UNIVERSITAT DE BARCELONA

Study of the acquisition and maintenance of antibody and cellular immune responses against SARS-CoV-2 in longitudinal cohorts

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Study of the acquisition and maintenance of antibody and cellular immune responses against SARS-CoV-2 in longitudinal cohorts

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The role of the infinitely small in nature is infinitely large.

Louis Pasteur

A la meua família,

per despertar en mi la curiositat

A Carlos Blázquez,

pel teu constant somriure

A totes les víctimes de la pandèmia de la COVID-19

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Abbreviations and Acronyms

aa	Amino acid
ACE2	Angiotensin-Converting Enzyme 2
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCD	Antibody-Dependent Complement Deposition
ADCP	Antibody-Dependent Cellular Phagocytosis
AE	Adverse Event
AIM	Activation-Induced Markers
APC	Antigen-Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
ASC	Antibody-Secreting Cell
BAL	Bronchoalveolar Lavage
BCG	Bacillus Calmette-Guérin
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
BTI	Breakthrough Infection
CD	Connecting Domain (Spike protein)
CDC	US Centers For Disease Control and Prevention
CH	Central Helix (Spike protein)
CI	Confidence Interval
CLR	C-Type Lectin Receptor
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus Disease 2019
CS	Cleavage Site
CSR	Class-Switch Recombination
CT	Cytoplasmic Tail (Spike protein)
CTD	C-Terminal Domain
CTL	Cytotoxic T Lymphocyte ($CD8^+$ Cytotoxic T Cell)
DC	Dendritic Cell
DMV	Double-Membrane Vesicle
DTP	Diphtheria-Tetanus-Pertussis (vaccine)
E	Envelope
ECDC	European Centre for Disease Control and Prevention
EMA	European Medicines Agency
ER	Endoplasmic Reticulum
ERGIC	ER-to-Golgi Intermediate Compartment
EUA	Emergence Use Authorization
Fab	Antigen-Binding Fragment
Fc	Crystallizable Fragment
FcR	Crystallizable Fragment Receptor
FC	Fold change
FDA	U.S. Food and Drug Administration
FL	Full length
FP	Fusion Peptide
GC	Germinal Center
GISAID	Global Initiative on Sharing All Influenza Data

hACE2	Human ACE2
HCoV	Human Coronavirus
HCW	Healthcare Worker
HI-FBS	Heat-Inactivated Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HR	Helix Region (SARS-CoV-2 genome)
ICU	Intensive Care Unit
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile Range
LDM	Lineage Defining Mutations
LLPC	Long-Lived Plasma Cell
LMM	Linear Mixed Model
M	Membrane
mAb	Monoclonal Antibody
MBC	Memory B Cell
MERS-CoV	Middle East Respiratory Syndrome-Related Coronavirus
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility Complex I (Class I) II (Class II)
MIS-C	Multisystem Inflammatory Syndrome in Children
Mpro	Main Protease
N	Nucleocapsid
NC	Negative Control
N CT	Nucleocapsid C-terminal
nAb	Neutralizing Antibody
NK	Natural Killer
NLR	Nod-Like Receptor
NSP	Non-Structural Protein
NTD	N-Terminal Domain
OAS	Original Antigenic Sin (immune imprinting)
ORF	Open Reading Frame
PAMPs	Pathogen-Associated Molecular Pattern
PASC	Post-Acute Sequelae of COVID-19 (long COVID)
PBMCs	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PC	Positive Control
PE	Phycoerythrin
PEI	Polyethylenimine
PHA	Phytohemagglutinin
PHEIC	Public Health Emergency of International Concern
PIMS-TS	Pediatric Inflammatory Multisystem Syndrome Temporally associated with SARS-CoV-2 (MIS-C)
PRR	Pattern Recognition Receptor
R_0	Basic reproduction number
RBD	Receptor-Binding Domain

RBM	Receptor-Binding Motif
RDT	Rapid Diagnostic Test
RLR	Rig-Like Receptor
RTC	Replicase-Transcriptase Complex
RT-PCR	Real-Time Reverse Transcription Polymerase Chain Reaction
S	Spike
SARS-CoV-1	Severe Acute Respiratory Syndrome Coronavirus 1
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SD	Subdomain (SARS-CoV-2 genome), Standard deviation (statistics)
SFU	Spot Forming Units
SHM	Somatic Hypermutation
sIgA	Secretory IgA
SIVIC	*Sistema d'Informació Per A La Vigilància d'Infeccions A Catalunya*
SiVIRA	*Sistema De Vigilancia De Infección Respiratoria Aguda*
SP	Signal Peptide
T	Timepoint
TAG-VE	Technical Advisory Group On COVID-19 Vaccine Composition
TCE	T Cell Epitope
TCR	T Cell Receptor
TLR	Toll-Like Receptor
TM	Transmembrane Domain (SARS-CoV-2 genome)
TMPRSS2	Transmembrane Serine Protease 2
TNF	Tumor Necrosis Factor
T_C	$CD8^+$ Cytotoxic T Cells
T_{CM}	Central Memory T Cells
T_{EM}	Effector Memory T Cells
T_{EMRA}	Terminally Differentiated Effector Memory T Cells re-expressing CD45RA
T_{FH}	Follicular Helper T Cells
T_H	$CD4^+$ Helper T Cells
T_{REG}	Regulatory T Cells
T_{RM}	Resident Memory T Cells
T_{SCM}	Stem Cell Memory T Cells
UTR	Untranslated Region
VOC	Variant of Concern
VOI	Variant of Interest
VUM	Variant under Monitoring
WHO	World Health Organization
WT	Wild type
Wu-1	Wuhan-Hu-1 Virus/ Wuhan-1 (Ancestral)
+ssRNA	Positive-Sense Single-Strand RNA

Glossary

Adverse event: An untoward medical occurrence after vaccine administration, which is not necessarily caused by that vaccine.

Ancestral virus: the original form of SARS-CoV-2 identified in December 2019; also referred to as the "wild type", "original", "Wuhan", or "Wuhan-Hu-1 (Wu-1)" virus (first fully sequenced genome isolated from a patient in Wuhan).

Antibody isotype: refers to the classification of an antibody (IgG, IgA, IgM, IgE, and IgD) based on the structure of its constant region within the heavy chain. This structural difference determines the antibody's biological function, effector mechanisms, and distribution in the body.

Antibody subclass: distinct categories within a specific antibody IgG or IgA isotypes that differ in their structural and functional properties.

Basic reproduction number (R_0): the average number of secondary infections caused by a single infectious individual introduced into a completely susceptible population.

Booster dose: additional dose of a vaccine given after the initial series (or primary doses) to enhance or restore the immune response.

Bivalent vaccine: a vaccine formulated to provide immunity against two distinct SARS-CoV-2 variants.

Breakthrough infection: infection following vaccination for the same infectious agent.

Bystander activation: activation of immune cells as a response to a different antigen or an unrelated pathogen due to the inflammatory environment and cytokine signals present.

Class switch recombination: is a process through which B cells replace the constant region of the immunoglobulin heavy chain from the initial μ (IgM) to other constant region segments (γ, α, ε), enabling the expression of different antibody isotypes (e.g., IgG, IgA and IgE) with distinct effector functions.

Clonal expansion: rapid proliferation (cell division) of B or T cells after they recognize a specific antigen, resulting in a large population of identical cells, or clones, that share the same antigen specificity as the original cell.

Comorbidity: the condition of having two or more diseases at the same time.

Control strategies (infectious disease): systematic and coordinated interventions implemented to reduce the transmission, impact, or prevalence of a pathogen within a population. They can include a combination of public health measures, medical treatments, vaccination, and behavioral interventions.

Convalescent individual: a person who is in the recovery phase following an illness or infection. During this period, the individual has overcome the acute stage of the disease and is no longer exhibiting active symptoms.

Convalescent plasma: acellular, antibody-rich component of blood collected from convalescent individuals, which contains polyclonal antibodies, including neutralizing antibodies, that target the pathogen responsible for the disease.

Cross-reactivity: immune system's ability to recognize and respond to one pathogen based on prior exposure to a different but related pathogen.

Cytokine storm: a severe immune reaction characterized by an uncontrolled overproduction of cytokines that leads to tissue damage, disseminated intravascular coagulation, acute respiratory distress syndrome, multiorgan failure and death.

Endemic: consistent presence or usual prevalence of a disease within a specific geographic area or population group.

Epidemic: the occurrence of a disease or health condition that spreads rapidly and affects a larger number of people within a specific community, region or population over a specific period.

Epidemic wave: dynamical behavior of epidemic curves as an oscillation between a very low and very high number of cases, deaths and/or hospitalizations throughout time.

Epitope: also known as antigenic determinant, the specific part of an antigen that is recognized by antibodies, B cells, or T cells.

Fc-effector functions: biological activities mediated by the Fc region of antibodies when interacts with Fc receptors on immune cells and components of the complement system.

First-infected: individuals whose first antigen encounter with SARS-CoV-2 virus was through infection, then received COVID-19 vaccination and for some cases subsequent breakthrough infections.

First-vaccinated: individuals whose first antigen encounter with SARS-CoV-2 virus was through vaccination, and in some cases with subsequent breakthrough infections.

Germinal center reaction: dynamic process which involves proliferation, differentiation, and selection of B cells that have encountered an antigen, leading to the generation of high-affinity antibodies and the formation of memory B cells.

Herd immunity: the indirect protection from infection conferred to susceptible individuals when a sufficiently large proportion of immune individuals exist in a population.

Heterologous immunization: use of different vaccines as immunization strategy.

Homologous immunization: use of the same vaccine as immunization strategy.

Hybrid immunity: individuals who have natural and vaccine-induced immunity due to infection and vaccination, regardless the order of occurrence.

Immune escape: the ability of a pathogen to partially or fully evade pre-existing immunity.

Immune imprinting: when the immune system preferentially relies on memory from its first encounter with an antigen when responding to a new, related variant. This often leads to a biased immune response skewed toward epitopes of the original antigen than those of the new variant. As a result, the immune response may be less effective or suboptimal to the new antigen.

Immune tolerance: immune system unresponsiveness to specific antigens.

Immunodominance: when the host immune response against an infectious agent is mostly focused on a few antigens.

Immunosenescence: gradual deterioration of the immune system function with aging, affecting particularly adaptive immune response, resulting in a reduced vaccine efficacy, increased susceptibility to infections, and chronic inflammation.

Incidence: the number of new cases of a disease in a population in a given period.

Incubation period: the time from initial exposure to an infectious agent to the onset of clinical signs and symptoms.

Lineage: group of closely related viruses with a common ancestor.

Lockdown: a state or period in which movement within or access to an area is restricted in the interests of public safety or health.

Long COVID: continuation or development of new symptoms three months after the initial SARS-CoV-2 infection, with symptoms lasting at least two months without explanation.

Memory cells: antigen experienced B and T cells that have previously encountered their specific antigen during a primary immune response and show enhanced functionality.

Monoclonal antibody: antibody recognizing a single epitope on an antigen, produced artificially from a single B-cell clone, with identical structure and antigen specificity.

Monovalent vaccine: vaccine designed to immunize against a single pathogen.

Mortality rate: percentage of people in a population who die out of the total population.

Multisystem inflammatory syndrome in children: hyperinflammatory complication of COVID-19 in children, characterized by a more inflammatory profile and severe clinical

phenotype, with cardiovascular, respiratory, neurologic, gastrointestinal, and mucocutaneous manifestations and multiorgan dysfunction.

Mutations: substitutions, insertions or deletions of one or more nucleotides in the virus RNA genome.

Mutation rate: the intrinsic rate at which genetic changes emerge per replication cycle.

Naïve cells: antigen-inexperienced B and T cells that have matured and left bone marrow and thymus, respectively, but have not yet encountered their specific antigen. Upon antigen encounter, they activate, proliferate and differentie into effector and memory cells.

Neutralizing antibodies: antibodies that interfere with pathogen binding to their host cells, thereby blocking infection.

Omicron subvariants: genetically distinct forms of the virus that are descendants from Omicron variant. For example, BA.1 and BA.2 are Omicron subvariants.

Omicron sublineages: further subdivision within the Omicron subvariants lineages. For example, BA.2.86 is a sublineage from the BA.2 Omicron subvariant.

Outbreak: sudden increase in the number of cases of a disease in a specific geographic area or population over a defined period. This rise exceeds what is normally expected based on past data or baseline levels.

Pandemic: epidemic that affects several countries across multiple continents or worldwide.

Polarizing cytokines: set of secreted cytokines in response to pathogens or immune signals to induce a specific local environment that drive the differentiation of naïve $CD4^+$ T cells into specific T helper cell subsets, T_H1, T_H2, T_H17, T_H22, T_H9, T_{FH}, and T_{REG}.

Prevalence: total number of people who have a disease (new and existing cases) in a population or in a given place at a given time.

Primary vaccination: initial series schedule of a vaccine to achieve sufficient immunity against a specific pathogen.

Protective immunity: relative ability to resist infection or attenuate an infectious disease or its clinical presentation.

Reservoir: any person, animal, arthropod, plant, soil, or substance, in which an infectious agent normally lives and multiplies, allowing transmission to a susceptible host.

SARS-CoV-2 reinfection: occurring ≥90 days after initial positive testing or ≥45 days with background information supporting contact with confirmed cases or the reappearance of COVID-19–like symptoms.

Serial interval: duration between symptom onset of a primary and secondary cases.

Seroconversion: change from negative to positive specific antibodies to an antigen.

Seroreversion: change from positive to negative specific antibodies to an antigen.

Somatic hypermutation: process in which B cells undergo extremely high mutation rates in the variable regions of an immunoglobulin to improve the affinity with the antigen.

Trained immunity: long-term functional modification of innate immune cells, which leads to an improved response to a second unrelated challenge.

Updated vaccine: vaccine targeting the last and prevailing SARS-CoV-2 emerged variant.

Vaccine effectiveness: ability of a vaccine to prevent a specific disease or outcome (such as infection, symptomatic disease, hospitalization, or death) in a real-world population, under routine conditions of use.

Vaccine hesitancy: delay in acceptance or refusal of vaccines despite availability of vaccination services and supporting evidence.

Variant: genetically distinct form of the SARS-CoV-2 virus.

Variant of concern: variant that meets the definition of a VOI and, through a risk assessment, conducted by WHO TAG-VE, and determined to be associated with a moderate or high level of confidence, meets at least one of the following criteria when compared with other variants: detrimental change in clinical disease severity; or change in COVID-19 epidemiology causing substantial impact on the ability of health systems to provide care to patients with COVID-19 or other illnesses and therefore requiring major public health interventions; or significant decrease in the effectiveness of available vaccines in protecting against severe disease.

Variant of interest: variant with genetic changes that are predicted or known to affect virus characteristics such as transmissibility, virulence, antibody evasion, susceptibility to therapeutics and detectability; and identified to have a growth advantage over other circulating variants in more than one WHO region with increasing relative prevalence alongside increasing number of cases over time, or other apparent epidemiological impacts to suggest an emerging risk to global public health.

Variant under monitoring: variant with genetic changes that are suspected to affect virus characteristics and early signals of growth advantage relative to other circulating variants (e.g. growth advantage which can occur globally or in only one WHO region), but for which evidence of phenotypic or epidemiological impact remains unclear, requiring enhanced monitoring and reassessment pending new evidence.

Viral fitness: replicative adaptability of a virus in a given environment.

