-17A expression (red colour) in a patient with active giant-cell arteritis (GCA). (B) IL-17A expression in a patient treated with 60 mg prednisone/day for 1 week. (C) Higher magnification of an inflammatory cell expressing only IL-17A (red, merged). (D) A magnified inflammatory cell intensively expressing both IL-17A and FoxP3 (orange, merged). (E) A magnified inflammatory cell from a treated patient with lower coexpression (orange, merged). (F) A magnified cell from a treated patient expressing primarily Foxp3 (green, merged). (G) IL-17A^{+} and IL-17A^{+}FoxP3^{+} double positive cells (mean±SD) in 10 different fields (200×) per specimen containing similar number of nuclei in lesions from an active patient versus two treated patients. $p<0.05$ (due to the small number of cases, statistics are only indicative).

clinical and follow-up data confirm preliminary observations performed in small series of patients, and support the participation of Th17 mechanisms in the pathogenesis of GCA.[3 12 13]

IL-17A, typically, but not exclusively, produced by Th17 cells, plays a central role in the development of tissue inflammation in a variety of experimental models and human diseases.[15 21] IL-17A exerts strong proinflammatory functions on a variety of cells, including endothelial cells, by inducing expression of classical proinflammatory cytokines IL-1β, IL-6 and TNFα, endothelial adhesion molecules, chemokines and metalloproteinases.[14 15 23] All these molecules are strongly expressed in GCA, and are thought to participate in proinflammatory amplification cascades.[2 9 16 23–25] The remarkable expression of IL-17A found in GCA supports a prominent role in vascular inflammation. Supporting this concept, deletion of IRF-4-binding protein leading to sustained activation of IRF-4, a transcription factor involved in Th17 differentiation, results in large-vessel vasculitis in mice.[26]

While IL-17A was remarkably upregulated in lesions, it was barely detectable in serum. Consistent with this finding, we could not find a relationship between IL-17A expression and systemic symptoms or acute-phase proteins, suggesting that IL-17A functions are predominantly exerted locally at the vascular lesions. IL-17A strongly amplifies the expression of other proinflammatory cytokines (IL-6, TNFα) which, in turn, are effectively released into the bloodstream and do correlate with the intensity of the systemic inflammatory response in GCA.[6 10]

As others and we have previously shown in small series,[3 12 13] IL-17A mRNA and IL-17A-expressing cells were dramatically reduced in specimens obtained from treated patients. This finding supports the prominent proinflammatory role of IL-17A in GCA, and suggests that downregulation of IL-17A may at least partially account for the dramatic effect of high-dose glucocorticoids in ameliorating disease-related symptoms, and in substantially decreasing vascular inflammation after long-term treatment.[3]

The most intriguing finding of this study was the association between strong initial expression of IL-17A and response to therapy. Patients with strong IL-17A expression tended to experience less relapses and were able to reduce and completely discontinue prednisone earlier than patients with weaker IL-17A expression. This may indicate that patients who develop a predominantly Th17 response are more sensitive to glucocorticoid treatment.

Recent studies have shown that, on one hand, Th17 cells are not the only producers of IL-17A[15 27] and that, on the other hand, there is remarkable plasticity among T cell lineages depending on the conditions of the microenvironment.[18–20] Particularly interesting is the fact that induced Tregs, which role was initially identified as immunosuppressive, are able to produce IL-17A when exposed to an inflammatory milieu.

We show that both RORα and RORc, markers and master regulators of Th17 are equally expressed in GCA and control arteries. Constitutive expression of RORα and RORc by non-inflamed arteries may indicate an important, previously unknown role of these factors in vascular biology. By contrast, FoxP3 was upregulated in GCA and decreased with treatment, consistent with our early findings demonstrating expression of CD25+ lymphocytes in lesions and their reduction upon treatment.[4] The lack of correlation between RORα/RORc and IL-17A expression also suggests that other cells, in addition to Th17 cells, may contribute to IL-17A production in GCA. FoxP3-positive cells contributed,

indeed, to IL-17A production. These preliminary findings suggest that induced Tregs may functionally evolve over time, and may have a role in limiting disease activity in GCA. Expanding Tregs by administration of low-dose IL-2 has been recently shown to ameliorate cryoglobulinemic-related vascular inflammation in humans.[28] Moreover, it has recently become apparent that not all Th17 cells are equally pathogenic, and that abundance of TGFβ expression may switch Th17cells to the alternative non-pathogenic phenotype.[29] We have recently shown that prednisone treatment increases TGFβ expression,[3] which may also limit the pathogenicity of Th17 cells.

In conclusion, strong IL-17A expression in involved arteries is a biomarker and predictor of response to therapy. Our preliminary data suggests that different cell lineages may contribute to IL-17A expression in GCA. However, the observational nature of this and other existing studies addressing IL-17A expression in GCA does not allow to draw strong mechanistic conclusions about the specific role of IL-17A and Th17 cells in GCA. Our findings suggest complex interplay among T cell lineages in delineating disease fate and response to therapy that deserves further investigation.

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Contributors GEF, MCC: designed the study and wrote the paper; GEF, MCB, EPR, EL, MS: performed experimental work; JHR, SPG, AGM, JMG, MCC: collected and analysed clinical data; MUR: designed the study, analysed data, provided important inputs to the writing of the manuscript and approved the final version. All the authors repeatedly discussed the results of the paper, made suggestions for improvement and contributed to generate further data. All the authors read and reviewed the drafts and approved the final version of the manuscript.

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Ethics approval Internal Review Board, Hospital Clínic.

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its inability to mediate complement-dependent cytotoxicity. Using rabbit serum and positively selected B cells, Kamburova et al show that RTX can induce complement-dependent killing. First, we are uncertain as to the relevance of this approach to human biology; we saw no loss of B cells when RTX was added to whole blood for 30 minutes (n = 32) or to PBMCs cultured in 10% normal human serum for 48 hours. Second, we appreciate the notion that detection of different CD19 epitopes might account for their findings but think this is unlikely as our data (as seen in Figure 5 of our article) demonstrate transfer of CD19 to the surface of monocytes and neutrophils. Additionally, investigators at other laboratories have also identified RTX-mediated loss of CD19 using different antibodies (Beum PV, Kennedy AD, Williams ME, Lindofer MA, Taylor RP. The shaving reaction: rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes. J Immunol 2006;176:2600–9).

Both Kamburova et al and Mei et al state that they saw a reduction in CD19 expression at >2 months in different patient populations. We do not disagree: RTX causes extensive peripheral B cell depletion. We stand by the observation that trogocytosis occurs in vivo as has been reported (Williams MD, Densmore JJ, Pawluczkowycz AW, Beum PV, Kennedy AD, Lindorfer MA, et al. Thrice-weekly low-dose rituximab decreases CD20 loss via shaving and promotes enhanced targeting in chronic lymphocytic leukemia. J Immunol 2006;177:7435–43) and which we have demonstrated in patients with RA (Figure 1). Finally, the fate of B cells subjected to trogocytosis (and therefore deficient in CD20 and CD19 expression) over time remains unknown.

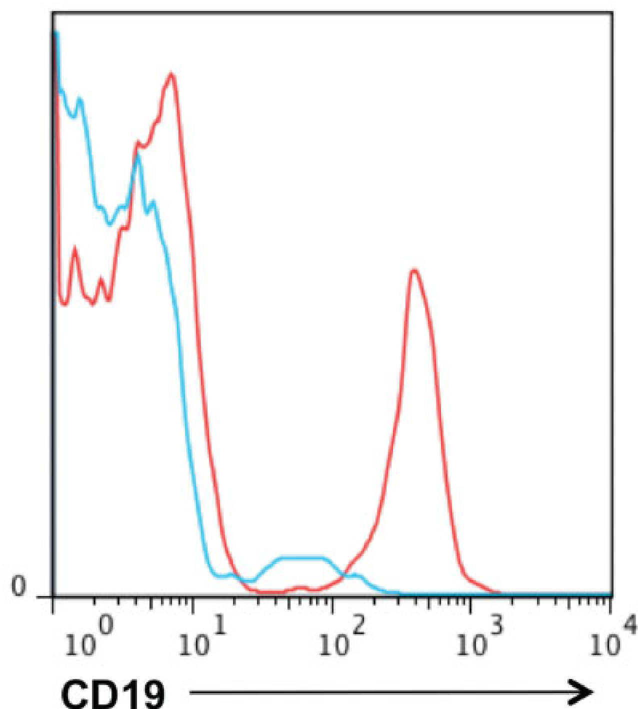

Figure 1. CD19 levels in the lymphocyte population of PBMCs isolated from whole blood drawn immediately preceding (red) and immediately following (blue) completion of a 1,000 mg intravenous RTX infusion in a patient with rheumatoid arthritis. Results show a significant drop in CD19+ cells, as well as a leftward shift in the mean fluorescence intensity of CD19, indicating emergence of a subpopulation of B cells that have undergone trogocytosis due to the binding of RTX to CD20.

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Functionally relevant Treg cells are present in giant cell arteritis lesions: comment on the article by Samson et al

To the Editor:

We read with great interest the recently published article by Samson et al (1) on the distribution of circulating CD4+ T lymphocyte subsets (Th1, Th17, CD161+ cells, which are common precursors of Th1, Th17, and Treg cells) in a cohort of 34 patients with active giant cell arteritis (GCA) or polymyalgia rheumatica and 31 healthy controls. Among other interesting and relevant findings, the authors observed an increase in circulating Th17 cells and common Th1/Th17 (CD4+CD161+) precursors, and a decrease in the number, but not function, of Treg cells in peripheral blood. The authors also investigated the presence of lymphocyte subsets in temporal artery lesions from 7 patients and concluded that there were no Treg cells (identified as FoxP3+ cells) in the inflammatory infiltrates of patients with GCA.

In a recent study, we found that FoxP3+ cells, although not predominant, are indeed present in GCA lesions (2). Using quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR), we also found significantly increased expression of FoxP3 messenger RNA (mRNA) in the temporal arteries of 38 untreated GCA patients compared to 19 controls, and demonstrated that expression of FoxP3 mRNA was significantly reduced in 19 specimens from glucocorticoid-treated patients with GCA (2). This accords with a previous study demonstrating the presence of a nonpredominant subset of CD25+ cells in GCA lesions and the decrease in the number of those cells after glucocorticoid treatment (3). Moreover, Treg cells present in GCA lesions are functionally relevant; they may express interleukin-17 (IL-17) in patients with active disease, as demonstrated by immunofluorescence and confocal microscopy, and IL-17 expression by FoxP3+ cells decreases with treatment (2). These observations highlight the functional plasticity of CD4+ T cells depending on inflammatory stimuli and cytokine predominance in the microenvironment (4,5), as well as the relevance of cells expressing FoxP3 in GCA.

The apparently opposite results described by Samson et al (1) may be due to technical issues. Transcription factors such as FoxP3 are usually expressed at low levels, and immunohistochemistry may not be sensitive enough to detect them in a nonpredominant cell subset. More sensitive techniques, such as real-time RT-PCR or immunofluorescence with confocal microscopy, may be needed to detect FoxP3-expressing cells in GCA-affected arteries. Furthermore, more samples need to be examined, given the wide variability in the density of inflammatory infiltrates in GCA.

A recent study showed that treatment with low-dose IL-2, which results in an increase in the number of Treg cells, reduces vascular inflammation in cryoglobulinemic vasculitis (5), suggesting that Treg cells may be a target for therapeutic manipulation in inflammatory diseases of blood vessels. Therefore, caution is needed before concluding that a potentially important functional T cell subset or biomarker is absent when using techniques with limited sensitivity and/or studying a small number of samples.

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Reply

To the Editor:

We thank Dr. Corbera-Bellalta and colleagues for their interesting comments regarding our work on the implication of Th1, Th17, and Treg cells in GCA and polymyalgia rheumatica (PMR). In our study, we assessed Treg cells in peripheral blood and in temporal artery biopsy samples. We demonstrated that circulating Treg cells were functionally not altered but that percentages of the cells were decreased in the blood of patients with GCA and patients with PMR in comparison with healthy controls. In temporal artery biopsy samples from GCA patients, Th1 cells and Th17 cells, identified by interferon-γ and IL-17 staining, respectively, massively infiltrated the entire arterial wall (Figures 1A and B), whereas FoxP3+ cells were detected at a very low level, especially at the adventitia–media junction, where the inflammatory infiltrate was the most prominent (Figures 1C and D).

We do agree with Corbera-Bellata et al that immunohistochemistry is not the most sensitive technique to detect FoxP3+ T cells. However, in our study, the same technique was used for detection of Treg cells, Th1 cells, and Th17 cells. Even if the number of FoxP3+ cells infiltrating the temporal artery may have been underestimated in our study, there is no doubt that there is a strong imbalance between Th1, Th17, and Treg cells. Furthermore, we demonstrated that a 1:2 ratio of Treg cells to effector T cells was required in vitro to observe efficient suppressive activity. Consequently, even if it is true that we did not detect all FoxP3+ cells because of a lack of sensitivity, these results suggest that their number was not sufficient to suppress Th1 and Th17 immune responses in the artery.

In a recent study, Corbera-Bellata and colleagues interestingly detected FoxP3+IL-17+ T cells in the arterial wall of GCA patients, demonstrating the functional plasticity of T cells regulated by cytokines produced in their microenvironment (1). However, human CD4+ T cells expressing FoxP3+ cannot be systematically considered to be suppressive cells. FoxP3 can indeed be induced in naive CD4+ T cells activated in vitro (2). FoxP3+CD4+ T cell subsets are delineated depending on the

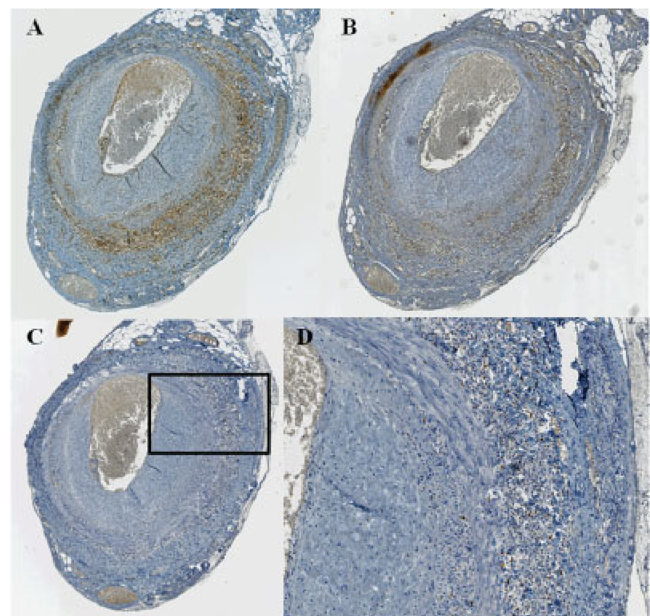

Figure 1. Analysis of one representative temporal artery biopsy sample from a patient with giant cell arteritis. **A,** Staining with anti–interferon-γ. **B,** Staining with anti–interleukin-17. **C** and **D,** Staining with anti-FoxP3. Boxed area in **C** is shown at higher magnification in **D**. Original magnification $\times$ 40 in **A–C**; $\times$ 120 in **D**.

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CONCISE REPORT

Positron emission tomography assessment of large vessel inflammation in patients with newly diagnosed, biopsy-proven giant cell arteritis: a prospective, case–control study

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ABSTRACT

Background Positron emission tomography (PET) scan is emerging as a promising imaging technique to detect large-vessel inflammation in giant cell arteritis (GCA). However, the lack of a standardised definition of arteritis based on ^{18}fluorodeoxyglucose (FDG) uptake is an important limitation to the use of PET scan for diagnostic purposes.

Objective To prospectively assess the intensity and distribution of FDG uptake at different vascular territories in patients with newly diagnosed GCA compared with controls.

Methods 32 consecutive, biopsy-proven, GCA patients treated with glucocorticoids for ≤3 days were included. The control group consisted of 20 individuals, who underwent PET/CT for cancer staging. Maximal standardised uptake value (SUVm) was calculated at four aortic segments, supraaortic branches and iliac-femoral territory. Sensitivity and specificity was calculated by receiver–operator characteristic curves (ROC) analysis.

Results Mean SUVm was significantly higher in patients than in controls in all vessels explored and correlated with acute-phase reactants and serum IL-6. Mean of the SUVm at all the vascular territories had an area under the curve (AUC) of 0.830, and a cut-off of 1.89 yielded a sensitivity of 80% and a specificity of 79% for GCA diagnosis. There were no significant differences in AUC among the vascular beds examined.