List of articles in the thesis

Thesis in compendium of publications format. The thesis consists of three objectives and five articles:

1. Dobaño C, Ramírez-Morros A, Alonso S, **Rubio R**, Ruiz-Olalla G, Vidal-Alaball J, Macià D, Catalina QM, Vidal M, Casanovas AF, Prados de la Torre E, Barrios D, Jiménez A, Zanoncello J, Melero NR, Carolis C, Izquierdo L, Aguilar R, Moncunill G, Ruiz-Comellas A. *Sustained seropositivity up to 20.5 months after COVID-19*. BMC Med. 2022 Oct 13;20(1):379. doi: 10.1186/s12916-022-02570-3.
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   Category: Medicine, General & Internal

2. Dobaño C, Ramírez-Morros A, Alonso S, Ruiz-Olalla G, **Rubio R**, Vidal M, Prados de la Torre E, Jairoce C, Mitchell RA, Barrios D, Jiménez A, Rodrigo Melero N, Carolis C, Izquierdo L, Zanoncello J, Aguilar R, Vidal-Alaball J, Moncunill G, Ruiz-Comellas A. *Eleven-month longitudinal study of antibodies in SARS-CoV-2 exposed and naïve primary health care workers upon COVID-19 vaccination*. Immunology. 2022 Dec;167(4):528-543. doi: 10.1111/imm.13551.
   Impact factor 2022: 6.4, Quartile 2
   Category: Immunology

3. **Rubio R**, Macià D, Barrios D, Vidal M, Jiménez A, Molinos-Albert LM, Díaz N, Canyelles M, Lara-Escandell M, Planchais C, Santamaria P, Carolis C, Izquierdo L, Aguilar R, Moncunill G, Dobaño C. *High-resolution kinetics and cellular determinants of SARS-CoV-2 antibody response over two years after COVID-19 vaccination*. Microbes Infect. 2024 Sep 17:105423. doi: 10.1016/j.micinf.2024.105423.
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Summary

The COVID-19 pandemic has underscored the importance of understanding adaptive immune responses to SARS-CoV-2 to optimize vaccination strategies and public health interventions. Adaptive immune responses generate immune memory, which is essential for preventing reinfections, facilitating rapid virus clearance and reducing disease severity. Neutralizing antibodies prevent viral infection and T cells support antibody production and eliminate infected cells. Despite extensive research on SARS-CoV-2 adaptive responses, evidence was scarce early in the COVID-19 pandemic, and knowledge gaps persist, such as their duration, factors influencing them, and the cross-recognition of emerging variants, key aspects to improve immunological protection. We characterized antibody and T-cell responses induced by SARS-CoV-2 infection, COVID-19 vaccination, or a combination of both against the ancestral virus and emerging variants over three years since the onset of the pandemic. Antibody levels against spike (S) and nucleocapsid (N) antigens were measured using Luminex assays, neutralizing capacity through pseudovirus assays, and T-cell responses to S, N and membrane antigens using IFN-γ/IL-2 FluoroSpot and activation induced marker assays. Antibody kinetics were modeled using linear mixed models, and determinants were analyzed with multivariable linear regression models. The results demonstrated that positive antibody and T-cell responses to S persisted for at least three years following infection during the first pandemic wave and two years after vaccination, with subsequent exposures (boosters and reinfections) supporting the maintenance. Antibody kinetics varied by isotype, antigen, and immunization type, showing greater durability in individuals with hybrid immunity and those receiving three vaccinations compared to two. These responses were heterogeneous, influenced by factors such as comorbidities and SARS-CoV-2 prior exposure. Infection as the first SARS-CoV-2 encounter was associated with weaker antibody responses against Omicron subvariants BA.1, BA.2, BA.4/5, BQ.1.1, and XBB, but with stronger T-cell responses after three years compared to vaccination as first antigen encounter. Although ancestral pre-existing antibodies showed reduced recognition of Beta, Gamma, Delta, and Omicron subvariants BA.1, BA.2, BA.4/5, BQ.1.1, XBB, with lost neutralization for BA.2.86, T cells effectively recognized BA.2.86. In conclusion, these findings contribute to a deeper knowledge of COVID-19 immunity for optimizing vaccination strategies to maximize protection against SARS-CoV-2 and future emerging variants.

Resumen

La pandemia de COVID-19 ha destacado la importancia de comprender las respuestas inmunitarias adaptativas frente al SARS-CoV-2 para optimizar las estrategias de vacunación e intervenciones de salud pública. Las respuestas inmunes adaptativas generan memoria inmunitaria, que es esencial para prevenir reinfecciones, facilitar una eliminación rápida del virus y reducir la gravedad de la enfermedad. Los anticuerpos neutralizantes previenen la infección viral, mientras que los linfocitos T contribuyen a la producción de anticuerpos y eliminan las células infectadas. A pesar de la extensa investigación, al inicio de la pandemia de COVID-19 las evidencias eran limitadas y aún persisten preguntas por resolver, como su duración, los factores que las influyen y el reconocimiento cruzado con las variantes emergentes, aspectos clave para mejorar la protección inmunológica. Hemos caracterizado las respuestas de anticuerpos y linfocitos T inducidas por la infección del SARS-CoV-2, vacunación COVID-19, o una combinación de ambas frente al virus ancestral y a las variantes emergentes durante los tres años transcurridos desde el inicio de la pandemia. Los niveles de anticuerpos frente los antígenos de la espícula (S) y la nucleocápside (N) se midieron con tecnología Luminex, la capacidad neutralizante mediante ensayos de pseudovirus, y las respuestas de linfocitos T a los antígenos S, N y membrana con ensayos de FluoroSpot IFN-γ/IL-2 e inducción de marcadores de activación. Las cinéticas de los anticuerpos se modelaron utilizando modelos lineales mixtos, y los factores influyentes se analizaron con modelos de regresión lineal multivariable. Los resultados demostraron que las respuestas positivas de anticuerpos y linfocitos T frente S persistieron al menos tres años después de la infección durante la primera ola en la pandemia, y dos años después de la vacunación, con exposiciones posteriores (dosis refuerzo y reinfecciones) contribuyendo al mantenimiento. Las cinéticas de los anticuerpos variaron según el isotipo, el antígeno y el tipo de inmunización, mostrando mayor durabilidad en individuos con inmunidad híbrida y aquellos que recibieron tres dosis de vacuna en comparación con dos. Estas respuestas fueron heterogéneas, influenciadas por factores como las comorbilidades y exposiciones previas al SARS-CoV-2. La infección como primer contacto con el SARS-CoV-2 se asoció con respuestas de anticuerpos más débiles frente a las subvariantes de Ómicron BA.1, BA.2, BA.4/5, BQ.1.1 y XBB, pero con respuestas de linfocitos T más robustas al cabo de tres años en comparación con la vacunación como primer contacto antigénico. Aunque los anticuerpos preexistentes dirigidos al virus ancestral mostraron un reconocimiento reducido a las variantes Beta, Gamma, Delta y subvariantes de Ómicron BA.1, BA.2, BA.4/5, BQ.1.1, XBB, con una pérdida de neutralización total a la variante BA.2.86, los linfocitos T reconocieron eficazmente BA.2.86. En conclusión, estos hallazgos contribuyen a un conocimiento más profundo de la inmunología de COVID-19 para optimizar las estrategias de vacunación y maximizar la protección frente al SARS-CoV-2 y futuras variantes emergentes.

Resum

La pandèmia de COVID-19 ha posat en manifest la importància de comprendre les respostes immunitàries adaptatives al SARS-CoV-2 per optimitzar les estratègies de vacunació i les intervencions de salut pública. Les respostes immunes adaptatives generen memòria immunitària, essencial per prevenir reinfeccions, eliminar el virus de manera ràpida i eficient i reduir la gravetat de la malaltia. Els anticossos neutralitzants impedeixen la infecció viral, mentre que els limfòcits T contribueixen a la producció d'anticossos i a l'eliminació de cèl·lules infectades. Tot i la investigació extensiva de les respostes adaptatives al SARS-CoV-2, les evidències eren escasses al començament de la pandèmia de COVID-19, i encara hi ha preguntes per resoldre, com ara la seva durada, els factors que les influeixen, i el reconeixement creuat a les variants emergents, aspectes clau per millorar la protecció immunològica. Hem caracteritzat les respostes d'anticossos i limfòcits T induïdes per la infecció del SARS-CoV-2, la vacunació COVID-19, o una combinació d'ambdues enfront el virus original i de les variants emergents al llarg de tres anys des de l'inici de la pandèmia. Els nivells d'anticossos enfront els antígens espícula (S) i nucleocàpsida (N) s'han mesurat mitjançant assajos de Luminex, la seva capacitat neutralitzant amb pseudovirus, i les respostes específiques dels limfòcits T als antígens S, N i membrana amb els assajos FluoroSpot d'IFN-γ/IL-2 i d'inducció de marcadors d'activació. La cinètica d'anticossos s'ha modelitzat amb models lineals mixtes, mentre que els factors influents s'han analitzat amb models de regressió lineal multivariable. Els resultats han mostrat que les respostes positives d'anticossos i limfòcits T a S persisteixen almenys tres anys després de la infecció durant la primera onada de la pandèmia i dos anys després de la vacunació, amb exposicions subseqüents (dosis reforç o reinfeccions) afavorint el manteniment. La cinètica d'anticossos va variar segons l'isotip, l'antigen i el tipus d'immunització, sent més duradores en individus amb immunitat híbrida i amb tres vacunacions en comparació a dues. Aquestes respostes van ser heterogènies, influenciades per factors com comorbiditats i l'exposició prèvia al SARS-CoV-2. La infecció com a primer encontre amb el SARS-CoV-2 es va associar a una menor resposta d'anticossos enfront les subvariants d'Òmicron BA.1, BA.2, BA.4/5, BQ.1.1 i XBB, però va generar major resposta de limfòcits T al cap de tres anys en comparació a la vacunació com a primer encontre antigènic. Tot i que els anticossos preexistents dirigits al virus original van disminuir el reconeixement a les variants Beta, Gamma, Delta, i les subvariants d'Òmicron BA.1, BA.2, BA.4/5, BQ.1.1, XBB, fins i tot perdent la capacitat de neutralització a la variant BA.2.86, els limfòcits T la van reconèixer de manera eficient. En conclusió, aquests resultats contribueixen a un coneixement més profund de la immunologia de la COVID-19 per optimitzar les estratègies de vacunació i maximitzar la protecció enfront del SARS-CoV-2 i futures variants emergents.

Introduction

Pandemics have devastated humanity throughout history, causing the loss of millions of lives. From ancient plagues like the Athenian and Antonine to the Black Death, Smallpox, and the Spanish Influenza, and more recent crises like Influenza A (2009) and COVID-19 (2019), pandemics continue to shape human existence. In recent decades, emerging and reemerging infectious diseases have increased, and outbreaks such as the SARS-CoV-1 (2002-2004), MERS-CoV (2012), Ebola (2014-2016), Zika (2015-2016), Monkeypox (2022-2024), and Marburg virus (2023-2024) have surged. Climate change and ecosystem disruption have heightened the frequency of disease spillovers. Additionally, geopolitical conflicts and wars exacerbate health risks by limiting access to care and increasing disease spread in overcrowded refugee settings. The host immune response is crucial in determining the outcome of a pandemic. It influences individual susceptibility, disease severity, and the development of immunologic memory and herd immunity, thus affecting the transmission dynamics of the pathogen. These interconnected global challenges underscore the need for integrated strategies to control pandemics. Understanding adaptive immune responses to SARS-CoV-2 is essential for developing effective control strategies and preparing for future pandemics.

The global impact of SARS-CoV-2

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the etiological agent of Coronavirus disease 2019 (COVID-19), the respiratory illness responsible for the COVID-19 pandemic [1]. First identified in Wuhan, China, in December 2019 [2], the World Health Organization (WHO) declared the outbreak a public health emergency of international concern (PHEIC) from January 30, 2020, to May 5, 2023 [3,4].

The global impact of SARS-CoV-2 has been profound and multifaceted [1]. It has generated a public health crisis with over 700 million confirmed cases and more than 7 million reported deaths globally [5,6], making COVID-19 one of the leading causes of death worldwide [7]. However, epidemiological data may be underreported, with estimates suggesting that the actual death toll may exceed 20 million [8]. Healthcare systems were overwhelmed, resulting in shortages of medical supplies, personnel, and hospital beds, especially during peak waves. The pandemic also disrupted routine healthcare services,

delaying treatments for non-COVID-19 diseases and elective surgeries, worsening overall health outcomes [9].

The pandemic caused economic contractions and exacerbated social and mental health crises [1]. Geopolitically, it fueled tensions between countries, particularly regarding the origins of the virus and vaccine distribution. Despite these challenges, it spurred unprecedented scientific collaboration, leading to the rapid development of vaccines and innovations in diagnostics, telemedicine, and health research data-sharing, opening new opportunities for tackling other diseases [1]. In addition, the lockdowns temporarily had a positive environmental impact, contributing to notable reductions in greenhouse gas emissions [1].

Although SARS-CoV-2 is no longer a PHEIC, the virus is still circulating among us, accumulating mutations that make it better at evading previous immunity. Therefore, public health authorities regard COVID-19 as a global health threat [8].

Understanding the SARS-CoV-2 and COVID-19

Phylogenetics

SARS-CoV-2 is a member of the *Betacoronavirus* genus within the *Coronaviridae* family [10]. Coronaviruses are named for the spike (S) proteins on the surface, which resemble a crown when viewed under electron microscopy (derived from the Latin "corona" meaning "crown") [11]. They are a large group of enveloped positive-sense single-stranded RNA viruses (+ssRNA) with a nucleocapsid of helical symmetry [12]. Coronaviruses infect and cause diseases in mammals (including humans, livestock, and pets) and avian species [12]. To date, seven human-infecting coronaviruses (HCoV) have been identified: the endemic HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, which typically cause "common cold" symptoms and account for 10% to 30% of upper respiratory tract infections in adults [13]; and the highly pathogenic Severe Acute Respiratory Syndrome Coronavirus 1 (SARS-CoV-1), Middle East Respiratory Syndrome-related coronavirus (MERS-CoV), and SARS-CoV-2, which have caused significant outbreaks and cause severe respiratory diseases affecting multiple tissues [14].

SARS-CoV-2 virology

Viral structure and genome

SARS-CoV-2 viral particles (**Figure 1**) have a diameter ranging from 60 to 140 nm [15]. The SARS-CoV-2 genome encodes 16 non-structural proteins (NSP-1 to NSP-16), nine accessory proteins, and four structural proteins: S (20-40 copies per virion), envelope (E) (~20 copies per virion), membrane (M) (~2,000 copies per virion), and nucleocapsid (N) (~1,000 copies per virion) proteins [15].

The virus possesses a large +ssRNA genome with about 30,000 bases, constituted by several open reading frames (ORFs) (**Figure 1**) that play an essential role in viral pathogenicity and infectivity [16]. The 5'-terminal region contains two ORFs, ORF1a and ORF1b, representing over two-thirds of the entire genome. These ORFs 1a-1b encode two precursor polyproteins (pp1a and pp1ab) co-translationally and post-translationally processed into NSPs 1–11 and 12–16, respectively [17]. These NSPs form the replicase-transcriptase complex (RTC) and are essential for viral replication. These proteins include an RNA-dependent RNA polymerase, RNA binding proteins, cofactors involved in the replication, an exonuclease for proofreading, a 3-chymotrypsin-like protease, a papain-like protease, a helicase, a 3'-5' endonuclease, N7 and 2'-O-ribose methyltransferases, and others [17]. The 3'-terminal region contains the four genes encoding the structural proteins (S, E, M, and N) and several ORFs encoding the accessory proteins (ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10) [17].

Structural proteins are crucial for the viral life cycle and are essential targets for immune responses and diagnostic tests [18]. The N protein encapsidates the RNA genome, forming the nucleocapsid structure. It is involved in viral replication and packaging of the viral RNA. The N protein elicits a strong immune response. It is frequently employed in diagnostic tests to detect SARS-CoV-2 infections because it is highly abundant in infected cells and relatively conserved among coronaviruses [18,19]. The M protein is an integral membrane protein and the most abundant structural protein. It is an essential component of the viral envelope, interacting with the other structural proteins to assemble viral particles, and it also contributes to viral budding [18,19]. The E protein is an

integral membrane protein, the smallest of the structural proteins, involved in the assembly and release of new virions. It also modulates host cell processes such as inflammation and contributes to viral pathogenesis [18,19]. The S is a transmembrane glycoprotein located on the virus surface. It mediates viral entry into host cells and determines the viral host range and tissue tropism. It is highly immunogenic and a primary target for neutralizing antibodies (nAbs), making it the critical component in COVID-19 vaccines and treatments. The S protein is also prone to mutations, impacting viral transmissibility and immune evasion [18,19].