Conclusions FDG uptake by large vessels has a substantial sensitivity and specificity for GCA diagnosis.

INTRODUCTION

Temporal artery biopsy is the gold standard for the diagnosis of giant cell arteritis (GCA) due to the tropism of GCA for the epicranial arteries.[1 2] With a few exceptions,[3] histopathological demonstration of temporal artery inflammation provides the most definitive evidence of GCA. Doppler ultrasonography (DUS) of temporal arteries has emerged as a useful alternative tool in centres where biopsy is not easily available.[1]

The diagnosis of GCA may be also supported by demonstrating extracranial artery involvement by imaging. Over the past recent years, positron emission tomography/CT (PET/CT), CT angiography, magnetic resonance imaging (MRI) angiography and DUS have revealed that extracranial involvement in GCA is more frequent than previously anticipated, occurring in 30–74% of patients.[4–7]

PET detection of large-vessel involvement in patients with fever of unknown origin, unexplained constitutional symptoms or apparently isolated polymyalgia rheumatica (PMR) has emphasised its diagnostic potential.[8 9] A limitation of PET as a diagnostic tool is the lack of a standardised definition of vascular inflammation based on the intensity of ^{18}fluorodeoxyglucose (FDG) uptake. While visual assessment of intensively positive cases may be clear, there is no consensus about the minimal intensity of FDG uptake necessary to define vascular inflammation. Conversely, atherosclerosis and ageing may increase vascular FDG uptake, potentially leading to vasculitis overdiagnosis.[10]

In this study, we measured FDG uptake by different vascular territories in a cohort of newly diagnosed patients and controls and performed receiver–operator characteristic curves (ROC) analysis to determine sensitivity and specificity of FDG uptake to detect inflammation at different vascular sites. As a secondary endpoint, we analysed potential correlation between FDG uptake and inflammatory biomarkers.

MATERIALS AND METHODS

Patients

Between November 2006 and March 2011, all patients diagnosed with biopsy-proven GCA[2] at our institution were assessed for potential participation in the study. Patients who had received glucocorticoid treatment for >3 days were excluded. Clinical and laboratory data recorded are detailed in the online supplementary methods. The study was approved by the ethics committee (Hospital Clínic, Barcelona).

The control group included 20 patients with no chronic inflammatory diseases, matched for gender, age and cardiovascular risk factors (CVRF), consecutively selected among patients who underwent PET/CT during the same timeframe for early lung cancer staging.

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PET/CT protocol

PET scans were performed using a hybrid PET/CT (Biograph, Siemens) with an ECAT EXACT HR+BGO PET and a helicoidal CT scanner (Somatom, Emotion). Patients fasted 4 h before injection of 370 MBq of 18F-FDG. Whole-body PET data were acquired 60 min after in three-dimensional mode and for 5 min per bed position. PET images were reconstructed both with and without CT data for attenuation correction. A region of interest (ROI) in 3-D around the vessel was placed manually in transaxial, sagittal and coronal slices. The standardised uptake value (SUV) was calculated based on the measured activity, decay-corrected injected dose and patient body weight. SUVm=maximal activity (ROI) (mBq/mL)/injected dose (mBq)/weight (g).

Four aortic segments (ascending thoracic aorta, aortic arch, descending thoracic aorta and abdominal aorta) and the main tributaries—carotid, subclavian, axillary, iliac and femoral arteries (each bilaterally)—were evaluated. The control group was subjected to the same PET/CT protocol. Assessment of PET data was carried out by two nuclear medicine specialists (FL and MD), who were blinded to clinical and pathological findings. However, unequivocal masked evaluation could not be guaranteed due to the controls' disease.

Statistical analysis

ROC were applied to each vascular territory to calculate sensitivity and specificity. Area under the curve (AUC) comparison was performed by Hanley and McNeil analysis. Cut-offs with best sensitivity and specificity were selected. Mann–Whitney U test or Student t test, when applicable, were used for quantitative data. Correlations were calculated using Pearson's or Spearman's test. Statistical significance was defined as $p<0.05$. Calculations were performed with the IBM SPSS Statistics (V.20.0, Armonk, New York, USA).

RESULTS

Clinical and laboratory findings of the GCA cohort

Seventy-one GCA patients were diagnosed during the recruitment period. Eight patients refused participation, and 31 had received glucocorticoid treatment for >3 days. The remaining 32 were included. Seventeen of them had been treated for ≤3 days at the time of imaging. Treatment consisted of oral prednisone at 1 mg/kg/day. Two patients received 250 mg intravenous methylprednisolone pulses (1 and 7 pulses, respectively) due to severe cranial ischaemic symptoms.

Online supplementary table S1 shows the clinical and laboratory data of the study group. There were no relevant differences in age, gender or CVRF between patients and controls (see online supplementary table S2).

FDG uptake cut-off for GCA diagnosis

SUVm at any vascular territory explored was significantly higher in GCA patients than in controls (table 1). ROC curves and AUCs are displayed in figure 1 and table 1, respectively. Mean of the SUVm observed at all the vascular territories had an AUC of 0.830 (0.715–0.946). A cut-off of 1.89 had a sensitivity of 80% and a specificity of 79%. Mean of the SUVm at supraaortic vessels showed the highest AUC (0.832). In this site, a cut-off of 1.70 achieved a sensitivity and specificity of 81 and 79%, respectively, for the diagnosis of GCA (95% CI 0.720 to 0.946). FDG uptake at the aorta showed lower AUC (0.738), with a sensitivity and specificity of 90 and 42, respectively, using a cut-off of 2.25, and a sensitivity of 58%, specificity of 90% with a cut-off of 2.65 (95% CI 0.598 to 0.881). However, differences in AUCs among territories did not reach statistical significance.

Vascular/liver uptake ratios were also significantly higher in patients than in controls at the right axillary and carotid arteries,

Table 1 SUVm and AUC at each vascular bed assessed

Territory	GCA patients (mean±SD)	Controls (mean±SD)	p Value	AUC (95% CI)
Ascending aorta	2.63±0.57	2.17±0.26	<0.001	0.778 (0.651 to 0.904)
Aortic arch	2.61±0.50	2.23±0.31	0.002	0.756 (0.621 to 0.891)
Descending thoracic aorta	2.78±0.65	2.39±0.33	0.007	0.739 (0.598 to 0.881)
Abdominal aorta	2.97±0.60	2.56±0.39	0.005	0.748 (0.608 to 0.888)
Right subclavian artery	2.46±0.54	2.14±0.40	0.030	0.763 (0.607 to 0.889)
Left subclavian artery	2.26±0.56	1.89±0.28	0.003	0.764 (0.610 to 0.891)
Right carotid artery	2.33±0.52	1.83±0.25	<0.001	0.812 (0.695 to 0.930)
Left carotid artery	2.32±0.51	1.97±0.30	0.004	0.733 (0.594 to 0.872)
Right axillary artery	1.21±0.31	0.88±0.17	<0.001	0.830 (0.725 to 0.940)
Left axillary artery	1.09±0.34	0.88±0.18	0.001	0.780 (0.627 to 0.886)
Right iliac artery	2.41±0.67	2.01±0.38	0.009	0.747 (0.606 to 0.888)
Left iliac artery	2.46±0.47	2.00±0.41	0.002	0.767 (0.628 to 0.905)
Right femoral artery	1.68±0.39	1.24±0.22	<0.001	0.817 (0.715 to 0.928)
Left femoral artery	1.50±0.37	1.14±0.18	<0.001	0.801 (0.679 to 0.922)
All territories*	2.15±0.37	1.79±0.17	<0.001	0.830 (0.715 to 0.946)
Aorta**	2.75±0.54	2.34±0.23	0.001	0.738 (0.612 to 0.874)
Supraaortic branches**	1.95±0.35	1.59±0.15	<0.001	0.832 (0.732 to 0.968)
Iliofemoral territory**	1.97±0.36	1.62±0.23	<0.001	0.802 (0.679 to 0.925)
Liver	2.76±0.57	2.52±0.42	0.119	0.635 (0.480 to 0.790)

Removal of the two patients who had received intravenous methylprednisolone pulses at the time of PET performance did not significantly modify the results (data not shown).
Differences in AUCs among different vascular territories did not reach statistical significance.
AUC, area under the curve; GCA, giant cell arteritis; PET, positron emission tomography.
*Values represent the mean of the SUVm observed at all the vascular beds assessed.
**Aorta, Supraaortic branches and Iliofemoral territory represents the mean of the SUVm observed at the different vessels of these areas.