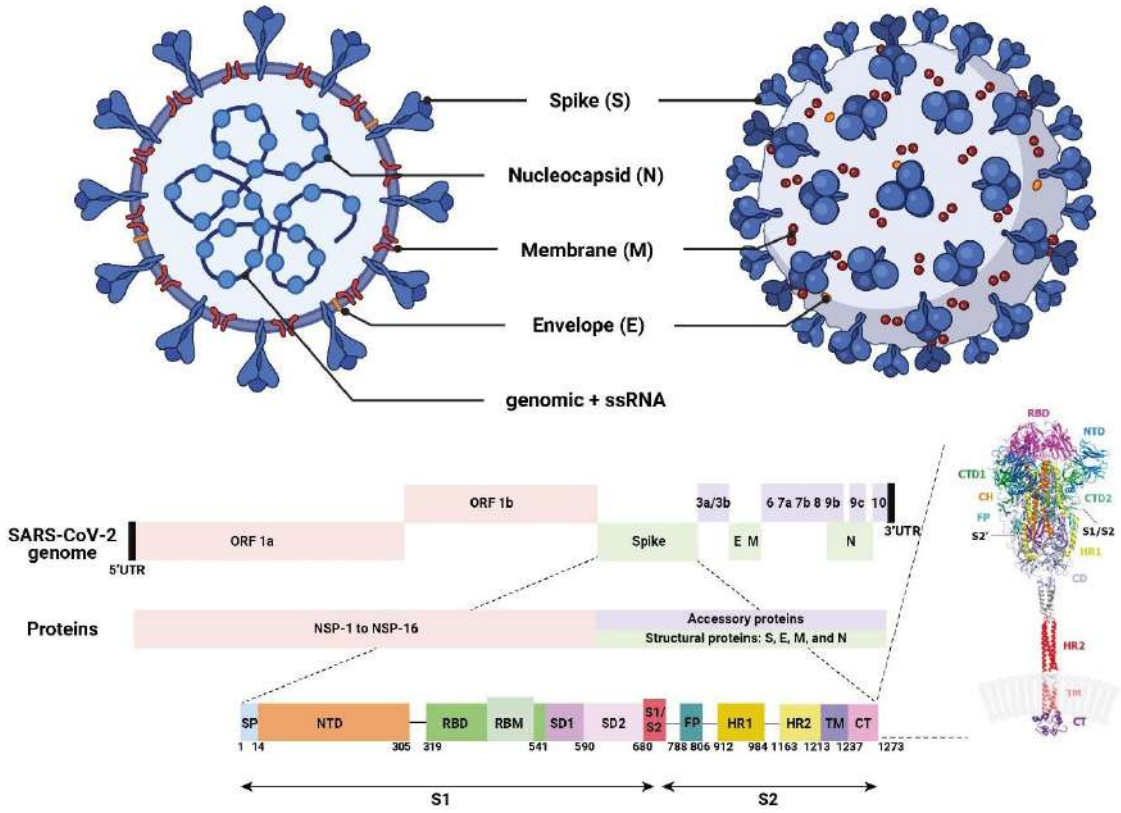

Figure 1 | SARS-CoV-2 structure and genome. The upper panel depicts a schematic representation of the SARS-CoV-2 viral particle. *Created in BioRender.com.* The lower panel illustrates SARS-CoV-2 genomic organization, detailing the spike (S) glycoprotein and its structure in the prefusion state. *Adapted with permission from Rani Rajpal et al.* [17] *and Casalino et al.* [20]. CD: Connecting domain; CH: Central Helix; CT: Cytoplasmic tail; CTD: C-terminal domain FP: Fusion peptide; HR: Helix region; NSP: Non-structural protein, NTD: N-terminal domain, ORF: Open reading frame; RBD: Receptor-binding domain; RBM: Receptor-binding motif; SD: Subdomain, SP: Signal peptide; ssRNA: single-strand RNA; TM: Transmembrane domain; UTR: Untranslated Region.

Accessory proteins are not essential for viral replication, but they play crucial roles in viral pathogenicity and modulate host immune responses [21]. For instance, ORF9c inhibits cytokine secretion while others (ORF3b, ORF6, ORF7a, ORF8, ORF9b) counteract the antiviral effects of type I interferons (IFNs). Moreover, these proteins influence several cellular processes such as autophagy and apoptosis (ORF3a), mitochondrial function (ORF9b), activation of the inflammasome (ORF9b), and regulation of major histocompatibility complex (MHC)-I molecules (ORF8) [18,21].

SARS-CoV-2 shares genomic homology with other HCoVs, including those responsible for the common cold (with ~50% or less homology) and the more severe acute respiratory diseases caused by SARS-CoV-1 (~80% homology) and MERS-CoV (~55% homology) [15].

Spike glycoprotein

The SARS-CoV-2 S glycoprotein (**Figure 1**) consists of 1273 amino acids (aa) and is a class I homotrimer fusion protein, like the hemagglutinin of influenza virus and the gp160 of human immunodeficiency virus (HIV) [22]. These proteins are typically found in an inactive precursor form and are activated by cleavage through host proteases. Once activated, they undergo a conformational change that exposes a hydrophobic region known as the "fusion peptide". This fusion peptide inserts into the host cell membrane, facilitating the fusion of the viral and host cell membranes, thereby enabling the virus to enter and infect the host cell [23].

Each S monomer (**Figure 1**) is 180-200 kDa in size and consists of a signal peptide (SP, aa 1-13), followed by two functionally distinct, non-covalently associated subunits: S1 and S2 [24]. The S1 subunit comprises the bulbous head and binds the host receptor, while the S2 subunit forms the stalk region and mediates membrane fusion [24]. The S1 subunit (aa 14-685) contains the N-terminal domain (NTD, aa 14-305), the receptor-binding domain (RBD, aa 319-541), which interacts with the host cell receptor, the angiotensin-converting enzyme 2 (ACE2). Within the RBD is the receptor binding motif (RBM, aa 437-508), the critical portion that directly contacts ACE2. Additionally, S1 contains two C-terminal domains (CTD1 and CTD2, aa 542-685) [17]. Importantly, unlike SARS-CoV-1 and other related coronaviruses, the SARS-CoV-2 S contains a polybasic cleavage site (CS) at

the S1/S2 boundary. This site enables efficient cleavage by furin-like proteases, facilitating S maturation and converting S into S1 and S2 subunits [24]. The S2 subunit (aa 686-1273) contains an NTD (aa 686-787) that is cleaved at the S2' site by serine proteases or cathepsins to release the fusion peptide (FP, aa 788-806). The S2 region also includes the central helix and helix region 1 (CH and HR1, aa 912-1035), followed by the connecting domain (CD, aa 1080-1135), HR 2 (HR2, aa 1163-1213), the transmembrane domain (TM, aa 1214-1237), and the cytoplasmic tail (CT, aa 1238-1273) [17].

The S protein is heavily glycosylated, with each trimer displaying 66 glycosylation sites, accounting for ~17% of its total weight and covering about ~40% of the weight of the protein surface [25]. These glycosylation sites, primarily N- and O-glycans, play a crucial role in viral pathobiology by shielding critical epitopes from recognition by the host immune system, thus contributing to immune evasion [25].

The S glycoprotein is relatively conserved among certain HCoVs. For example, the S from SARS-CoV-2 shares ~76% homology with that of SARS-CoV-1 and ~35% homology with MERS-CoV [26]. Notably, although SARS-CoV-1 and SARS-CoV-2 are highly homologous and use the same host cell receptor, SARS-CoV-2 exhibits more efficient transmission and infection. This higher infectivity of SARS-CoV-2 is mainly due to its higher binding affinity (2 to 20-fold greater) for ACE2 [26]. The SARS-CoV-2 RBD has a more compact conformation than that of SARS-CoV-1, which, along with several residue changes, likely stabilizes the hotspots of the ACE2-RBD binding interface [27]. Another difference between S of SARS-CoV-1 and SARS-CoV-2 lies in the cleavage process. The cleavage of SARS-CoV-2 S involves furin-like enzymes, a feature not observed in SARS-CoV-1 [24]. Mutations in the S protein occur more frequently in the S1 subunit, particularly in the RBD region, facilitating virus evolution and, thus, the emergence of new variants with improved transmissibility and immune evasion. However, the S2 subunit, along with the N protein, is more conserved across coronaviruses and less prone to mutations than the S1 subunit [28], resulting in higher immune cross-reactivity, which influences seropositivity thresholds.

Replication cycle

Viruses are non-living infectious entities incapable of autonomous reproduction. Thus, they rely on the host molecular mechanisms to replicate and generate new viral particles. The replication cycle for SARS-CoV-2 is estimated to take approximately 10 hours [15]. The SARS-CoV-2 replication cycle (**Figure 2**) begins recognizing and binding the S protein, specifically its RBD, to the host cell surface receptor ACE2 [29]. While other potential receptors and co-receptors have been proposed, their exact contribution to SARS-CoV-2 pathogenesis remains uncertain [29–31]. The RBD-ACE2 binding induces conformational changes in the S1 subunit, exposing the S2' cleavage site within the S2 subunit. Subsequently, a serine protease such as transmembrane serine protease 2 (TMPRSS2) cleaves the S2' site, triggering further conformational changes in the S protein that anchor it into the host cell membrane. The viral and host membranes then fuse, enabling the viral RNA genome to enter the host cell cytoplasm [29].

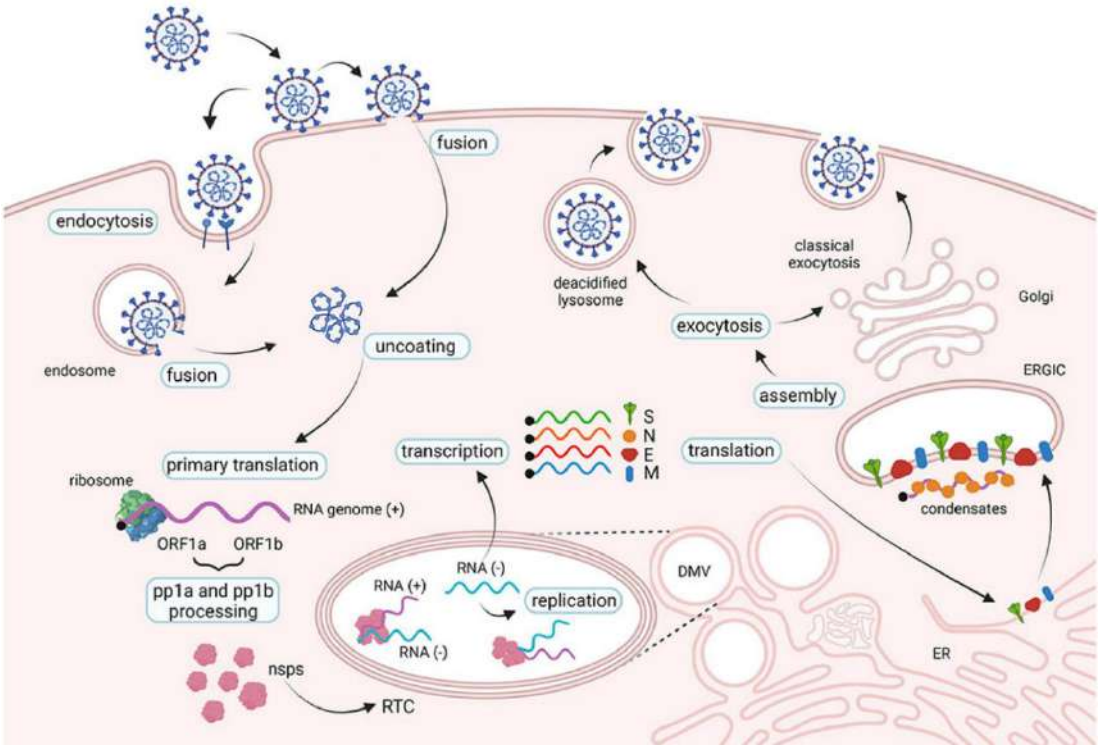

Figure 2 | SARS-CoV-2 replication cycle. *Adapted with permission from Pizzato et al.* [32]
DMV: Double-membrane vesicle, E: envelope; ER: Endoplasmic-reticulum; ERGIC: Endoplasmic-reticulum-Golgi intermediate; M: Membrane; N: Nucleocapsid; nsps: Non-structural proteins; ORF: Open reading frame; RTC: Replicase-transcriptase complex; S: Spike, pp1ab instead of pp1b

An alternative entry pathway for SARS-CoV-2 involves the endocytic route (**Figures 2 and 3**), used when the target cell expresses insufficient TMPRSS2 or when the virus-ACE2 complex does not encounter TMPRSS2. In this case, the virus-ACE2 complex is internalized via clathrin-mediated endocytosis into the endolysosomes. Inside the endolysosomes, S2' cleavage is carried out by cathepsins, which are activated in the acidic environment. This cleavage induces a conformational change in the S protein, enabling membrane fusion and subsequent release of the viral RNA genome into the host cell cytoplasm [29].

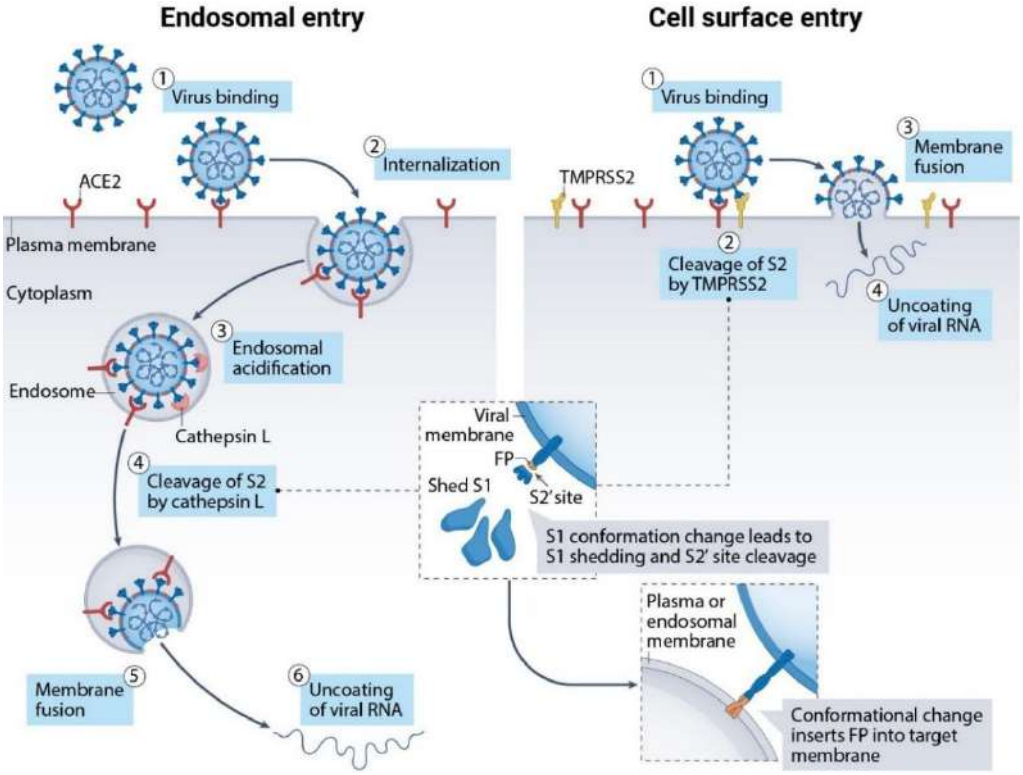

Figure 3 | SARS-CoV-2 entry pathways. The left panel shows the endosomal entry and the right panel the cell surface entry. *Adapted with permission from Jackson et al*. [29] ACE2: angiotensin-converting enzyme 2; FP: Fusion protein; TMPRSS2: Transmembrane serine protease 2.

Once inside the cell cytoplasm, ribosomes immediately translate ORF1a and ORF1b into the polyproteins pp1a and pp1ab, processed into individual NSPs that form the viral RTC [29,32] (**Figure 2**). Viral genomic RNA replication takes place within protective double-membrane vesicles (DMVs). The negative-strand RNA serves as a template for the transcription and translation of the structural proteins. Those are inserted into the

endoplasmic reticulum (ER) membrane and transit through the ER-to-Golgi intermediate compartment (ERGIC) [32], where co-translational and post-translational modifications of the S protein, including extensive glycosylation, signal peptide removal, and trimerization occur [33].

In the ERGIC, condensates of newly synthesized genomic RNA and N proteins interact with E and M proteins, leading to the assembly of viral particles, which bud into the lumen of secretory vesicular compartments [32]. Finally, virions are released from the infected cell by exocytosis through the classical Golgi-mediated pathway or by incorporating into deacidified lysosomes that fuse with cellular surface membrane [32].

Transmission

Current evidence suggests SARS-CoV-2 likely originated from wildlife, although the precise mechanisms of its emergence, timing, and location remain elusive [34,35]. The prevailing hypothesis posits that the virus was transmitted from bats or pangolins to an intermediary host species (e.g., raccoon dogs, foxes, minks) and subsequently to humans [36–39]. The Huanan Seafood Wholesale Market in Wuhan, China, is the epicenter of the COVID-19 pandemic [40]. Retrospective studies suggest that SARS-CoV-2 likely spilled into humans between early October and mid-November 2019 [41,42].

SARS-CoV-2 is primarily transmitted via airborne droplets (>5 µm diameter, quickly fall to the ground within 1-2 m) and aerosols (≤5 µm diameter, can remain suspended in the air for more extended periods and travel greater distances) [43,44]. These particles are generated when an infected person coughs, sneezes, talks, or breathes, and transmission occurs when another person inhales them or enters the respiratory tract [43,44]. Although less common, it can be transmitted through contact with contaminated surfaces (fomites), followed by touching the mouth, nose, or eyes [44]. Other potential but rare transmission routes include fecal-oral and vertical transmission from the mother to the fetus, though the evidence remains limited and unclear [44–46].

The basic reproduction number (R_0) —expected number of new cases that can be caused by one infected person during the infectious period in a naïve, uninfected population— for the ancestral SARS-CoV-2 is about 2-3, comparable to the Spanish Influenza in 1918 [15,47,48]. Additionally, the serial interval, which measures the time between the illness

onset in primary and secondary cases, is estimated to range from four to eight days. This relatively short serial interval highlights the potential for rapid transmission of ancestral SARS-CoV-2 within communities [49]. Several factors, including asymptomatic individuals, viral load, viral variants, and the implementation of preventive measures, influence the transmissibility of SARS-CoV-2, changing its R_0 and serial interval [50,51]. Actions taken to control the spread during the COVID-19 pandemic —such as mask-wearing, physical distancing, hand hygiene, improved ventilation, lockdowns, and vaccination campaigns— significantly helped reduce transmission [52]. Remarkably, despite the SARS-CoV-2 putative zoonotic origin and the susceptibility of many animal species to infection, whether these species can act as long-term viral reservoirs remains unclear [53].

COVID-19 disease

SARS-CoV-2 is capable of infecting various human cells due to the widespread expression of its receptor, ACE2, in multiple organs and tissues, including the lungs (highly expressed in alveolar epithelial cells), heart (cardiomyocytes, endothelial cells, and fibroblasts), kidneys (proximal tubular cells), intestines (enterocytes), liver (lowly expressed in bile duct cells), blood vessels (endothelial cells), brain (neurons and glial cells), testes (Leydig and Sertoli cells), and the placenta. ACE2 is critical in regulating blood pressure, explaining its broad distribution throughout the body [54–56]. While ACE2 expression is essential for SARS-CoV-2 entry into host cells, other factors such as tissue accessibility, ACE2 expression levels, the presence of cofactors, and host immune responses also influence the virus cell tropism. SARS-CoV-2 primarily targets the lungs, particularly cells in the upper and lower respiratory tract, due to its entry route and high ACE2 expression levels in these tissues. However, the widespread expression of ACE2 contributes to the multiorgan effects observed in COVID-19 [54].

Following SARS-CoV-2 infection, there is an incubation period (the time from infection to the onset of clinical signs and symptoms) characterized by low viral titters, lasting approximately four to seven days [49]. After this period, the virus undergoes exponential replication, and the nasopharyngeal viral load peak is detected by day ~7 from symptom onset [57]. At this peak, an infected individual is estimated to carry between 1 and 100 billion ancestral SARS-CoV-2 virions [15].

COVID-19 symptoms, severity, and outcomes after recovery are highly variable. The infection can be asymptomatic or develop a mild, moderate, severe, or critical disease that eventually can provoke death [58,59]. In mild-to-moderate cases, symptoms typically last about one to two weeks. In severe cases, especially those requiring hospitalization, symptoms may persist for three to six weeks [58,59]. Additionally, some individuals experience long-term symptoms and complications, a condition referred to as "long COVID", also known as post-COVID-19 condition or Post-Acute Sequelae of COVID-19 (PASC), where symptoms can last for months after the initial SARS-CoV-2 infection [60].

Research indicates that 20% to 40% of SARS-CoV-2 infections during the first waves were asymptomatic [61,62]. Among patients who present clinical symptoms, the majority (80%) typically experienced mild-to-moderate disease, characterized by cough, sore throat, fever, fatigue, breathing difficulties (dyspnea), myalgias, loss of smell (anosmia), loss of taste (dysgeusia), chest pain and gastrointestinal symptoms [63,64]. Approximately 15% developed severe COVID-19, requiring oxygen support [64]. A smaller proportion, about 5%, experienced critical illness with complications such as respiratory failure, acute respiratory distress syndrome (ARDS), sepsis and septic shock, thromboembolism, and multiorgan failure, including acute kidney and heart injuries [64]. Significantly, these numbers vary across studies, likely due to methodological differences, demographic variance, and for other SARS-CoV-2 variants, vaccination status, or other factors. The mechanisms underlying the diverse manifestations of SARS-CoV-2 infection still need to be understood [54].

The natural course of ancestral SARS-CoV-2 infection comprises three stages (**Figure 4**): 1) early infection, 2) pulmonary, and 3) hyperinflammatory phases. The first phase is characterized by viral replication, often accompanied by mild-to-moderate symptoms (or asymptomatic) and a strong antiviral innate immune response. During the second phase, viral replication continues, and more severe symptoms, such as pneumonia and dyspnea, may develop. Then, if the immune system fails to control viral replication effectively, the infection progresses to the third phase, marked by an excessive proinflammatory host immune response, a cytokine storm. This stage can lead to complications such as sepsis, respiratory failure, ARDS, and ultimately death [65,66]. Severe disease and worse prognosis are associated with lymphopenia, thrombocytopenia, and

elevated levels of D-dimer, lactate dehydrogenase, C-reactive protein, ferritin, liver enzymes, interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), troponin, creatine phosphokinase, and prolonged prothrombin time [63].

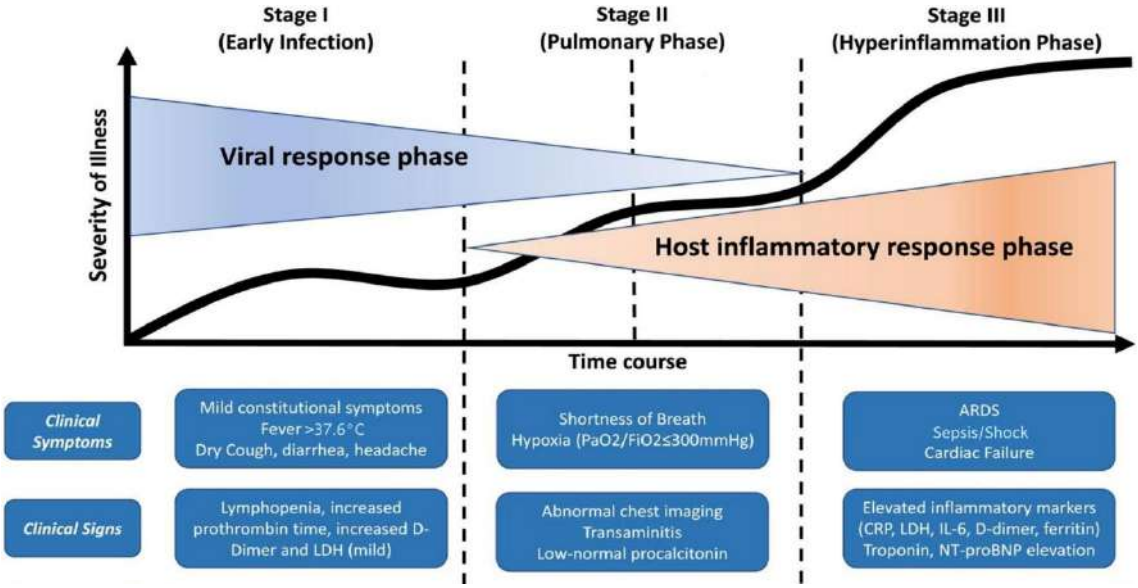

Figure 4 | COVID-19 pathogenic phases. Three escalating phases of COVID-19 disease progression, with associated signs and symptoms. *Adapted with permission from Nile et al.* [66] ARDS: Acute respiratory distress syndrome; CRP: C-reactive protein; LDH: Lactate dehydrogenase.

Throughout the pandemic, the main risk factors for COVID-19 have been identified through all research progress, including our contributions. Age is one of the main risk factors, especially for individuals over 65, as well as for some children who develop multisystem inflammatory syndrome in children (MIS-C) [67,68]. Other risk factors include male sex and smoking [67–69]. Comorbidities, including cardiovascular disease, diabetes type 1 and 2, chronic respiratory diseases (e.g., chronic obstructive pulmonary disease (COPD) or asthma), obesity, chronic kidney disease, liver disease, cancer, neurological conditions (e.g., dementia), and immunocompromised status, are associated with an increased risk of severe COVID-19 [70,71]. With an estimated 1.7 billion people —about a quarter of the world's population— having at least one comorbidity, these conditions pose significant concerns for their impact on COVID-19 progression and outcomes [70]. Additionally, ethnicity, pregnancy, socioeconomic and environmental factors, and

occupational exposure, particularly among healthcare workers (HCW), further contribute to heightened risk [72–77].

Long COVID

The WHO defines long COVID as the continuation or development of new symptoms three months after the initial SARS-CoV-2 infection, with symptoms lasting at least two months without explanation [78]. Common symptoms include fatigue, shortness of breath, and cognitive dysfunction, though more than 200 symptoms affecting multiple organ systems and daily functioning have been reported [60]. Long COVID affects an estimated 5-20% of people after SARS-CoV-2 infection, and its underlying causes are still unknown [60,78,79]. Several hypotheses have been proposed, including the presence of persistent reservoirs of SARS-CoV-2 in tissues, immune dysregulation with or without reactivation of underlying pathogens, effects on the microbiota, autoimmunity triggered by molecular mimicry, microvascular blood clotting with endothelial dysfunction, and dysfunctional signaling in the brainstem and vague nerve [60]. Current diagnostic and treatment options are insufficient. Thus, longitudinal studies are required to shed light on the long-term effects of the disease, biomarkers and its underlying mechanisms.

MIS-C

MIS-C, or pediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 (PIMS-TS), is a hyperinflammatory complication of COVID-19 in children [80]. It is characterized by a more inflammatory profile and severe clinical phenotype, with cardiovascular, respiratory, neurologic, gastrointestinal, and mucocutaneous manifestations and multiorgan dysfunction [80,81]. MIS-C has an estimated incidence of 0.05% of SARS-CoV-2 infections in children <15 years old, typically appearing four to six weeks following SARS-CoV-2 infection [80]. More than 50% of affected children require admission to the intensive care unit (ICU) [81]. This syndrome leads to severe and life-threatening illness in previously healthy children and adolescents [81]. While the exact pathophysiology is poorly understood, research suggests it results from immune dysregulation. However, the specific mechanisms by which an abnormal immune response to SARS-CoV-2 is triggered in children remain unknown [80,82]. Early diagnosis and prompt treatment are critical and result in favorable outcomes [80].

SARS-CoV-2 variants

Viruses are a remarkable example of Darwin's theory of evolution by natural selection, as they constantly adapt to environmental pressures —such as immune responses, vaccination campaigns, and treatments— by accumulating mutations that enhance their survival [83]. Mutations can be nucleotide substitutions, insertions, or deletions in the viral genome. Nucleotide substitutions may be synonymous, where the aa remains unchanged, or non-synonymous, resulting in an aa change [84]. RNA viruses are characterized by having high mutation rates [85]. However, coronaviruses generally have a comparatively lower mutational rate than other RNA viruses.

The estimated mutation rate of SARS-CoV-2 is ~$1x10^{-6}$ – $2x10^{-6}$ mutations per nucleotide per replication cycle, which is consistent with other *Betacoronavirus* [86]. In contrast to the higher mutation rates observed in other RNA viruses such as hepatitis C, influenza A, and HIV, which are ~$1x10^{-5}$ – $1x10^{-6}$, ~$2x10^{-4}$ – $2x10^{-6}$, and ~$1x10^{-4}$ – $1x10^{-6}$ mutations per nucleotide per replication cycle, respectively [86,87]. This relatively lower mutation rate in SARS-CoV-2 is due to an exonuclease for proofreading in their replicases, ensuring the correction of mismatches [88,89]. Although SARS-CoV-2 exhibits a relatively low mutation rate compared to other RNA viruses, and most viral mutations are deleterious (making the virions unable to replicate successfully), we have witnessed its evolution into more transmissible and immune-escaping variants, leading to successive COVID-19 waves (refer to *Impact of SARS-CoV-2 variants on the dynamics of the COVID-19 pandemic*) [86,90].

Throughout the COVID-19 pandemic, the scientific community has extensively monitored the evolution of SARS-CoV-2 and its impact on public health, including its transmissibility, pathogenesis, immunity, treatments, and diagnosis. As of January 2025, over 15 million SARS-CoV-2 genomic sequences have been recorded in the Global Initiative on Sharing All Influenza Data (GISAID) database, making it one of the most massively sequenced human viruses in history [91,92].

WHO has categorized the SARS-CoV-2 variants dynamically based on their impact on global public health into variants of concern (VOC), variants of interest (VOI), and variants under monitoring (VUM). These classifications are made by evaluating several parameters that influence the properties of the virus, such as changes (evident,

predictable, or suspected) in transmissibility, pathogenesis, and their impact on public health and social measures, immune responses, diagnosis, treatments, and vaccines [93]. For more detailed definitions, refer to Glossary.

According to WHO, no VOCs have been circulating since March 15, 2023. The currently circulating VOI (last update as of December 2, 2024) is JN.1, while the VUMs (last update as of December 9, 2024) are KP.2, KP.3, KP.3.1.1, JN.1.18, LB.1 and XEC [93]. The European Centre for Disease Control and Prevention (ECDC) considers (last update as of December 20, 2024) BA.2.86 and KP.3 as VOIs and XEC as VUM [94].

Some variants, particularly the Omicron subvariants (first emerged in October 2021), have accumulated many mutations, significantly diverging them from the ancestral virus (**Figure 5**). Mutations in the S protein are especially critical, as they enhance infectivity and escape from nAbs [90]. Notably, the genetic distance of these variants predicts vaccine effectiveness against symptomatic SARS-CoV-2 infection [95].

D614G

The D614G was not considered a new variant or a separate strain of SARS-CoV-2. Instead, it was a mutation —a substitution of the aspartic acid (D) at position 614 by a glycine (G) in the S protein— within the ancestral Wuhan-Hu-1 (Wu-1) virus. This mutation emerged during the first months of the outbreak (January – February 2020, **Figure 5**) and rapidly became the globally dominant form, replacing the ancestral Wu-1 virus [96]. The D614G SARS-CoV-2 exhibited increased infectivity and transmissibility compared to Wu-1 [96–101]. It was associated with higher viral loads at the nasopharyngeal region and enhanced viral replication in pulmonary epithelial cells, although it did not result in more severe disease [96–101]. Furthermore, this mutation promotes a more open conformation of the S protein, enhancing the exposure of the RBD epitopes and making them more accessible to nAbs, resulting in a moderately increased sensitivity to neutralization compared to the Wu-1 virus [100–102]. Nonetheless, the D614G mutation is critical for the survival of SARS-CoV-2 and is preserved across all subsequent variants [103].

Alpha variant

The Alpha variant (B.1.1.7 lineage) emerged in the United Kingdom in September 2020

(**Figure 5 and Table 1**) and was designated VOC from December 2020 till September 2021 [93,94]. Compared to the D614G, it carried 17 mutations, with eight (Δ69-70, Δ144, N501Y, A570D, P681H, T716I, S982A, D1118H) occurring in the S protein [104]. This variant exhibited increased transmissibility and a higher R_0 and was associated with higher hospitalization and mortality rates [104–107]. Key mutations (in addition to D614G) were Δ69-70, N501Y, and P681H, which improved infectivity and transmission by enhancing binding to the ACE2 receptor, assembly, and viral entry [108]. Notably, N501Y is one of the key contact residues within the RBM, and this mutation is conserved in most major SARS-CoV-2 variants, except the Delta variant [103].