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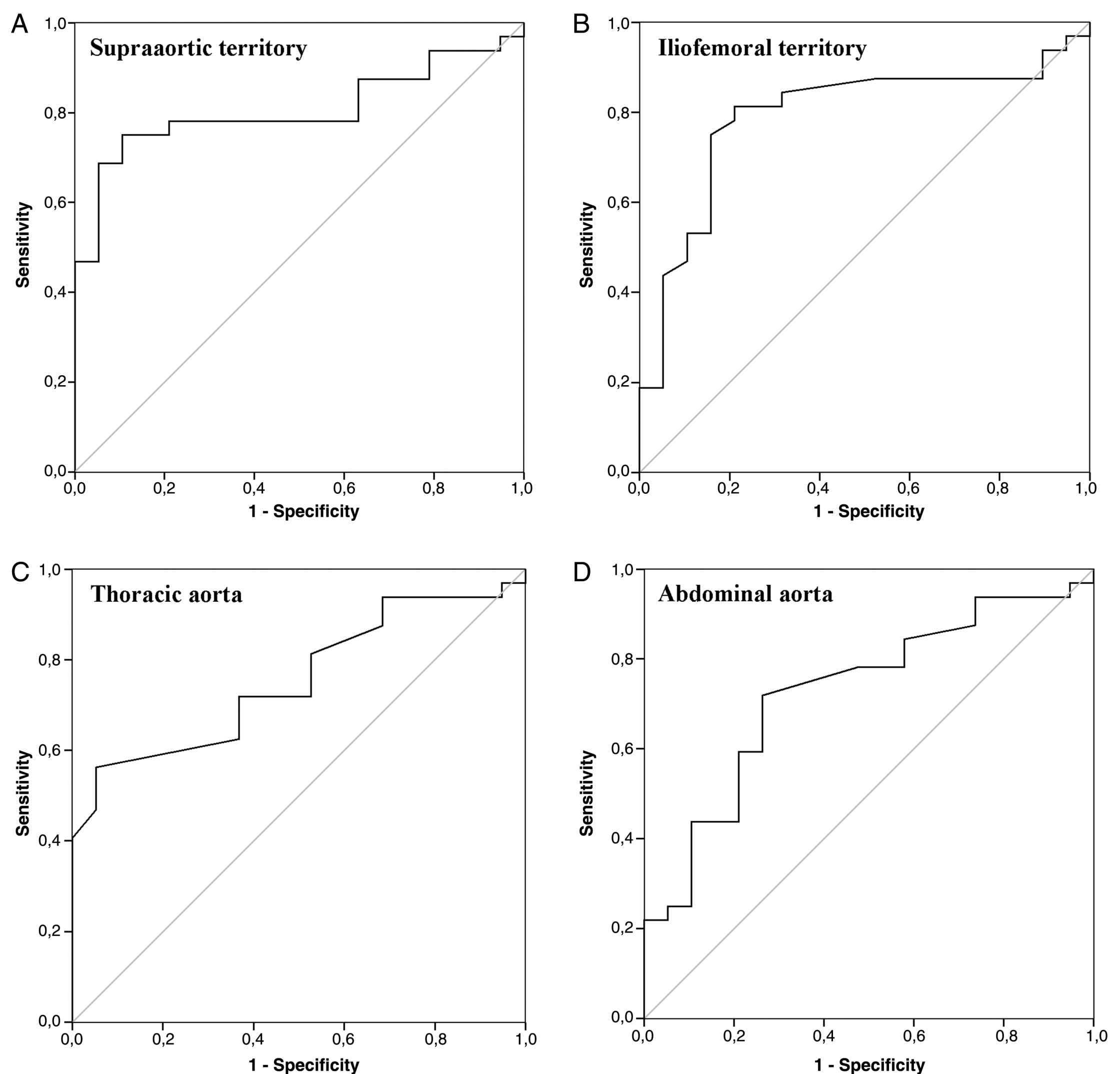

Figure 1 Receiver–operator characteristic curves of standardised uptake value at different vascular regions.

but the overall discriminatory performance was much lower (see online supplementary table S3).

Relationship between FDG uptake and clinical and laboratory findings

Patients with cranial symptoms presented significantly higher values of maximal and mean SUVm (combined average of all vascular territories) than patients lacking cranial manifestations. No relationship between the intensity of FDG uptake and other clinical findings was observed (table 2). No differences in maximal or mean SUVm were observed between treatment-naïve patients and those who had received glucocorticoids. The maximal and mean SUVm correlated with acute-phase reactants and serum IL-6 concentrations (table 2).

DISCUSSION

The present prospective study, performed in an unselected patient cohort with unequivocal GCA, shows that FDG uptake is significantly stronger in patients than in controls in all vascular territories tested, confirming the diagnostic potential of PET/CT.[4 8 9 11] PET/CT allows rapid, reproducible and broad vascular evaluation. Nevertheless, there is no standardised definition of vasculitis based on an objective FDG uptake measure, and strategies employed to establish a PET-based diagnosis of GCA has been heterogeneous. Most studies have used qualitative visual assessment or a semiquantitative score using liver uptake as a reference. Visual scoring has a remarkable investigator dependency and interobserver variability. Liver uptake is influenced by individual metabolic activity, glucocorticoid treatment and the time lapse between injection and scanning.[12]

We tried to overcome this limitation by objectively quantifying FDG uptake by different vascular beds in patients and controls and performing ROC analysis to determine the optimal cut-off for GCA diagnosis at different vascular territories. FDG uptake by supraaortic branches had the highest AUC, in accordance with a pioneer study showing that supraaortic branches were the most frequently involved when assessed by PET.[4] In this area, an FDG uptake cut-off value of 1.70 had the best sensitivity and specificity. A similar value, in the same territory but with lower performance (AUC=0.72), was reported in a retrospective study of 17 patients with GCA and 3 Takayasu arteritis patients.[13] This observation may be useful to differentiate GCA from other inflammatory aortic diseases that may produce systemic complaints and active aortic FDG uptake, including idiopathic aortitis, periaortitis, IgG4 disease and severe atherosclerosis.[10 14–17] This is crucial since a positive PET/CT may be accepted in the near future as a diagnostic criterion and is currently accepted as such in an ongoing clinical trial with tocilizumab in GCA.[18]

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Table 2 Relationship between clinical and laboratory data and maximal SUV at any vascular territory (SUVm) and mean of the SUVm obtained at every vascular bed assessed (mean SUVm).

	Maximal SUVm		Mean SUVm	
		p Value		p Value
Cranial symptoms (P/A)	3.21±0.65/2.50±0.52	0.021	2.24±0.32/1.77±0.36	0.004
Systemic symptoms (P/A)	3.12±0.61/2.98±0.82	0.589	2.20±0.35/2.07±0.43	0.354
Ischaemic symptoms (P/A)	2.91±0.61/3.14±0.71	0.402	2.11±0.39/2.17±0.37	0.708
PMR (P/A)	3.11±0.62/3.06±0.71	0.886	2.27±0.35/2.12±0.38	0.321
GC treatment (Y/N)	2.97±0.44/3.20±0.81	0.385	2.14±0.36/2.17±0.40	0.858
CRP, mg/dL	r=0.551	0.001	r=0.476	0.034
ESR, mm/h	r=0.442	0.011	r=0.335	0.050
Haptoglobin, mg/dL	r=0.585	0.008	r=0.358	0.050
IL-6, pg/mL	r=0.616	0.002	r=0.544	0.007

Values are mean±SD.
Removal of the two patients who had received intravenous methylprednisolone pulses at the time of PET performance did not substantially modify the results (data not shown).
A, absence; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; F, female; GC, glucocorticoid; IL-6, interleukin 6; M, male; N, no; NS, not significant; P, presence; PET, positron emission tomography; PMR, polymyalgia rheumatica; SUV, standardized uptake value; Y, yes.