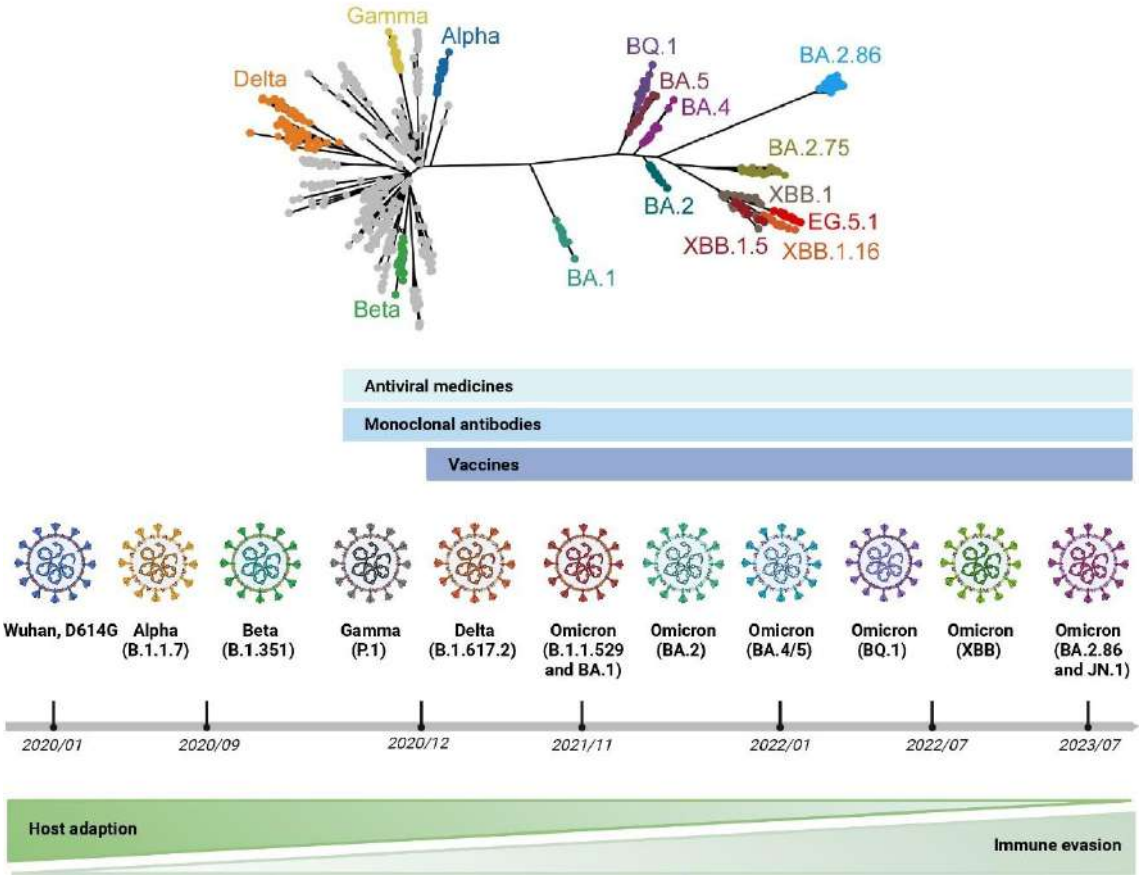

Figure 5 | SARS-CoV-2 evolution. The upper panel illustrates a maximum likelihood phylogenetic tree of SARS-CoV-2. *Reproduced with permission from Tamura et al.* [109]. The lower panel charts the evolution of the major SARS-CoV-2 variants over time, highlighting their emergence in parallel with advancements in treatments and vaccines, and its adaptive trajectory, transitioning from initial host adaptation in early variants to immune evasion mechanisms in more recent variants. *Created in https://BioRender.com.*

Beta variant

The Beta variant (B.1.351 lineage) was first detected in South Africa in September 2020 (**Figure 5 and Table 1**) and was designated VOC from January 2021 to March 2022 [93,94]. Compared to the D614G, it carried eight mutations (L18F, D80A, D215G, R246I, K417N, E484K, N501Y, and A701V) in the S protein. K417N, E484K, and N501Y were particularly significant as they enhanced infectivity and transmission by improving binding to the ACE2 receptor and altering antibody binding to RBD [110]. E484K, a hotspot for binding nAbs, contributed to reduced neutralization activity [111,112]. Beta also contained the N501Y mutation, like the Alpha variant, although the two variants arose from different viral lineages.

Gamma variant

The Gamma variant (P.1 lineage) was first reported in Brazil in December 2020 (**Figure 5 and Table 1**) and was designated VOC from January 2021 to March 2022 [93,94]. Compared to the D614G, it carried 11 mutations (L18F, T20N, P26S, D138Y, R190S, H655Y, T1027I V1176, K417T, E484K, and N501Y) in the S protein. It included key mutations K417T, E484K and N501Y. At position 417, unlike the Beta variant, where a lysine was replaced by an asparagine (K417N), in the Gamma variant, the lysine was replaced by a threonine (K417T) [103]. Combining these three mutations resulted in a 5.3-fold improvement in binding affinity to ACE2 compared to Wu-1 [113]. Despite having virtually identical mutations with the Beta variant, the sensitivity to neutralization was higher [114]. It also evolved separately from Alpha and Beta variants.

Delta variant

The Delta variant (B.1.617.2 Lineage) emerged in India in December 2020 (**Figure 5 and Table 1**) and was designated VOC from May 2021 to June 2022 [93,94]. Compared to D614G, it harbored nine mutations in the S protein (T19R, G142D*, Δ156, Δ157, R158G, L452R, T478K, P681R, D950N), with L452R, T478K and P681R identified as critical mutations. L452R and T478K were novel, and P681R was exclusive from the Delta variant, as the P681H mutation occurred in Alpha and the subsequent Omicron subvariants [103]. These mutations enhanced binding affinity to the ACE2 receptor, increasing infectivity and

Table 1 | Characteristics of major SARS-CoV-2 variants

	Lineage	Notable mutations*	First Reported	Designation	Transm.#	Immune evasion#	Severity#
Alpha	B.1.1.7	Δ69-70, N501Y, P681H	2020/10 UK	VOC 2020/12 - 2021/09	↑	~	↑
Beta	B.1.351	K417N, E484K, N501Y	2020/10 SA	VOC 2021/01 - 2022/03	↑	↑	↑
Gamma	P.1	K417T, E484K, N501Y, H655Y	2020/12 BR	VOC 2021/01 - 2022/03	↑	↑	↑
Delta	B.1.617.2	L452R, T478K, P681R	2020/12 IN	VOC 2021/05 - 2022/06	↑	↑	↑
Omicron	B.1.1.529	R346X, K444X, N460X, F490X, and many more	2021/11 SA	VOC 2021/11 - 2022/12	↑	↑	↓
	BA.1	A67V, Δ69-70, T95I, G142D, Δ143-145, N211I, Δ212, ins215EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F	2021/11 SA & BOT	VOC 2021/11 - 2022/08	↑	↑	↓
	BA.2	G142D, N211I, Δ212, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R, Q498R, N501Y, Y505H, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K	2021/11 SA	VOC 2021/11 - 2023/03	↑	↑	↓
	XBB	BA.2 + V83A, Y144-, H146Q, Q183E, V213E, G252V, G339H, R346T, L368I, V445P, G446S, N460K, F486S, F490S	2022/08 SG & IN	VOI 2022/12 - 2024/06	↑	↑	?
	BA.2.86	BA.2 + I332V, D339H, R403K, V445H, G446S, N450D, L452W, N481K, 483del, E484K, F486P	2023/07 DK	VOI 2023/11 - Curr.	NE	NE	NE
	JN.1	BA.2.86 + L455S	2023/08 US	VOI 2023/12 - Curr.	NE	NE	NE
	KP.3	JN.1 + F456L, Q493E, V1104L	2024/02 US	VUM 2024/05 - Curr.	NE	NE	NE
	XEC	JN.1 + T22N, F59S, F456L, Q493E, V1104L	2024/05 GER	VUM 2024/09 - Curr.	NE	NE	NE
	BA.4/5	L452R, F486V, R493Q	2022/01 SA	VOC 2022/05 - 2023/03	NE	↑	?
	BQ.1.1	BA.5 + R346T, K444T, N460K	2022/07 NRA	VOI 2022/10 - 2023/08	↑	↑	?

Information from [6,94]. *Plus D614G. #Compared to D614G. Transmissibility (Transm.), Currently (Curr.), No Evidence (NE), Similar (~), Unclear (?), Botswana (BOT), Brazil (BR), Denmark (DK), Germany (GER), India (IN), Nigeria (NRA), Singapore (SG), South Africa (SA), United Kingdom (UK), United States (US)

transmissibility [115]. Delta variant also showed greater escape from T-cell immunity and neutralization by antibodies [115,116]. It exhibited 1.5-fold increased transmissibility and a 20% reduction in sensitivity to host immune response compared to non-Delta earlier variants [117]. It was also associated with a higher risk of severe disease and hospitalization and higher R_0 than the Alpha, Beta, and Gamma variants [118–120]. These characteristics allowed Delta to replace preexisting variants and become the dominant variant worldwide between June and December 2021.

The Omicron subvariants

The Omicron variant (B.1.1.529 Lineage) was first reported in South Africa and Botswana in November 2021 (**Figure 5 and Table 1**) [93,94]. Within the same month, it was designated VOC and quickly replaced all previous variants to become the predominant globally [103]. Omicron accumulated more than 50 mutations, with over 30 of them in the S protein [103]. Following the original B.1.1.529 variant, BA.1, BA.2, BA.3, BA.4, and BA.5 subvariants of Omicron emerged, along with many sublineages from these subvariants, like BQ.1 from BA.5 or XBB and BA.2.86 from BA.2 (**Figure 5**). The current circulating VOIs and VUMs are descendants of this lineage [93,94].

Most Omicron subvariants shared key mutations [121], as detailed in **Table 1**. This array of mutations conferred a remarkable immune evasion, high transmissibility, and a shift toward using the TMPRSS2-independent endosomal entry pathway, which favors viral entry [122–126]. For example, the N501Y–Q498R combination of mutations increased the binding to the ACE2 receptor by almost 20 times [127], and it had a 5-fold higher relative risk of reinfection than the Delta variant [128]. Despite these adaptations, Omicron generally caused less severe disease than earlier variants [129].

The emergence of so many mutations was unexpected, and there is no clear transmission path linking Omicron directly to its predecessors. Several theories have been proposed to explain its evolution, including chronic infection in an immunocompromised individual, animal reservoir (most probably mouse or rat), and undetected spread in a population with limited genomic surveillance. However, the exact origin of Omicron remains unknown [130].

Within the Omicron lineage, several subvariants and sublineages raised alarms in the scientific community, including BA.1, BA.2, BA.4/5, BQ.1, XBB, and BA.2.86 [93,94,131]. **BA.1**, was the first Omicron subvariant, notable for its high transmissibility and immune evasion, leading to a rapid global spread in early 2022. **BA.2**, emerged shortly after BA.1, and became dominant in many regions by early to mid-2022, displaying increased transmissibility compared to BA.1., but with similar immune escape [94,132].

BA.4 and **BA.5** subvariants were quite similar and drove waves of reinfections during mid-to-late 2022 due to even greater immune escape than BA.1 and BA.2. **BQ.1/BQ.1.1**, descendants of BA.5, carried further mutations that enhanced immune escape and were responsible for significant spread between late 2022 and early 2023. **XBB/XBB.1.5**, recombinant variants derived from two BA.2 lineages (BA.2.75 and BJ), became widespread in early 2023 due to their immune evasive properties and increased transmissibility [94,133–135].

Finally, the hypermutated **BA.2.86**, also known as Pirola, emerged with substantial genetic divergence from its predecessor, the BA.2 variant, harboring 38 aa changes in the S protein. This magnitude of change is comparable to the genetic leap between the Delta and Omicron variants, raising concerns over potential immune escape [136–138]. BA.2.86 and its immediate descendants, such as KP.3 and XEC, are responsible for 99% of COVID-19 cases during November 2023 till January 2025 [139].

Several other SARS-CoV-2 variants emerged, including Kappa (B.1.617.1), Delta plus (AY.4.2), Epsilon (B.1.427/B.1.429), Zeta (P.2), Eta (B.1.525), Theta (P.3), Iota (B.1.526), Lambda (C.37), and Mu (B.1.621). However, none of these variants had a significant global impact and were never classified as VOCs [93,94].

The prevalence of different variants has varied across regions (**Figure 6**), and it is essential to consider that not all countries have equal resources for monitoring these variants [140,141]. The Alpha variant affected Asia, Europe, North America, and Africa. The Beta variant was primarily prevalent in South Africa but also spread to Asia and Europe. The Gamma variant was mainly concentrated in South America, with some spread to Europe and North America. However, the Delta and Omicron subvariants had a global impact [140].

Figure 6 | Global prevalence of SARS-CoV-2 variants. *Retrieved from https://nextstrain.org with data provided by GISAID* [140,141].

Impact of SARS-CoV-2 variants on the dynamics of the COVID-19 pandemic

The dynamics of a pandemic are shaped by a complex interplay of factors, encompassing the pathogen infectiousness and transmissibility, the population susceptibility, the effectiveness and availability of interventions such as vaccines and treatments, the speed, extent, and compliance with public health measures, as well as socio-economic and environmental elements including population density, mobility, and access to healthcare [142]. Additional determinants that influenced COVID-19 pandemic dynamics included the emergence and spread of new viral variants, the degree of cross-immunity conferred by prior infections or vaccinations, and the extent of public awareness and perception of the threat posed by the virus [143,144].

The COVID-19 pandemic has been characterized by multiple global waves, driven by the emergence of new SARS-CoV-2 variants better adapted to population contexts (**Figure 7**). Emerging variants with immune escape capabilities, combined with waning immunity from previous infections and vaccinations, have led to reinfections —defined by the US Centre for Disease Control and Prevention (CDC) as occurring ≥90 days after initial positive testing or ≥45 days with background information supporting contact with confirmed cases or the reappearance of COVID-19–like symptoms [145]— and

breakthrough infections (BTIs) —infections occurring in an individual after receiving vaccination.

The evolution of SARS-CoV-2 initially advanced slowly but accelerated over time, adapting to the population immunity (**Figure 5**) [146]. Early variants showed limited immune evasion capabilities, with mutations primarily enhancing viral reproductive success as D614G and Alpha [100,102,108]. This viral fitness enhancement may have been facilitated by the relatively low population immunity at that stage; however, as population immunity started to build through natural infection, variants began to exhibit more immune resistance, as seen with Beta, Gamma, and Delta [111,112,114–116,147,148]. The most pronounced immune evasion strategies emerged with the Omicron variant after the global population had developed immunity through widespread vaccination [122,123,146].

COVID-19 waves in Spain

SARS-CoV-2 has resulted in nearly 14 million confirmed cases and over 120,000 deaths in Spain between the start of the COVID-19 pandemic and June 2023, when the WHO declared the end of COVID-19 as PHEIC and universal surveillance was discontinued [149,150]. During the first months of the pandemic (February-April 2020), Spain, alongside San Marino and Italy, was one of the hardest-hit countries globally, with 750 daily deaths [6,151,152]. By contrast, between September 2022 and April 2023, the pandemic in Spain appeared to be under control, with ~20-30 daily deaths, representing a reduction of more than 97% [151,152]. Catalonia recorded the highest number of COVID-19 cases in Spain, exceeding 2.5 million, and together with Madrid, accounted for the most deaths, with over 20,000 fatalities [149]. Notably, Barcelona and its metropolitan area and central Catalonia —including the Berguedà, Bages, Anoia, and Osona regions— were among the most affected by COVID-19 [153]. Spain experienced seven distinct epidemic waves (**Figure 7 and Table 2**).

The First Wave (March 2020 – June 2020): During this period, Spain had one of the highest incidences of SARS-CoV-2 in Europe, with a geographical variability in seroprevalence ranging from 1.2% to 14.4% and higher prevalence in Madrid and Barcelona [154]. The first weeks of the pandemic were dominated by the ancestral strain,

with the D614G mutation being introduced in March 2020, coinciding with a strict lockdown on non-essential services and movements under the state of alarm. Pre-variant SARS-CoV-2 strains, including clades 19A, 19B, 20A, and 20B, were predominant during this first wave [155]. The country experienced a peak of ~180 cases and ~18 deaths per million population per day, resulting in over 250,000 cases and nearly 30,000 deaths [151]. There were no recordings of COVID-19 hospital admissions and ICU requirements. The state of alarm in Spain comprised this entire period, from March 14, 2020, to June 21, 2020 [155]. It is important to highlight the outbreak in the Conca d'Òdena in Anoia (Catalonia), which caused 745 cases and 147 deaths [153].

The Second Wave (June 2020 – December 2020): In June 2020, Spain began lifting some of the confinement measures, and pre-variant SARS-CoV-2 strains, including clades 20A and 20E, were predominant during this period [156]. Clade 20E was first identified in Spain and became prevalent in Western Europe by the end of 2020. However, due to its characteristics, it was not classified as a VOC but rather as an opportunistic variant [157]. In this wave, a peak of 435 cases and seven deaths per million population per day were reported, with a cumulative of more than 1.7 million cases and over 50,000 deaths [151]. More than 300 hospital admissions and 60 patients required ICU per million population per day [151].

The Third Wave (December 2020 – March 2021): The vaccination rollout in Spain began on December 27, 2020 [158]. In this period, there was a codominance of clade 20E and the Alpha variant [156]. During this wave, a peak of 745 cases and 12 deaths per million population per day were reported, resulting in a total of over 3 million cases and nearly 77,000 deaths [151]. The number of hospital admissions exceeded 470, and 100 ICU patients per million population per day [151].