The sensitivity and specificity of PET/CT obtained in this study is close to that calculated in a recent systematic review/meta-analysis of heterogeneous, mostly retrospective studies, and those reported in a retrospective analysis evaluating the impact of PET on the management of patients with suspected large-vessel vasculitis.[19]

Interestingly, FDG uptake by the aorta showed a lower AUC, being worse in the abdominal segment where atherosclerosis is more prevalent in the general population. This fact highlights the diagnostic limitation of PET in this territory since aortic FDG uptake may be markedly influenced by ageing or atheroma plaques. Hautzel *et al* reported a higher sensitivity and specificity of thoracic aorta FDG uptake to detect large-vessel inflammation in a cohort of 18 patients with GCA.[20] A thoracic aorta/liver ratio of 1.0 had a sensitivity and specificity of 88% and 93%, respectively (AUC = 0.932). However, a substantial proportion of the patients assembled in this cohort were selected on the basis of previously known large-vessel involvement demonstrated by other techniques. In our study, direct, territory-focused comparison of SUVm between patients and controls discriminated better than vascular/liver ratios.

A retrospective study evaluating how PET/CT results influenced management of patients with suspected GCA suggested that previous glucocorticoid (GC) treatment decreased the diagnostic yield of PET/CT.[19] Sequential assessments have demonstrated, indeed, that FDG uptake decreases after 3 months of treatment.[4] The present study suggests that a short course of therapy (≤3 days) may not substantially reduce the diagnostic accuracy of PET/CT.

In conclusion, this study provides sensitive and specific, territory-focused cut-off values to detect vascular inflammation by PET/CT. A limitation of the study is that while patients were prospectively recruited, controls were retrospectively selected. Another limitation is the relatively small number of patients analysed, although our cohort is among the largest investigated. Further prospective studies using objective cut-offs are necessary to confirm their diagnostic performance in patients with suspected GCA.

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Contributors MCC had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study design: SP-G, FL and MCC. Acquisition of data: SP-G, MD, AG-M, GE-F, IT-B, FL, MC-B, EP-R, MAA, JMG, JH-R and MCC. Analysis and interpretation of data: SP-G, MD, GE-F, FL, JH-R and MCC. Manuscript preparation: SP-G, FL, JH-R, JMG and MCC. Statistical analysis: SP-G, MAA, MCC.

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Ethics approval The study was approved by the ethics committee of our institution (Hospital Clínic, Barcelona).

Provenance and peer review Not commissioned; externally peer reviewed.

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Positron emission tomography assessment of large vessel inflammation in patients with newly diagnosed, biopsy-proven giant cell arteritis: a prospective, case–control study

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Relapses in Patients With Giant Cell Arteritis

Prevalence, Characteristics, and Associated Clinical Findings in a Longitudinally Followed Cohort of 106 Patients

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Abstract: Giant cell arteritis (GCA) is a relapsing disease. However, the nature, chronology, therapeutic impact, and clinical consequences of relapses have been scarcely addressed. We conducted the present study to investigate the prevalence, timing, and characteristics of relapses in patients with GCA and to analyze whether a relapsing course is associated with disease-related complications, increased glucocorticoid (GC) doses, and GC-related adverse effects. The study cohort included 106 patients, longitudinally followed by the authors for 7.8 ± 3.3 years. Relapses were defined as reappearance of disease-related symptoms requiring treatment adjustment. Relapses were classified into 4 categories: polymyalgia rheumatica (PMR), cranial symptoms (including ischemic complications), systemic disease, or symptomatic large vessel involvement. Cumulated GC dose during the first year of treatment, time required to achieve a maintenance prednisone dose <10 mg/d (T10), <5 mg/d (T5), or complete prednisone discontinuation (T0), and GC-related side effects were recorded. Sixty-eight patients (64%) experienced at least 1 relapse, and 38 (36%) experienced 2 or more. First relapse consisted of PMR in 51%, cranial symptoms in 31%, and systemic complaints in 18%. Relapses appeared predominantly, but not exclusively, within the first 2 years of treatment, and only 1 patient developed visual loss. T10, T5, and T0 were significantly longer in patients with relapses than in patients without relapse (median, 40 vs 27 wk, $p < 0.0001$; 163 vs 89.5 wk, $p = 0.004$; and 340 vs 190 wk, $p = 0.001$, respectively). Cumulated prednisone dose during the first year was significantly higher in relapsing patients (6.2 ± 1.7 g vs 5.4 ± 0.78 g, $p = 0.015$). Osteoporosis was more common in patients with relapses compared to those without (65% vs 32%, $p = 0.001$). In conclusion, the results of the present study provide evidence that a relapsing course is associated with higher and prolonged GC requirements and a higher frequency of osteoporosis in GCA.

(*Medicine* 2014;93: 194–201)

Abbreviations: CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, GC = glucocorticoids, GCA = giant cell arteritis, Hb = hemoglobin, IQR = interquartile range, PDN = prednisone, PMR = polymyalgia rheumatica, SD = standard deviation, SIR = systemic inflammatory response, TNF = tumor necrosis factor.

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INTRODUCTION

Giant cell arteritis (GCA) is a granulomatous arteritis predominantly affecting large and medium-sized vessels.[18,27] Treatment with high-dose glucocorticoids (GC) results in prompt and remarkable improvement of symptoms and reduces the risk of ischemic complications.[2] However, reduced GC doses do not completely abolish essential pathways involved in disease persistence, and consequently, the course of GCA may be troubled by relapses.[5,8,27] Recrudescence of GCA activity is common, occurring in at least 43% of patients in population-based studies[3,26] and up to 80% in clinical trials with adjuvant therapies.[15,16,19,20,22] The remarkable variability in the reported prevalence of relapses may be related to heterogeneity in the definition of relapses and to variability in the GC-tapering schedules. Definition of relapse, flare, or recurrence considerably varies across different studies.[16,21–23,26] While in some publications definition of relapse has been based on clinical grounds,[15,16,19] in others, isolated increases in acute-phase reactants have been considered disease flares.[24]

In addition, although this has not been formally evaluated, initial doses and tapering schedules seem to influence relapse rate in GCA.[17,20] In this regard, it is noteworthy that the higher relapse rates have been observed in the context of clinical trials with adjuvant therapies where GC tapering is more aggressive than in standard of care settings, and when alternate-day GC tapering is applied.[15,16,19,20,22] Consistently, a detailed review of treatments received by patients with isolated polymyalgia rheumatica (PMR) suggests that starting with lower GC doses is associated with higher relapse rates.[13]

Relapse rate is a commonly used primary endpoint in clinical trials with patients with GCA. However, although frequency of relapses has been reported in various studies,[15,16,19,20,22,26] limited information exists regarding the

clinical characteristics and predictors of relapses, and accompanying blood test abnormalities, which have been only specifically addressed in a previous study.[23] Moreover, it has not been clearly demonstrated whether a relapsing course results in increased disease or treatment-related morbidity in these patients. Therefore, we conducted the present study to investigate the prevalence, timing, predictors, and main features of relapses in a longitudinally followed cohort of patients with GCA with long-term follow-up. In addition, we analyzed whether a relapsing course was associated with disease-related ischemic complications, higher cumulated GC doses, more prolonged treatment periods, and/or higher frequency of GC-related adverse effects.

PATIENTS AND METHODS

Between 1995 and 2007, 187 individuals were diagnosed with biopsy-proven GCA at our institution (Hospital Clínic, Barcelona, Spain). Among them, patients treated by the authors who underwent a regular follow-up for at least 4 years were selected. From the initial 187 patients diagnosed, 81 were excluded for the following reasons: 31 were subsequently treated at other departments or institutions, 19 died early during follow-up, 14 were transferred to nursing homes for advanced dementia, and 17 moved to other regions or had deficient compliance with the scheduled follow-up visits.

The remaining 106 patients were uniformly evaluated, treated, and longitudinally followed by the authors for an average of 7.8 ± 3.3 years (range, 4–15 yr). Clinical and laboratory findings at disease diagnosis were recorded. A combination of clinical and blood test abnormalities was used to evaluate the intensity of the systemic inflammatory response (SIR) as previously reported.[6,7,14] These included fever $>38\,°C$, weight loss ≥ 4 kg, hemoglobin (Hb) <11 g/L, and erythrocyte sedimentation rate (ESR) ≥ 85 mm/h. Patients with 3 or 4 of these items were considered to have a strong SIR, whereas patients with ≤ 2 were considered to have a weak SIR. Patients underwent clinical assessments in our outpatient facility every 3 months for the first 2 years after diagnosis and approximately every 4–6 months thereafter. ESR, C-reactive protein (CRP), blood cell counts, and Hb concentration were determined at each visit. The treatment protocol consisted of an initial prednisone (PDN) dose of 1 mg/kg per day (up to 60 mg/d) for 1 month. Intravenous methylprednisolone pulse therapy (1 g daily for 3 d) was initially administered to patients with recent (<48 h) visual loss. PDN was subsequently tapered at 10 mg/wk. When reaching 20 mg/d, this dose was maintained for 1–2 weeks and then reduced to 15 mg/d, which was maintained for 1 month. A further reduction to a maintenance dose of 10 mg/d was attempted. If tolerated, a reduction to 7.5 mg/d was tried after 3–6 months. Subsequent tapering was more variable. In general, a reduction to 5 mg/d was attempted approximately 3–6 months later and maintained for 1 year, after which a reduction of 1.25 mg/d was attempted every 6 months. Methotrexate at 15 mg/wk was added when patients experienced ≥ 2 relapses or had developed GC side effects. Reduction in PDN dose was performed 1 month before the scheduled follow-up visit to evaluate tolerance to the adjustment and to avoid severe relapses. If disease-related symptoms (cranial manifestations or PMR), fever, weight loss, or anemia not attributable to other reasons after the necessary work-up occurred, PDN dose was increased by 10–15 mg/d above the previous effective dose. If asymptomatic increases in acute-phase reactants were detected, PDN dose was held until the next visit. When a relapse could be defined, patients were managed as discussed above. If not, a reduction was attempted regardless of the ESR or CRP levels.

We used a consensus definition of relapse established in the context of international multicenter clinical trials.[15,16,19] Relapse or recurrence were indistinctly defined as reappearance of disease-related symptoms, usually accompanied by elevation of acute-phase reactants that required treatment adjustment. Relapses were categorized according to the clinical manifestation into 4 categories: 1) PMR, 2) cranial symptoms (headache, scalp tenderness, jaw claudication, cranial ischemic complications), 3) systemic disease (anemia, fever, and/or weight loss), or 4) symptomatic large vessel involvement (extremity claudication). Cranial ischemic manifestations included stroke, transient ischemic attacks, amaurosis fugax, GCA-related visual loss, or diplopia. Number of relapses, time (in weeks) from the initiation of treatment to first relapse, time required to reach a PDN maintenance dose <10 mg/d (T10), <5 mg/d (T5), and time required to complete GC withdrawal (T0), not followed by a relapse for at least 3 months, were recorded. Cumulated PDN doses received after the first year of treatment were calculated. For each episode of relapsing activity, the ESR, serum CRP, and Hb concentrations were determined, as well as the PDN dose received at that time. In addition, GC-related adverse effects including new or worsening hypertension, diabetes mellitus, hypercholesterolemia, osteoporosis, cataracts, and Cushing appearance were recorded. Measurement of bone mineral density with dual energy X-ray absorptiometry was performed at disease diagnosis and thereafter approximately every 2 years. Osteoporosis was diagnosed using the World Health Organization criteria–that is, bone mineral density T-score of 2.5 standard deviations (SDs) or more below the young adult mean.[4] For screening of diabetes and hyperlipidemia, patients had blood tests prior to each visit, and blood pressure was periodically assessed both at their scheduled visits and by their primary care physicians. We recorded events as adverse events when they appeared or worsened after GC treatment and required new treatment or intensification of previous therapy. The study was approved by the Ethics Committee of Hospital Clínic (Barcelona, Spain).

Statistical Analysis

Continuous variables are presented as mean±SD and/or median and interquartile range (IQR) and categorical data as percentages. Association between relapses and selected covariates was analyzed using the T-test (paired and unpaired) for quantitative variables and the chi-square test for categorical data. Time required to achieve maintenance PDN dose <10 mg/d, <5 mg/d, and time to treatment discontinuation were compared between patients with and without relapses by the Kaplan-Meier survival analysis method. Statistical significance was defined as $p < 0.05$. Calculations were performed with the statistical package PAWS statistics v 18 (SPSS Inc, Chicago, IL) and GraphPad Prism v 5.04 for Windows (GraphPad Software, La Jolla, CA).

RESULTS

We analyzed 106 patients. Mean age at diagnosis was 75 ± 7 years (range, 58–89 yr) with a male to female ratio of 1:2.6. Demographic data and main clinical features at disease onset are depicted in Table 1.

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TABLE 1. Baseline Characteristics at Diagnosis (*n=106*)

General Characteristics:	
Age, mean ± SD (range), years	75 ± 7 (58–89)
Sex, No. Male/female (%)	29/77 (27/73)
Duration of Symptoms, Mean ± SD, Weeks	16 ± 21
Cranial Symptoms at Diagnosis, N (%)	
Headache	83 (78)
Jaw Claudication	47 (44)
Scalp Tenderness	49 (46)
Facial Pain+	50 (47)
Cranial Ischemic Complications†	26 (24.5)
Polymyalgia Rheumatica	54 (51)
Systemic Manifestations, N (%)	
Fever	40 (38)
Anorexia	12 (11)
Weight Loss	54 (51)
Laboratory Parameters	
ESR, mm/hour	90 ± 30.2
CRP, mg/dL	11.2 ± 17.6
Haptoglobin, g/L	3.6 ± 1.5
Hemoglobin, g/dL	11.3 ± 1.4

+Includes ocular pain, tongue pain, toothache, earache, odynophagia and carotidynia.

† Includes stroke, transitory ischemic attacks, amaurosis fugax, blindness and diplopia.

Chronology and Characteristics of Relapses

Sixty-eight patients (64%) relapsed during follow-up (mean, 7.8 ± 3.3 yr; range, 4–15 yr) (Figure 1). Mean time to first relapse was 79 ± 75 weeks (range, 11–339 wk) with a median of 51 (IQR, 89) weeks. Thirty-four of the 68 patients (50%) relapsed during the first year after diagnosis (Figure 2A).

PMR was the most frequent clinical manifestation observed during the first flare (Figure 2B). Of note, severe ischemic complications were not developed by any patient as part of the first relapsing episode. Most patients relapsed with the same features originally present at GCA diagnosis (n = 52, 78%). For those who developed a different clinical manifestation, PMR was the most frequent new feature (n = 9, 75%). No patients in this series relapsed with symptomatic large vessel involvement.

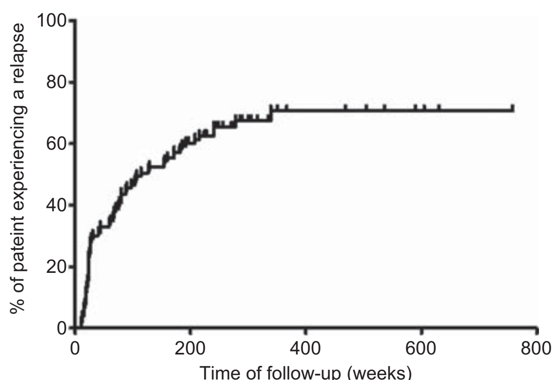

FIGURE 1. Kaplan-Meier plot of the entire series showing the probability of relapse over time.

Figure 2A shows the mean±SD and median (IQR) of PDN used by patients at the time of relapse during the first 5 years of follow-up. Mean PDN dose received by the 68 patients at the first relapse was of 5.3 ± 6.5 mg/d with a median of 2.5 (IQR, 7.5) mg/d. Fifty-two percent were receiving doses ≤2.5 mg/d (Figure 2C). PDN doses at the time of relapse tended to decrease over time (Figure 2A). Patients who relapsed during the first year received 8.3 ± 8.2 mg/d, median 7.5 mg (IQR, 15), whereas patients who relapsed during the second year were receiving 3.7 ± 2.3 mg, median 5 mg (IQR, 2.5).

Mean ESR, CRP, and Hb levels at the time of the first relapse were 61 ± 29 mm/h, 4.0 ± 3.8 mg/dL, and 12 ± 1.4 g/L, respectively. The inflammatory response at that time was comparatively lower than that observed at GCA onset (ESR 88 ± 33 vs 61 ± 29 mm/h, $p < 0.0001$; CRP 11 ± 19 vs 4.0 ± 3.8 mg/dL, $p = 0.001$; and Hb 11.3 ± 1.6 vs 12 ± 1.4 g/L, $p < 0.0001$). We observed an increase of 33 ± 14 mm/hr in ESR level, an increase of 2.9 ± 2.2 mg/dL in CRP concentration, and a decrease of 0.7 ± 0.1 g/L for Hb values between the previous laboratory tests while in remission and the ones performed at disease relapse.