The Fourth Wave (March 2021 – June 2021): The vaccination campaign significantly mitigated the pandemic's impact, reducing the cases and deaths to 700,000 and 5,000, respectively, during this period [151]. At its peak, there were 180 cases and two deaths per million population per day, bringing the cumulative total to nearly 4 million cases and over 80,000 deaths [151]. Hospital admissions reached 150 and 50 ICU patients per million population per day [151]. Alpha was the predominant variant, with the first cases of the Delta variant detected at the end of the period [156].

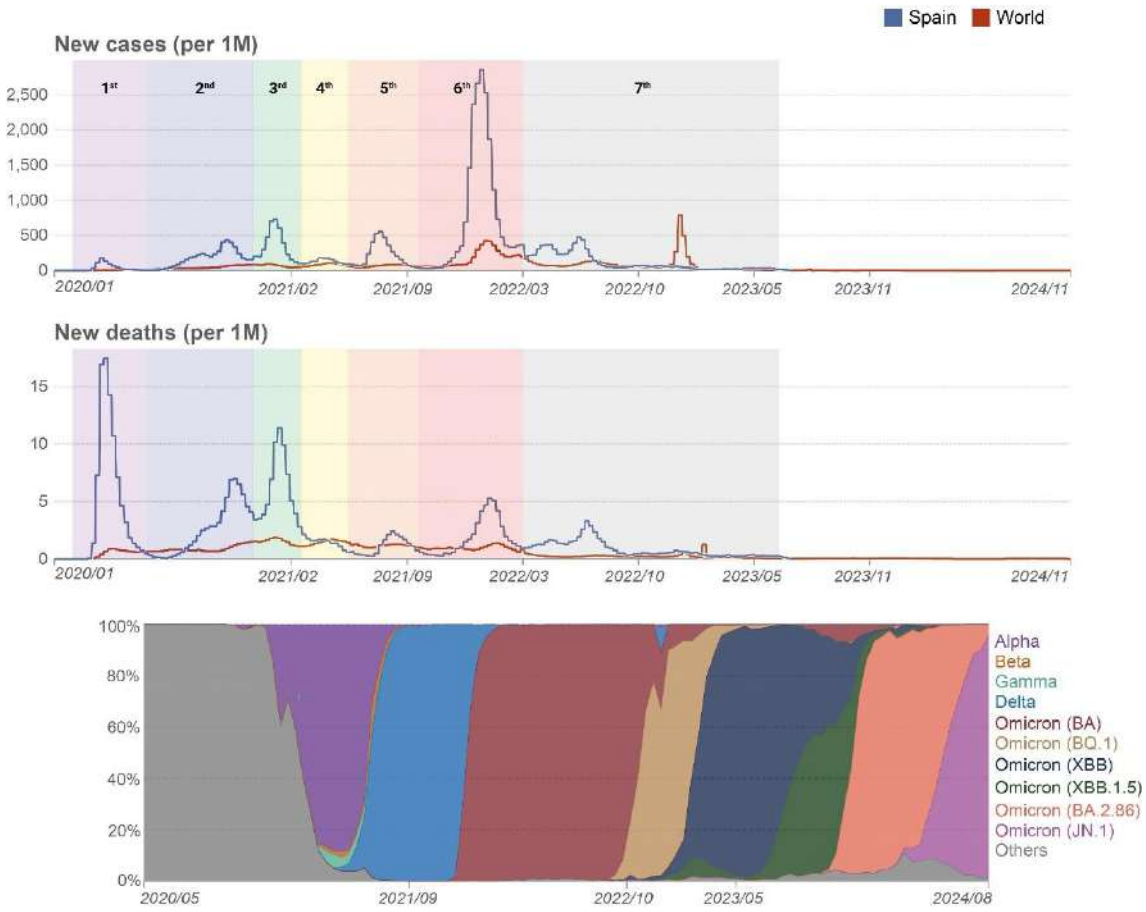

Figure 7 | COVID-19 cases, deaths, and variants over time. The upper and middle panels show the weekly confirmed COVID-19 cases and deaths per million people, respectively, from January to November 2024, in Spain and globally. The seven waves observed in Spain are highlighted in distinct colors. The lower panel illustrates the relative frequency of SARS-CoV-2 variants in Spain from May 2020 to August 2024. It should be noted that most countries ceased reporting COVID-19 cases and deaths after April 2024. *Retrieved from https:/ourworldindata.org.*

The Fifth Wave (June 2021 – October 2021): The "EU Digital COVID-19 Certificate" entered into application on July 1, 2021, facilitating traveling within the European Union for immunized individuals [159]. Spain experienced a peak of 560 cases and two deaths per million population per day, leading to a total cumulative of nearly 5 million cases and 88,000 deaths [151]. The number of hospital admissions exceeded 170, and 40 ICU patients per million population per day [151]. In this wave, Delta was the prevailing variant [156].

Table 2 | Summary of the characteristics of COVID-19 Waves in Spain.

Wave	Range and duration	Predominant variant	Cases	Deaths	Cumulative cases	Cumulative deaths
1st	Start - 21/06/2020 (99 days)	pre-variants 19A, 19B, 20A, 20B	250,000	30,000	250,000	30,000
2nd	22/06/2020 - 06/12/2020 (167 days)	pre-variants 20A, 20E	1,450,000	20,000	1,700,000	50,000
3rd	07/12/2020 - 14/03/2021 (97 days)	20E and Alpha	1,300,000	27,000	3,000,000	77,000
4th	15/03/2021 - 19/06/2021 (96 days)	Alpha	700,000	5,000	3,700,000	82,000
5th	20/06/2021 - 13/10/2021 (115 days)	Delta	1,300,000	6,000	5,000,000	88,000
6th	14/10/2021 - 27/03/2022 (164 days)	Delta, Omicron BA.1	6,500,000	16,000	11,500,000	104,000
7th	28/03/2022 - 05/07/2023 (464 days)	Omicron BA.2, BA.5, BQ.1, XBB	2,500,000	17,700	14,000,000	121,700

Approximate numbers of cases and deaths are presented in the table.

The Sixth Wave (October 2021 – March 2022): The COVID-19 booster dose was introduced during this period. This wave reached a peak of nearly 3,000 cases and five deaths per million population per day, marking it as the worst wave in number of cases. Approximately 6.5 million cases and 16,000 deaths were reported, leading to a cumulative incidence of 11.5 million cases and over 100,000 deaths [151]. More than 300 hospital admissions and 40 ICU patients per million population per day were exceeded [151]. The beginning of the wave was characterized by the Delta variant, later replaced by the first Omicron subvariant, BA.1 [156].

The Seventh Wave (March 2022 – July 2023): On March/April 2022, Spain implemented a new surveillance strategy —SiVIRA (*Sistema de Vigilancia de Infección Respiratoria Aguda*) [160] at national level and SIVIC (*Sistema d'Informació per a la Vigilància d'Infeccions a Catalunya*) [161] in Catalonia— that only records diagnoses in people aged ≥60 years and hospitalized cases of all ages. Control measures were updated,

establishing that mild and asymptomatic confirmed cases will not be isolated and close contacts will not be quarantined [162]. In this period, there was a peak of 476 cases and three deaths per million population per day, resulting in a cumulative incidence of 14 million cases and over 120,000 deaths [151]. There were more than 220 hospital admissions and 10 ICU patients per million population per day. This last epidemic period was characterized by the emergence and dominance of several successive Omicron subvariants, including BA.2, BA.5, BQ.1, and XBB [156].

Diagnostic tools

The symptoms caused by SARS-CoV-2 are very similar to those produced by other respiratory viruses, making it clinically challenging to distinguish between them [163]. Therefore, diagnostic tools for SARS-CoV-2 are essential for confirming active infections and detecting past exposure.

Real-time reverse transcription polymerase chain reaction (RT-PCR) is the gold standard for diagnosing SARS-CoV-2 infections [164]. This method detects the presence of viral RNA in samples collected from nasopharyngeal swab, saliva, or less common from other respiratory tract specimens (oropharyngeal swab, middle turbinate and anterior nares, sputum, tracheal aspirate or bronchoalveolar lavage (BAL)) [165–167]. RT-PCR is highly sensitive and specific, making it reliable for early-stage detection when viral load peaks. Although rarely, false negatives can still occur, especially in cases where viral load is low, or the sample is improperly collected. Results can take several hours to days and require specialized equipment and trained personnel [164].

SARS-CoV-2 antigen-detecting rapid diagnostic tests (RDT) have been widely adopted as a primary screening tool for SARS-CoV-2 infections in non-hospitalized populations due to their broad availability, ease of use, rapid results, and low cost [168,169]. These tests detect specific viral antigens, most commonly targeting the N protein (which is highly immunogenic, abundantly expressed during infection, and less variable than the S protein, although there is some cross-reactivity with HCoVs) [170]. Antigen RDTs are typically conducted using nasal, nasopharyngeal, and oropharyngeal swabs and may

begin to be positive some days after symptomatology [171]. However, antigen RDTs false negatives occur, in cases of low viral load or asymptomatic individuals [171].

Serological tests indirectly detect SARS-CoV-2 by measuring the antibodies produced in viral response [172]. These tests are not used for diagnosing acute infections, as antibodies take time to develop. Indeed, they are primarily used for epidemiological studies and to determine or confirm past exposures [172]. Vaccination status and the time from the last infection must be considered when interpreting serological results. Given that most of the population has received S-based COVID-19 vaccines, the presence of anti-S antibodies alone is not a reliable marker of natural infection. To address this limitation, countries like China uses a 4-fold increase in IgG levels against SARS-CoV-2 during the convalescent phase to retrospectively diagnose past infections [173].

Treatments and therapies

Throughout the pandemic, numerous potential treatments for SARS-CoV-2 have been evaluated, but only a few have demonstrated significant efficacy, and some become obsolete with the emergence of new viral variants. Current treatments and therapies to avoid severe COVID-19 include antivirals, monoclonal antibodies (mAbs), and immunomodulators (**Table 3**).

Antivirals

The antiviral drugs for SARS-CoV-2 authorized by European Medicines Agency (EMA) or U.S. Food and Drug Administration (FDA) are Paxlovid (nirmatrelvir and ritonavir), Veklury (remdesivir), and Lagevrio (molnupiravir) (**Table 3**), which also work against emerged variants. All three drugs are used for adults with mild-to-moderate COVID-19 cases to reduce the risk of hospitalization and death. In addition, Veklury is used for adults and children with pneumonia requiring supplemental oxygen [174,175].

Paxlovid inhibits the main protease (Mpro) of SARS-CoV-2 through its nirmatrelvir active compound. The co-administered ritonavir acts as a pharmacokinetic enhancer, inhibiting liver enzymes that would otherwise metabolize nirmatrelvir too quickly, thereby maintaining adequate drug levels in the body. Studies have demonstrated that Paxlovid has an efficacy of >85% in preventing severe disease, hospitalization, and death

[176–180]. In addition, a study has reported that Paxlovid reduces the risk of long COVID by 26% [181]. However, mutations in the SARS-CoV-2 Mpro may confer resistance to nirmatrelvir and similar compounds [182,183].

Remdesivir is a nucleotide analog that works by inhibiting viral RNA polymerases. Initially developed to treat hepatitis C, it was later repurposed for use against Ebola and Marburg virus infections, and it showed activity against SARS-CoV-1 and MERS-CoV [184,185]. Marketed as Veklury, remdesivir has shown promising efficacy in reducing severe COVID-19, although its impact on mortality remains uncertain in specific cases [186–189].

Molnupiravir inhibits viral replication by inducing mutations in the viral genome during replication, most of which appear deleterious and result in non-viable viral progeny. Studies have shown that molnupiravir is less effective in reducing the risk of hospitalization than previously mentioned treatments [180,190,191]. In addition, a multicenter trial concluded that Lagevrio does not significantly reduce COVID-19 hospitalization or deaths among vaccinated individuals at high risk [192]. Notably, one study suggests that molnupiravir might, in certain cases, contribute to the evolution of viral lineages. Due to these limitations, the FDA recommends its use only when alternative options are unavailable [175].

Several drugs with antiviral potential have been evaluated against COVID-19 and have not shown significant clinical benefit. These include favipiravir [193,194], ribavirin [195,196], fluvoxamine [197–199], lopinavir plus ritonavir [200,201], arbidol [202], nitazoxanide [203] and camostat mesylate [204–206], the antiparasitic ivermectin [207–212], and the antimalarials chloroquine and hydroxychloroquine [213–215].

Currently, numerous antiviral drugs are under investigation, including metformin, traditionally used for type 2 diabetes mellitus [216,217], sabizabulin [217], azvudine, xiannuoxin, simnotrelvir plus ritonavir, VV116, and RAY1216, which are approved in China [218], and researchers are looking for new compound candidates against Mpro enzyme [219,220].

mAbs

Anti-S mAbs have been shown to decrease the risk of symptomatic SARS-CoV-2 infection

when used as pre- or post-exposure prophylaxis and reduce the risk of progression to severe disease, hospitalization, and death if used early in the course of infection [221–223]. However, their usefulness has been hampered by the emergence of SARS-CoV-2 variants resistant to neutralization [224–226]. As of December 2024, four anti-S mAb are authorized by EMA and/or FDA for individuals >12 years old who do not require supplemental oxygen and are at increased risk of severe disease. These include Ronapreve (casirivimab/imdevimab), Regkirona (regdanvimab), Evusheld (tixagevimab/cilgavimab), and Xevudy (sotrovimab) (**Table 3**)**.** However, both agencies no longer recommend using these mAbs due to their reduced or no efficacy against dominant Omicron subvariants [175,224].

Recently, many studies have described mAbs with broad neutralizing activity across multiple variants, demonstrating efficacy even against Omicron subvariants [227–235]. Other approaches with mAbs are being investigated, such as the use of ACE2 agonists, which block the infection but do not affect ACE2 expression or enzymatic activity in mice [229,236–239]. Anti-ACE2 mAbs may have potential therapeutic and/or prophylactic agents capable of circumventing the mutations found in the emerging variants.

Immunomodulators

The FDA and EMA treatment guidelines for COVID-19 include four immunomodulators (**Table 3**): RoActemra (tocilizumab), Kineret (anakinra), Olumiant (baricitinib), and Gohibic (vilobelimab), to reduce the excessive pro-inflammatory response in COVID-19 in patients requiring supplemental oxygen and who are at risk of developing severe respiratory failure [174,175].

Tocilizumab, an anti-IL-6 receptor mAb, treats rheumatoid arthritis and other inflammatory conditions. Clinical trials have demonstrated that tocilizumab, when administered with corticosteroids —potent anti-inflammatory agents— provides a moderate survival benefit in patients with severe or critical COVID-19 [240–242]. However, other studies have not found a significant benefit [243–245].

Anakinra, a drug used for rheumatoid arthritis, is a recombinant protein that mimics the human IL-1 receptor antagonist. Some studies suggested that anakinra benefits severe COVID-19 patients by reducing the need for invasive mechanical ventilation and the

mortality risk of hospitalized non-intubated patients [246–248]. However, other studies reported no significant effect in SARS-CoV-2 infected hospitalized adults [249–251].

Baricitinib is a janus kinase inhibitor used to treat rheumatoid arthritis, alopecia areata, and COVID-19. It offers potential benefits for inpatients with COVID-19, particularly the elderly [252–254]. Vilobelimab is an anti-C5A mAb that blocks the complement system. The clinical trial showed that participants treated with vilobelimab had a lower risk of death compared to placebo [255]. The FDA has authorized it for hospitalized adults with ARDS receiving corticosteroids [175].

Table 3 | Summary of COVID-19 treatments authorized by EMA and FDA.

Company	Name	Type	EMA	FDA	Use*	Given
Pfizer	Paxlovid (nirmatrelvir and ritonavir)	Antiviral	YES	YES	adults, EUA for >12 yo	Oral
Gilead Sciences	Veklury (remdesivir)	Antiviral	YES	YES	adults and children	I.v.
Merck Sharp & Dohme B.V.	Lagevrio (molnupiravir)	Antiviral	NO	EUA	adults	Oral
Roche/ Regeneron	Ronapreve (casirivimab / imdevimab)	mAb	YES	NO	>12 yo	I.v. / I.m.
Celltrion	Regkirona (regdanvimab)	mAb	YES	NO	certain adults	I.v.
AstraZeneca	Evusheld (tixagevimab / cilgavimab)	mAb	YES	NO	>12 yo	I.m.
GSK/Vir Biotechnology	Xevudy (sotrovimab)	mAb	YES	NO	>12 yo	I.v.
Roche/ Genentech	RoActemra (Tocilizumab)	Immunom.	YES	YES	adults, EUA for children	I.v.
Sobi	Kineret (anakinra)	Immunom.	YES	EUA	adults	I.m.
Eli Lilly	Olumiant (baricitinib)	Immunom.	NO	YES	adults, EUA for children	Oral
InflaRx	Gohibic (vilobelimab)	Immunom.	NO	EUA	adults	I.v

*Usage in certain populations. Emergence Use Authorization (EUA), Immunomodulator (Immunom.), Intramuscular (I.m.), Intravenous (I.v.), monoclonal antibody (mAb), years old (yo).

Many treatments have been tested for COVID-19, but most have failed to show significant clinical benefits in trials. For instance, treatments involving IFNs or supplements such as vitamin C, vitamin D, and zinc gluconate have shown no consistent efficacy. Similarly, convalescent plasma and hyperimmune immunoglobulin (Ig) —a concentrated preparation of purified antibodies derived from pooled convalescent plasma or immunized animal sources— have not yielded promising results [256]. In light

of these limitations, innovative treatment approaches are emerging, including CAR-T cells engineered to target viral components [257,258], ACE2-Fc decoy fusion protein to neutralize the virus and induce Fc-effector functions [259–261], allogenic natural killer (NK) cells immunotherapy [262], and mRNA vaccines as a treatment encoding a broadly nAb potent inducer of Fc-effector functions. These strategies hold significant potential for advancing COVID-19 treatment and prevention.

COVID-19 vaccines

Vaccines, one of the oldest and most cost-effective medical interventions in modern medicine, are among our most powerful tools in the fight against infectious diseases, having saved countless lives throughout human history [263,264]. Vaccination campaigns declared smallpox and rinderpest eradicated worldwide in 1980 and 2011, respectively [265]. Other infectious diseases, such as poliomyelitis, measles, mumps, and rubella, are also nearing eradication due to continued immunization efforts [265]. Despite the 27 licensed vaccines protecting against more than 20 life-threatening infectious diseases in humans, the development of vaccines for many pathogens remains a significant challenge, such as for HIV, hepatitis C, Epstein-Barr, Zika and Marburg viruses, syphilis (*Treponema pallidum*), Leishmaniasis, and gonorrhea (*Neisseria gonorrhoeae*) [266].

The rapid development of the COVID-19 vaccines was made possible by decades of progress in pathogen surveillance, genomics, structure-based antigen design, delivery technology, and human immunology, which converged during the pandemic, as well as significant investments by pharmaceutical companies that produced large quantities of vaccines before they were approved for distribution [263]. According to the WHO, by the end of December 2023, over 13 billion vaccine doses had been administered globally. Approximately 67% of the global population has received a complete primary vaccination series, while 32% received at least one vaccine dose [6]. However, vaccine distribution remains highly unequal (**Figure 8**). High-income countries such as the United Arab Emirates, Qatar, Puerto Rico, and Chile have achieved vaccination rates exceeding 90% of their populations with the initial full vaccination schedule, while low and middle-income countries such as Haiti, Yemen, Papua New Guinea, and Senegal yet to reach even 5% [6].

As of March 2023 (last update), 50 COVID-19 vaccines, based on different technologies, including adenoviral vectors, mRNA, protein subunit, and inactivated vaccines, have been authorized for use in at least one country. Additionally, more than 180 vaccine candidates were under development as of March 2023 [267,268]. The WHO has authorized or included in its emergency use list 11 vaccines, the EMA authorized four vaccines for use within the European Union, and the FDA three vaccines for use in the United States [174,267,269]. All approved COVID-19 vaccines by the FDA and/or EMA are administered via intramuscular injection. Authorized COVID-19 vaccines are detailed in **Table 4**.

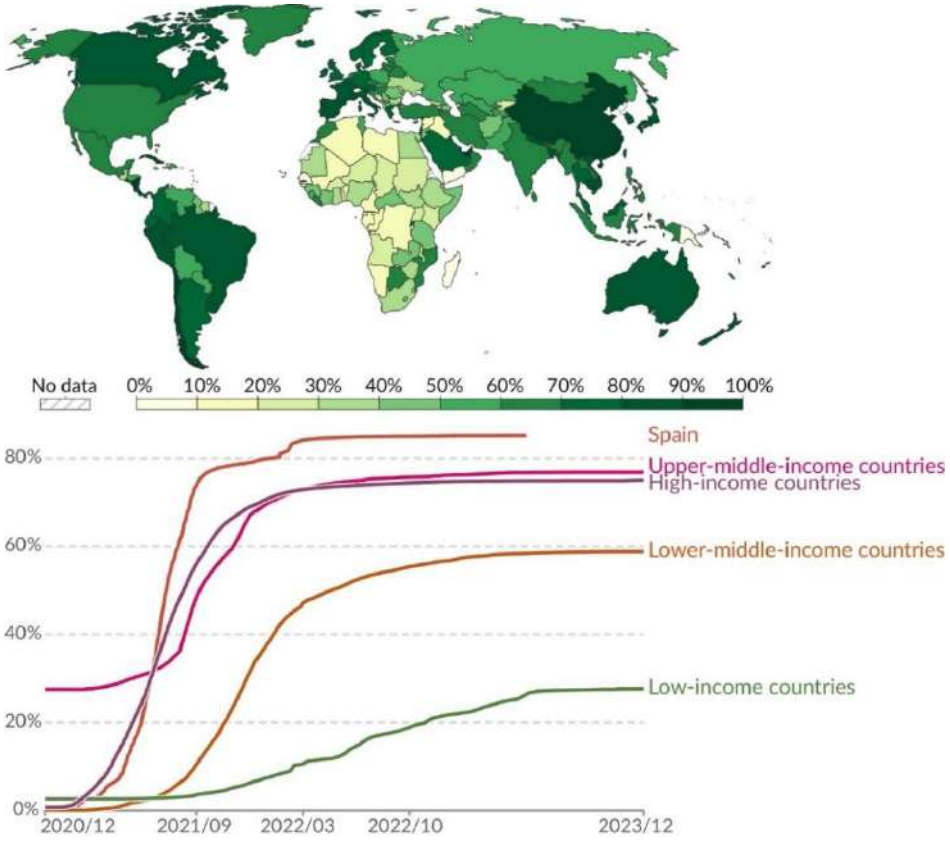

Figure 8 | Share of people who completed the initial COVID-19 vaccination schedule worldwide. Total number of individuals who completed the initial vaccination protocol, divided by the total population of the country. The upper panel illustrates the global vaccination coverage (%) for each country globally as of December 2023. The lower panel shows the progression of vaccination coverage in Spain and across regions categorized by income level from December 2020 to December 2023. *Retrieved from https:/ourworldindata.org.*

COVID-19 vaccine types being administered include: i) adenoviral vector vaccines, which use a harmless adenovirus as a delivery system to carry the genetic material encoding the S protein into human cells. Once inside, cells produce the S protein, triggering an immune response. ii) mRNA vaccines, which deliver a synthetic mRNA sequence encoding the S protein into cells. Once inside, cells produce the S protein, triggering an immune response. iii) Protein subunit vaccines, which contain purified pieces of the S protein combined with an adjuvant to boost the immune response. iv) inactivated vaccines, which use the whole virus chemically or physically inactivated so they can no longer cause infection and can elicit an immune response against multiple viral components.

COVID-19 vaccines have significantly mitigated the health and economic burden of the pandemic by preventing severe disease, hospitalizations, and deaths. It is estimated that nearly 20 million potential COVID-19 deaths were averted globally within the first year of vaccine rollouts [270]. Vaccination has also been shown to reduce the transmission of SARS-CoV-2 and the probability of reinfection [271–274]. Moreover, COVID-19 vaccination has been associated with reduced severity and duration of long COVID symptoms and lower risk of developing MIS-C [60,82]. Importantly, COVID-19 vaccines have proven to be safe, with most adverse events being mild and transient, with infrequent serious reactions [6]. However, adenoviral vector vaccines, AZD1222-Oxford/AstraZeneca and Ad26.COV2.S-Janssen/J&J, have demonstrated a slightly higher risk (though low incidence) of thrombosis and thrombocytopenia, linked to serum antibodies against platelet factor 4 complexes [275–279].

Initial studies reported 94-95% protection against symptomatic COVID-19 provided by mRNA vaccines [280,281]. However, vaccine effectiveness decreased over time and was further impacted by the emergence of more immuno-evasive variants [282–285]. This led to the promotion of booster dose administration and the development of updated vaccines incorporating the S protein from new variants as a monovalent dose or combined with the ancestral S protein as a bivalent dose. Both strategies have shown evidence of restoring the effectiveness of primary vaccination and offering protection against the new SARS-CoV-2 variants [284–286]. Nevertheless, booster doses are mostly recommended

for the elderly, immunocompromised, and individuals with high risk of developing COVID-19 or high exposure, such as HCWs [6].

Table 4 | Summary of COVID-19 vaccines authorized by WHO, EMA and FDA.

Company	Name	Type	Doses	Virus	WHO	EMA	FDA	USE
Pfizer/ BioNTech	Comirnaty (BNT162b2)	mRNA	2	S Ancestral	Yes	Yes	Yes	>6m
	Original/Omicron BA.1		Boost.	S Anc. + BA.1				
	Original/Omicron BA.4/5			S Anc. + BA.4/5				
	Omicron XBB.1.5			S XBB.1.5				
	Omicron JN.1			S JN.1				
	Omicron KP.2			S KP.2				
Moderna	Spikevax (mRNA-1273)	mRNA	2	S Ancestral	Yes	Yes	Yes	>6m
	mRNA-1273.214		Boost.	S Anc + BA.1				
	mRNA-1273.222			S Anc + BA.4/5				
	mRNA-1273.815			S XBB.1.5				
	mRNA-1273.167			S JN.2				
Novavax	Nuvaxovid [Covovax in India] (NVX-CoV2373)	Protein Subunit	2	S Ancestral	Yes	Yes	Yes	>12 yo
	XBB.1.5		Boost.	S XBB.1.5				
	JN.1			S JN.1				
HIPRA	Bimervax (PHH-1V)	Protein Subunit	Boost.	RBD Alpha + Beta	No	Yes	No	>16 yo
Janssen/ J&J	Jcovden (Ad26.COV2.S)	Viral Vector	1	S Ancestral	Yes	No	No	>18 yo
Oxford/ AstraZeneca	Vaxzevria [Covishield in India] (AZD1222)	Viral Vector	2	S Ancestral	Yes	Yes	No	>18 yo
CanSino Biologics	Convidecia (Ad5-nCoV)	Viral Vector	1	S Ancestral	Yes	No	No	>12 yo
Biological E. Limited	Corbevax	Protein Subunit	2	S Ancestral	Yes	No	No	>12 yo
SK Bioscience	Skycovione	Protein Subunit	2	S Ancestral	Yes	No	No	>18 yo
Bharat Biotech	Covaxin (BBV152)	Inactivated	2	Ancestral	Yes	No	No	>18 yo
Sinopharm	Covilo (BBIBP-CorV)	Inactivated	2	Ancestral	Yes	No	No	>18 yo
Sinovac	CoronaVac	Inactivated	2	Ancestral	Yes	No	No	>6 yo
Valneva	COVID-19 Vaccine (VLA2001)	Inactivated	2	Ancestral	No	Yes	No	18-50 yo

All vaccines are administered intramuscularly. Ancestral (Anc.), Booster (Boost.), Months (m), Years old (yo).

In addition to the decline in vaccine effectiveness due to the antibody waning and the emergence of new SARS-CoV-2 variants, other major concerns, such as global vaccine accessibility and vaccine hesitancy, have had a significant impact on the course of the pandemic [287–289]. Vaccine hesitancy has led to stark disparities in vaccination rates, especially among children [290–293]. For instance, in Chile, which boasts one of the highest

COVID-19 vaccination rates globally, approximately 90% of children aged 3 to 17 years are fully immunized. In contrast, Spain has achieved a vaccination rate of just over 50% in the same age group. In other European countries, including France, Switzerland, and England, fewer than 5% of children have been vaccinated against SARS-CoV-2 [294]. This vaccine hesitancy raises concerns given the widespread availability and importance of authorized vaccines for this specific age group, as well as for other non-COVID-19 immunizations [81,295–297].

As explained in section Adaptive immunity to SARS-CoV-2, COVID-19 vaccines elicit a robust immune response —binding antibodies, with neutralizing activity and Fc-effector functions, generation of memory B cells (MBCs), germinal centers (GCs) reactions, and memory $CD4^+$ and $CD8^+$ T cells— that confers protection. However, intramuscularly administered COVID-19 vaccines have shown limitations in inducing adequate mucosal immunity, which is critical for preventing initial infection in the respiratory tract. Thus, ongoing research is exploring the development of intranasal vaccines to improve mucosal immunity [298–306]. Notably, in the last quarter of 2022, the first intranasal COVID-19 vaccines were approved as booster doses. These include Convidecia Air-CanSino Biologics in China and iNCOVACC-Bharat Biotech in India [307–312]. Additionally, most currently approved vaccines are based on the S protein, which is prone to mutations in emerging variants. To overcome this limitation, researchers are actively working on the development of vaccines targeting alternative antigens beyond the S glycoprotein, aiming to broaden and enhance the immune response [313–317]. Additionally, other vaccine types and compositions are currently under investigation. These include self-amplifying mRNA vaccines [318,319], single-cycle infection SARS-CoV-2 viruses [320], protein subunit vaccines including RBD-Fc [321] or RBD with hotspot mutations [322].

The host immune response to SARS-CoV-2

The human immune system is a sophisticated network of organs, tissues, cells, and molecules coordinated to protect the body from internal and external threats such as cancer and pathogens, respectively. Broadly, immunity is classified into innate and adaptive (**Figure 9**) [323].

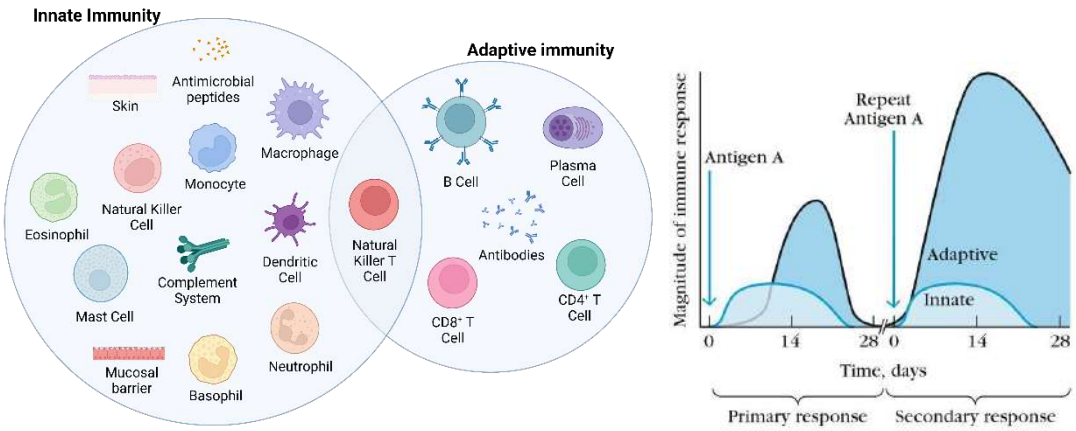

Figure 9 | Innate and adaptive immunity. The left panel shows the components of innate and adaptive immunity. *Created in BioRender.com.* The right panel illustrates the innate and adaptive immune responses during primary and secondary responses. *Retrieved with permission from Kuby Immunology 7th edition* [324].

Respiratory viruses predominantly induce acute infections within the upper respiratory tract, typically controlled and eliminated by the immune system [325,326]. Nevertheless, the mechanisms by which the immune system recognizes, fights, and eliminates viruses in the human body have yet to be fully elucidated [326,327]. This gap in understanding hinders the development of efficacious vaccines or therapeutic interventions for numerous respiratory viruses.

Overview of innate immunity

Innate immunity (**Figure 9**) is the first line of defense, offering rapid and non-specific protection against invading pathogens [323,328,329]. It involves anatomical barriers: physical (e.g., epithelial layers of the skin and of the mucosal) and chemical (e.g., pH, antimicrobial peptides, and enzymes) [328]. However, if these barriers are breached, the cellular innate responses are activated. The cellular innate component includes various immune cells such as monocytes, macrophages, neutrophils, basophils, eosinophils, mast cells, dendritic cells (DCs), and NK cells. These cells carry out multiple essential functions like phagocytosis, inflammation, production of antimicrobials, and elimination of infected cells [328]. Despite its non-specific nature, the innate immune system distinguishes between self and non-self-entities using pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs), RIG-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptor (CLRs) that bind pathogen-associated

molecular patterns (PAMPs) present on microbes [328,329]. In addition, innate immunity has the capacity for trained immunity, in which innate cells undergo long-term functional reprogramming in response to exogenous or endogenous stimuli, leading to an altered response to succeeding challenges [330]. Furthermore, the innate systems also include the complement system, proteins that cooperate to eliminate pathogens. Despite the multiple layers of the innate immune system, some pathogens can evade its defenses. In such cases, the adaptive immune system —triggered and shaped by the innate response— comes into play to provide specific and long-lasting protection [328].