There were significant differences in the PDN doses used to treat relapses according to the type of recurrence (Table 2). The lowest doses were used to treat PMR symptoms (14.5 ± 6.8 mg/d) whereas higher doses were employed to treat cranial manifestations (23.7 ± 12.9 mg/d). ESR levels and Hb concentrations were significantly more deviated from normal values in patients who relapsed with systemic manifestations (see Table 2).

Thirty-eight patients (36%) had 2 or more relapses. Distribution of relapse types was similar to that observed at the first episode (Figure 2D). One patient developed a severe ischemic complication (anterior ischemic optic neuritis) as part of her second recurrence. This patient was treated for 3.5 years and regularly followed for 5 years. She subsequently abandoned regular visits and presented with a relapse including malaise, ischemic optic neuritis, and elevation of ESR, 4 years later. The mean PDN dose at the second flare was 4.3 ± 2.7 mg/d with a median of 5 (IQR 2.5) mg. ESR (mm/h), CRP (mg/dL), and Hb (g/L) levels were 55 ± 30, 3.9 ± 6.1, and 12.1 ± 1.4 respectively.

Predictors of Relapse

To search for predictors of relapses, we compared initial clinical and laboratory findings between patients with or without recurrent disease. At disease onset, PMR and scalp tenderness were more frequently observed in relapsing patients (Table 3). Acute-phase reactants tended to be higher in patients with recurrences, but only haptoglobin reached statistical significance (3.8 ± 1.6 g/L vs 3.0 ± 1.3 g/L, $p = 0.042$). When the intensity of the SIR was evaluated combining clinical and laboratory findings, patients with multiple relapses were significantly more frequent among those with a strong SIR: 23 of 27 (85%) patients with a strong SIR relapsed 2 or more times compared with 15 of 41 (37%) of those with a weak SIR. No other predictors of recurrences could be identified.

Glucocorticoid Requirements and Side Effects According to Disease Recurrences

Treatment requirements were different in both groups of patients (Table 4). Patients with relapses required

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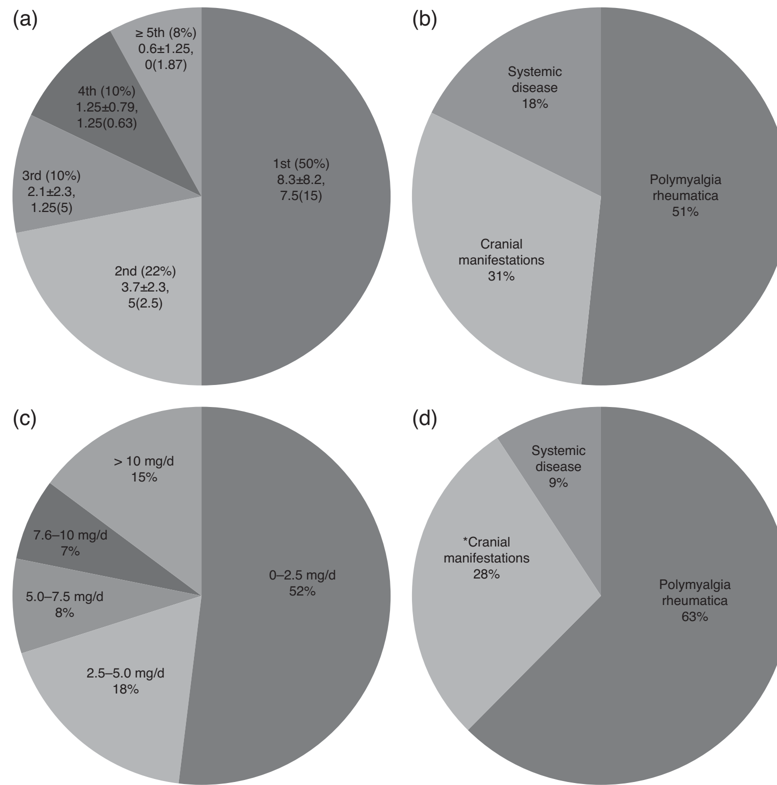

FIGURE 2. Characteristics and timing of flares in relapsing patients (n = 68). A) Percentage of patients relapsing per year of follow-up and mean±SD and median (IQR) prednisone dose (mg/d) received at the time of relapse. B) Percentage of relapsing patients with a given clinical type of relapse. C) Percentage of patients receiving the indicated dose of prednisone (mg/d) at the time of the first

significantly longer periods of time to reach a maintenance dose of PDN <10 mg/d, <5 mg/d, and to completely discontinue GC therapy (Figure 3). Cumulated PDN dose during the first year was significantly higher in relapsing patients (6.2 ± 1.7 g vs 5.4 ± 0.78 g, $p = 0.015$). Relapsing patients had an increased prevalence of osteoporosis (65% for relapsing patients vs 32% for nonrelapsing, $p = 0.001$). Other adverse effects also tended to be more frequent in patients with relapses, but differences did not reach statistical significance. As expected, methotrexate was administered more frequently in patients with relapses than in patients in sustained remission (22% vs 3%, $p = 0.009$).

DISCUSSION

Limited information about the characteristics of recurrences occurring in patients with GCA is available.[23] Here, we present detailed data about clinical and laboratory characteristics of relapses from a cohort of uniformly treated patients with GCA with long-term follow-up. The definition of relapse used in the present study was similar to that used in randomized, controlled clinical trials evaluating adjunctive therapies for GCA.[15,16,19]

In spite of the satisfactory initial response to GC treatment, 64% of patients relapsed in the present series. This percentage is somewhat higher than that reported in population-based studies,[3,26] possibly due to the more extended follow-up of our patient cohort, but some selection bias cannot be excluded. Although most relapses occurred within the first 2 years of treatment, recurrences also developed subsequently. PMR was the most frequent symptom (51%) at the time of relapse, followed by cranial manifestations (31%). In previous studies[16,22,23] headache was the leading feature (44%-60%), followed by PMR (19%-30%),[16,23] and constitutional syndrome (28%).[23] Therefore, the distribution found in the current cohort is close to that found in other studies. It is noteworthy that disease-related

TABLE 2. Laboratory Characteristics and PDN Dose at Each Relapse Type

	Polymyalgia Rheumatica	Cranial Manifestations	Systemic Disease	*p*
ESR (mm/h)	55 ± 28	59 ± 28	80 ± 26	0.038
CRP (mg/L)	3.9 ± 3.5	4.5 ± 4.2	3.6 ± 4.1	ns
Hemoglobin (g/L)	12.2 ± 1.3	12.3 ± 1.4	11.7 ± 1.2	0.017
Dose of prednisone (mg/d) used to treat relapse	14.5 ± 6.8	23.7 ± 12.9	17.9 ± 5.6	0.002
Increment in prednisone dose (mg/d)	10 ± 6.5	17 ± 12	14 ± 11	0.009

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TABLE 3. Clinical Manifestations at Diagnosis in Patients With and without Relapses

Clinical characteristics	*Relapse (n=68)*	*No relapse (n=38)*	*p*
General characteristics:			
Sex, no. female/male	52/16 (76.5/23.5)	25/13 (66/34)	ns
Age, mean, yr	74 ± 6.5	76 ± 8	ns
Cranial symptoms, n (%)			
Headache	56 (82)	27 (71)	ns
Jaw claudication	34 (50)	13 (34)	ns
Scalp tenderness	40 (59)	9 (24)	0.001
Ischemic complications	17 (25)	9 (24)	ns
Stroke	2 (3)	0 (0)	ns
Transitory ischemic attack	4 (6)	0 (0)	ns
Amaurosis fugax	9 (13)	6 (16)	ns
Diplopia	9 (13)	2 (5)	ns
Permanent visual loss	6 (9)	6 (16)	ns
Polymyalgia rheumatica	40 (59)	14 (37)	0.042
Systemic manifestations, n (%)			
Fever	24 (35)	16 (42)	ns
Anorexia	7 (10)	5 (13)	ns
Weight loss	36 (53)	18 (47)	ns
Baseline laboratory parameters, mean ± SD			
ESR, mm/hour	88 ± 33	92 ± 24	ns
CRP, mg/dL	11.4 ± 18.6	10.7 ± 15.9	ns
Haptoglobin, g/L	3.8 ± 1.6	3.0 ± 1.3	0.049
Hemoglobin, g/dL	11.3 ± 1.5	11.2 ± 1.2	ns

ischemic complications seem to be extremely infrequent in the context of controlled relapses. In previous studies the occurrence of ischemic manifestations has been also found to be infrequent during follow-up (0%-6%).[1,16,23] Only 1 patient in the current series suffered anterior ischemic optic neuropathy in the context of a delayed relapse, but this patient had interrupted regular control visits at the time of disease recurrence. No patient in our series relapsed with symptomatic involvement of large vessels.

Relapses were usually accompanied by elevated levels of ESR and CRP that were, nevertheless, lower than those observed at disease onset. In accordance, PDN doses much lower than the starting doses were usually effective for treating controlled relapses. However, it must be stressed that the reported features were obtained from patients who were closely followed with reassessments performed approximately every 3 months during the first 2 years after diagnosis. We cannot exclude that severe relapses requiring higher GC doses may occur in patients controlled less tightly. These findings indicate that patients with GCA need to be indefinitely observed even after successful GC discontinuation.

As for the time at greatest risk for relapse, in 50% of patients who relapsed, recurrences occurred during the first year. Mean time to first relapse was 19.7 ± 18.7 months, similar to what has been reported by others.[21,23,26] However delayed relapses also occurred.

As shown in Figure 2, PDN dose received at the time when relapses occurred decreased over time, suggesting that

TABLE 4. Treatment Requirements and Side Effects During Follow-up

Clinical characteristic	*Relapse (n=68)*	*No relapse (n=38)*	*p*
Treatment requirements			
*Intravenous methylprednisolone pulse**	3 (4)	4 (10.5)	ns
Cumulated dose first year, mean ± SD (g)	6.2 ± 1.7	5.4 ± 0.78	0.015
Methotrexate (15 mg/week)	15 (22)	1 (3)	0.009
Glucocorticoid related adverse effects n, (%)†			
Diabetes mellitus	7 (10)	2 (5)	ns
Systemic hypertension	37 (54)	17 (45)	ns
Hypercholesterolemia	42 (62)	26 (69)	ns
Osteoporosis	44 (65)	12 (32)	0.001
Cushing appearance	8 (12)	1 (3)	ns
Cataracts	16 (23.5)	3 (8)	ns

* At disease onset.
† 100% of patients presented at least 1 side effect.

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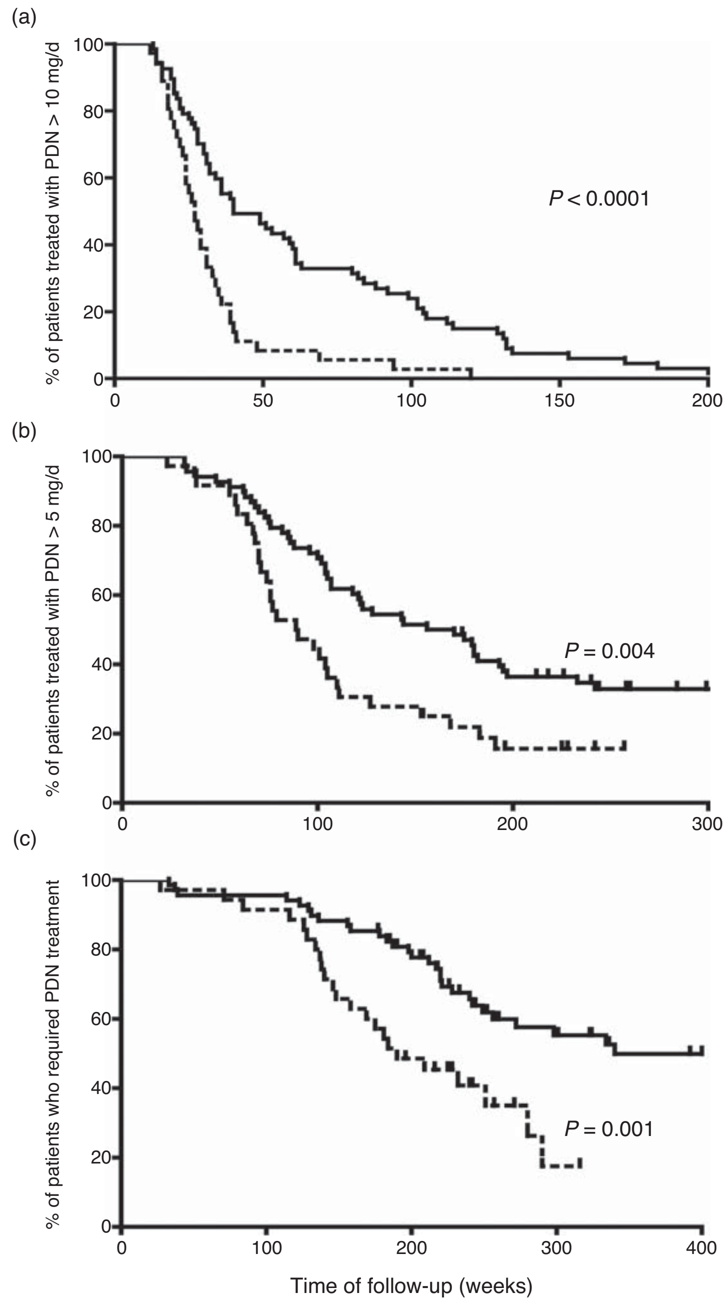

FIGURE 3. Survival curves showing the time required to reach a stable dose of prednisone <10 mg/d (A), <5 mg/d (B), and 0 mg/d (C) in patients with relapses (solid line) and with sustained remission (broken line). [Note the scale for time of follow-up is different among the 3 figure parts].

disease activity progressively decreases and, over the years, lower PDN doses are required to maintain remission. Overall, relapses occurred when patients were receiving a mean PDN dose of 5.3 ± 6.5 mg/d with median 2.5 mg/d (IQR, 7.5). This dose is lower than that reported in other series. This may be due to variability in the rate of initial PDN tapering across different studies or to other reasons. No patient in our cohort relapsed with PDN higher than 25 mg/d.

Patients with relapses required longer periods of treatment and were exposed to higher cumulated PDN doses, similar to what was found in a previous study.[23] All patients in our cohort experienced at least 1 GC-related side effect. Other studies have reported GC adverse effects in 90%-95% of GCA patients within the first 3 years of therapy.[24,26] These include new or worsening hypertension (22%-84%),[19,24,26] infections (22%-56%),[19,22,24,26] osteoporosis and bone fractures (8%-38%),[19,22–24,26] new or worsening diabetes mellitus (7%-37%),[19,22–24,26] and cataracts (4%-41%).[19,24,26] The higher frequency of side effects in our patient cohort may be related to the longer follow-up period. We observed that patients with

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recurrences presented more GC-related toxicity, in particular osteoporosis despite the administration of calcium supplements, vitamin D, and bisphosphonates. These data highlight the need for more efficient and safer therapies.

In the current cohort, relapses could not be attributed to insufficient treatment because relapsing patients received more GC and for more extended periods of time. This observation indicates that some patients have a more resistant disease. From the clinical standpoint, an intense acute-phase response was associated with higher risk of relapse. Other investigators have also observed that abnormalities related to the acute-phase response are predictors of relapse.[6,9,12,14,23,25,28] Among other findings, only scalp tenderness and PMR were slightly more frequent in relapsing patients. Although this association may be spurious, a similar trend has been observed in other studies.[23] Previous studies have investigated tissue and serum biomarkers associated with persistent disease activity and relapsing course. Elevated serum concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and soluble intercellular adhesion molecule (ICAM)-1 are associated with relapsing disease.[10,12,28] Increased expression of TNF-α or chemokine (C-C motif) ligand (CCL)-2 mRNA in involved arteries is associated with recurrent disease and higher GC requirements. However, TNF-α blockade was not sufficient to reduce relapses and spare corticosteroids,[16] indicating that association does not imply causality and suggesting that TNF-α effects may be compensated by other cytokines and that upstream mediators may be more relevant to perpetuate disease activity. Elevated mRNA concentrations of Th1 cytokines IL-12/23p40 and interferon (IFN)-γ have been observed after 1 year of treatment in second temporal artery biopsies of relapsing patients, suggesting reactivation of initial events able to drive subsequent inflammatory cascades.[28] In contrast, increased IL-17 expression in GCA lesions is a predictor of sustained response to GC.[11] Further research is needed to elucidate the mechanisms involved in disease persistence, to enable the design of more specific and efficient targeted therapies.

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