Innate immunity to SARS-CoV-2

Innate immune cells recognize the SARS-CoV-2 through TLRs (TLR 2, 3, 4, 7, 8, and 9), RLRs (RIG-I and MDA5), and NLRs (NLRP3). These PRRs initiate signaling cascades with subsequent production of various cytokines, which alert the immune system and recruit additional cells to the site of the infection. The main cytokines produced with antiviral function are type I (IFN-α and IFN-β) and type III (IFN-λ) IFNs, and with inflammatory functions are TNF-α, IL-1, IL-6, IL-12, IL-1β, and IL-18 [326,331,332].

Current evidence suggests that type I IFN-mediated immunity is critical to the clinical course of COVID-19 and shapes the subsequent immune responses [333–335]. Impaired type I and III IFN responses have been associated with severe disease and increased risk of critical pneumonia and mortality [336]. However, the excessive release of cytokines contributes to the COVID-19 pathogenesis, promoting inflammatory cell death and perpetuating the cytokine storm [337–339].

NK cells are critical effectors of antiviral immunity, primarily due to their cytotoxicity activity and their production of IFN-γ —type II IFN, potent macrophage activator and help activating and shaping adaptive immune responses— and TNF-α. Individuals with COVID-19 showed reduced NK cell counts, likely due to cell death and the redistribution to infected sites. In addition, NK cell cytotoxicity is negatively correlated with COVID-19 severity [331,340].

Macrophages, the most abundant cell type in the lungs under homeostatic conditions, play a key role in mediating rapid responses by recruiting other immune cells, regulating inflammation, and serving as antigen-presenting cells (APCs). Alongside DCs, which are

professional APCs, both cell types are essential for activating antigen-specific T cells and amplifying inflammation [331].

Although vaccines aim to generate memory through adaptive immune responses, they also stimulate innate immunity. For example, it has been shown that COVID-19 mRNA, adenoviral, and inactivated vaccines induce the production of type-I IFN, and recombinant-protein-based vaccines produce IL-1β and IL-18 [331].

As stated in the Viral structure and genome section, SARS-CoV-2 and its emerging variants have developed multiple strategies to evade innate host immune responses, including masking its genome, blocking host recognition, blocking IFN signaling, and reducing protein trafficking [332]. For example, NSP15, ORF3a, ORF3c, ORF7a, ORF10, E, and M proteins have been reported to manipulate and antagonize autophagy, while ORF3b, ORF6, ORF7a, ORF8, ORF9b, ORF10, NSP3, NSP13, NSP14, and NSP15 interfere with IFNs [341]. Furthermore, studies indicate that the virus can increase pro-apoptotic pathways in DCs and reduce MHC expression on monocytes, macrophages, and DCs, thereby impairing antigen presentation [331,342].

Overview of adaptive immunity

Adaptive immunity (**Figure 9**) is the second line of defense, providing specific protection and developing immunological memory against invading pathogens. This memory is crucial for mounting a faster and more effective response upon a pathogen re-exposure. The adaptive immune system comprises T and B lymphocytes, plasma cells, and their secreted antibodies. Adaptive immune cells are predominantly localized within tissues and circulation, while antibodies are soluble mediators found mainly in blood and bodily secretions [343]. For practical purposes, adaptive immunity is generally divided into two complementary branches: cell-mediated immunity (T cells) and humoral immunity (B cells, plasma cells, and antibodies) [344]. Its specificity lies in recognizing antigens through specific receptors: T- and B-cell receptors (TCR and BRC, respectively). The specific part of the antigen these receptors recognize is called epitope or antigen determinant. Thus, TCR and BCR recognize T- and B-cell epitopes, respectively.

During a primary encounter with a pathogen, adaptive immunity, consisting of antigen-inexperienced (naïve) cells, requires time to develop. However, upon re-exposure

(secondary response), memory T cells are rapidly activated, proliferate, and produce effector molecules at faster rates and greater magnitudes. Similarly, MBCs are quickly activated and differentiated into antibody-secreting cells (ASCs) more swiftly and undergo processes that enhance their antigen affinity and functionality [343,344]. The capacity of T and B lymphocytes to form memory cells following antigen exposure is the rationale behind vaccination. Understanding immune memory is essential for designing more efficacious vaccines and developing immunotherapies for infectious diseases and cancer [343].

T-cell mediated responses

T lymphocytes are categorized into two major subsets based on the expression of surface proteins: $CD4^+$ helper T (T_H) cells and $CD8^+$ cytotoxic T (T_C or CTLs) cells. $CD4^+$ T_H cells help B cells to produce antibodies and activate other cellular types, while $CD8^+$ Tc cells kill infected and cancerous cells and recruit other immune cells [324]. T cells can only recognize —through TCRs— antigen fragments when they are presented on the surface of other cells via MHC molecules. Specifically, MHC-I, expressed on all nucleated cells, presents processed endogenous (intracellular) antigens to $CD8^+$ Tc cells. In contrast, MHC-II, expressed on APCs (DCs, macrophages, and B cells), presents processed exogenous (extracellular) antigens to $CD4^+$ T_H cells [324].

During a primary immune response, APCs have coordinated interactions with T and B lymphocytes in secondary lymphoid tissues near the site of infection. After the innate immune response, DCs bearing the virus migrate to nearby lymph nodes (e.g., lung-associated lymph nodes in a respiratory infection). Once there, DCs present viral antigens on their surface via the MHC molecules to antigen-specific naïve T cells. TCR and costimulatory engagement lead to T-cell activation, IL-2 production (which stimulates T-cell proliferation), and, together with local cytokines, differentiation into effector $CD4^+$ or $CD8^+$ T cells [324,343].

These local cytokines, known as polarizing cytokines, drive the differentiation of $CD4^+$ T lymphocytes into distinct effector $CD4^+$ T_H subsets: T_H1, T_H2, T_H17, T_H9, T_H22, T follicular helper (T_{FH}), and T regulatory (T_{REG}). Each subset secretes a signature set of cytokines and orchestrates specific immune functions within the body [324,345–348], as outlined in

Figure 10. This functional diversity enables the immune system to tailor responses to a particular pathogen. For example, viral infections induce IL-12, IFN-γ, and IL-18 polarizing cytokines (produced by DCs, NKs, T cells, etc.) to promote the differentiation of activated $CD4^+$ T cells into T_H1 cells, which, in turn, will encourage B cells to produce antibodies that contribute to innate cell-mediated immunity —supporting phagocytosis and complement fixation— (e.g., IgG1 and IgG3), and enhance $CD8^+$ T cells, which will kill infected cells. In addition, T_{REG} suppress immune responses.

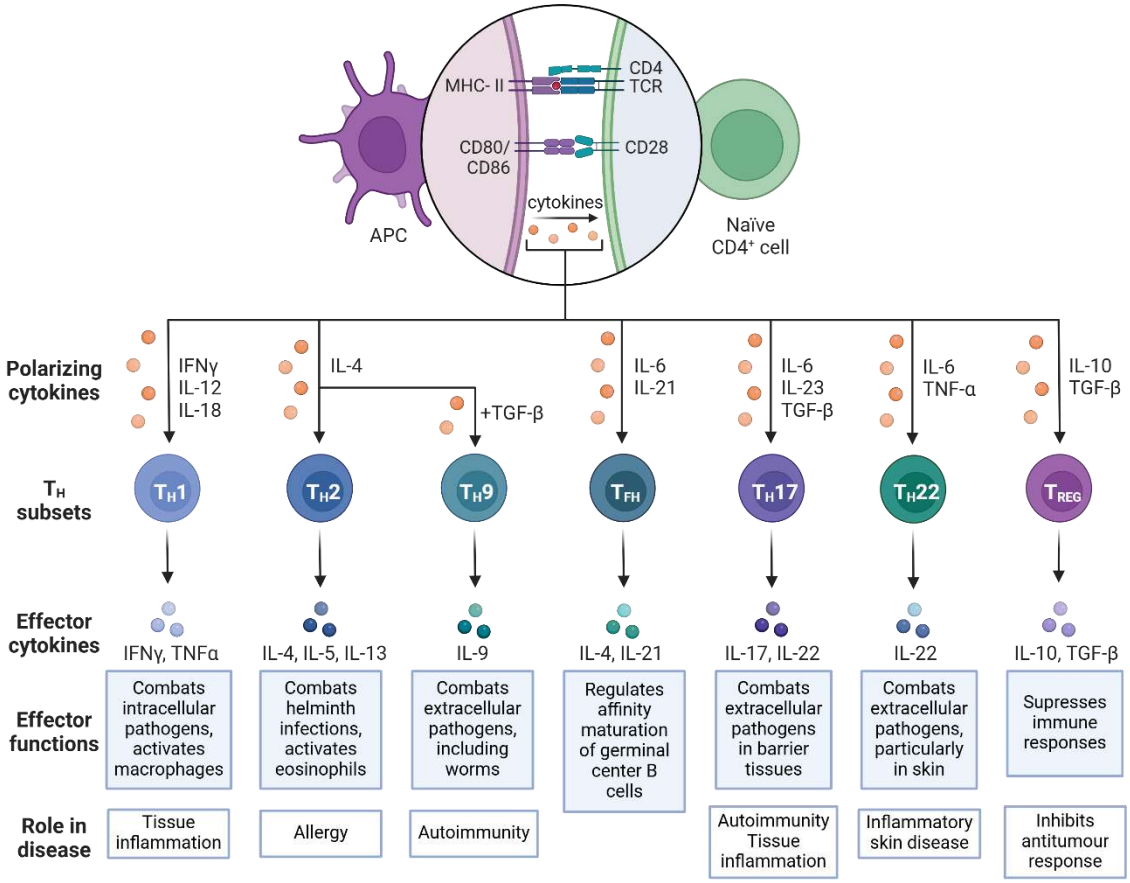

Figure 10 | T helper (T_H) subset differentiation. *Created in BioRender.com.* APC: Antigen-presenting cell; IFN: Interferon; IL: Interleukin; MHC: Major histocompatibility complex; TCR: T-cell receptor; TGF: Transforming growth factor; TNF: Tumor necrosis factor.

Effector T lymphocytes rapidly proliferate —clonal expansion— and circulate through the bloodstream and various tissue sites to eliminate the pathogen. Once the pathogen is cleared, most of the expanded immune effector T-cell population undergoes apoptosis during the contraction phase of the immune response (**Figure 11**). However, a small fraction persists as memory $CD4^+$ and $CD8^+$ T cells, which are more easily

activated and responsible for secondary responses [324,343]. Memory $CD8^+$ T cells are usually more abundant than their $CD4^+$ counterparts due to their more robust proliferation and longer lifespan than $CD4^+$ T cells [324,343]. Different memory T-cell subsets can be identified based on surface markers, and they differ in phenotype, location, and function. The T-cell memory subsets include stem cell memory T cells (T_{SCM}), central memory T cells (T_{CM}), effector memory T cells (T_{EM}), terminally differentiated effector memory T cells re-expressing CD45RA (T_{EMRA}), and resident memory T cells (T_{RM}). As memory cells differentiate, there is a progressive loss of self-renewal potential and lifespan from T_{SCM} to T_{EMRA} [324,343,349–351].

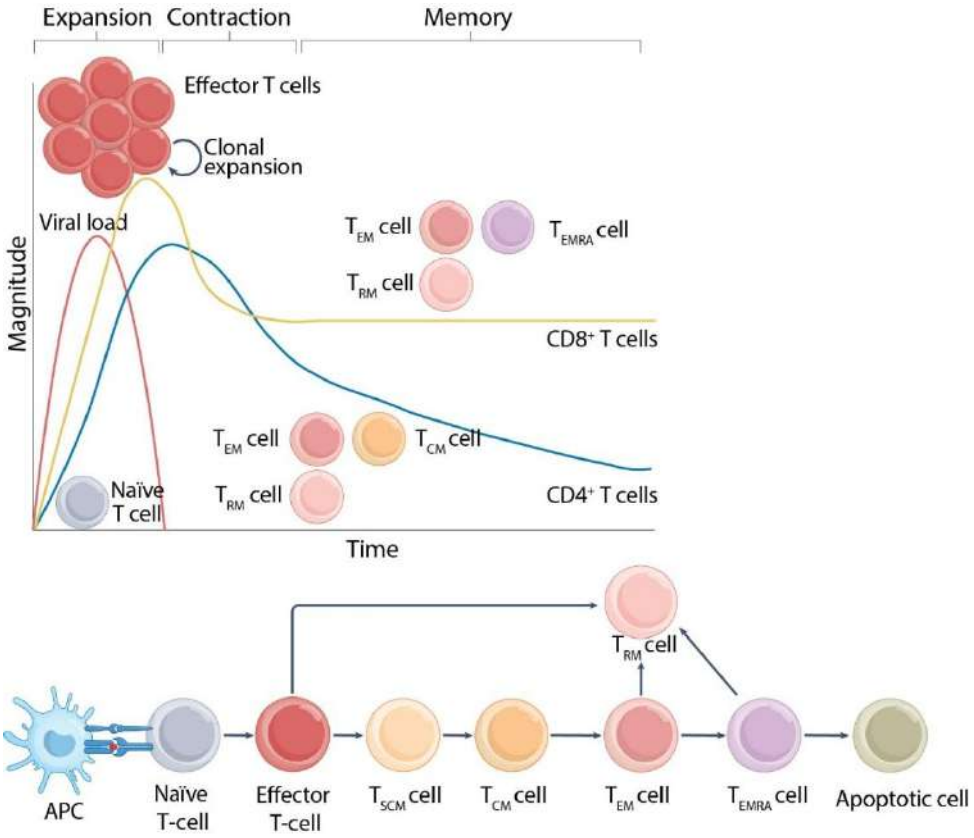

Figure 11 | Memory T-cell kinetics and differentiation after viral infection. The upper panel illustrates the T-cell response in the three phases known as expansion, contraction and memory phases. The lower panel displays the progressive differentiation model for the generation of memory T cells. *Adapted with permission from Lam et al.* [343] . T_{SCM}: stem cell, T_{CM}: central memory, T_{EM}: effector memory T_{EMRA}: terminally differentiated re-expressing CD45RA; T_{RM}: resident memory T cells.

T_{SCM} are the least differentiated memory cells found in lymphoid tissues and can develop into other memory T-cell subsets (**Figure 11**). T_{CM}, mostly $CD4^+$, also reside in lymphoid tissues, generating a high amount of IL-2 but lower amounts of other effector cytokines (compared to T_{EM}). T_{EM} are found in lymphoid and mucosa tissues and secrete low levels of IL-2 but high levels of effector cytokines (compared to T_{CM}). T_{EMRA}, primarily $CD8^+$, are found in circulation (the lung, blood, and spleen) and produce inflammatory cytokines. Finally, T_{RM} reside in mucosal tissues and rapidly produce IFN-γ, IL-2, and IL-17 to provide localized protection at the tissue site [324,343,349,351]. The precise signals inducing the commitment to memory T-cell differentiation remain unknown, although the strength and duration of antigen stimulation are critical factors in its formation [324,352].

Humoral responses

Antibodies, also called Igs (**Figure 12**), are the main players of humoral immunity. These glycoproteins have two general structural regions: the variable region, forming the antigen-binding site, that defines antigen specificity, and the constant region, which determines the antibody isotype (IgD, IgM, IgG, IgA, IgE) and subclass (IgG1, IgG2, IgG3, IgG4, IgA1, IgA2) [324]. These regions constitute three fragments in a monomer: two identical antigen-binding fragments (Fab) and one crystallizable fragment (Fc). The Fc fragment confers functional specialization through binding to cellular receptors and the complement system. Each Fab can be coupled to any of the different Fc fragments, tailoring the effector functions to suit the pathogen best [324]. Glycosylation in Fab and Fc regions can significantly influence antigen binding and effector functions by mediating optimal binding. Notably, glycosylation patterns vary across different antibody isotypes. In addition, glycans are involved in antibody solubility, conformation, transport, secretion, and clearance [353].

Antibodies recognize and bind unprocessed antigens and mediate protection through neutralization and Fc-effector functions, including phagocytosis, cytotoxicity, and complement fixation (**Figure 13**) [324,354]. Neutralizing antibodies prevent pathogens from infecting their host cells (e.g., in SARS-CoV-2, inhibiting the interaction of S with ACE2, inhibiting S conformational changes, binding to S key sites during membrane fusion or causing aggregation of viral particles) [355,356]. Opsonizing antibodies —antibodies coat the surface of a pathogen, making it more attractive to phagocytes— trigger phagocytosis

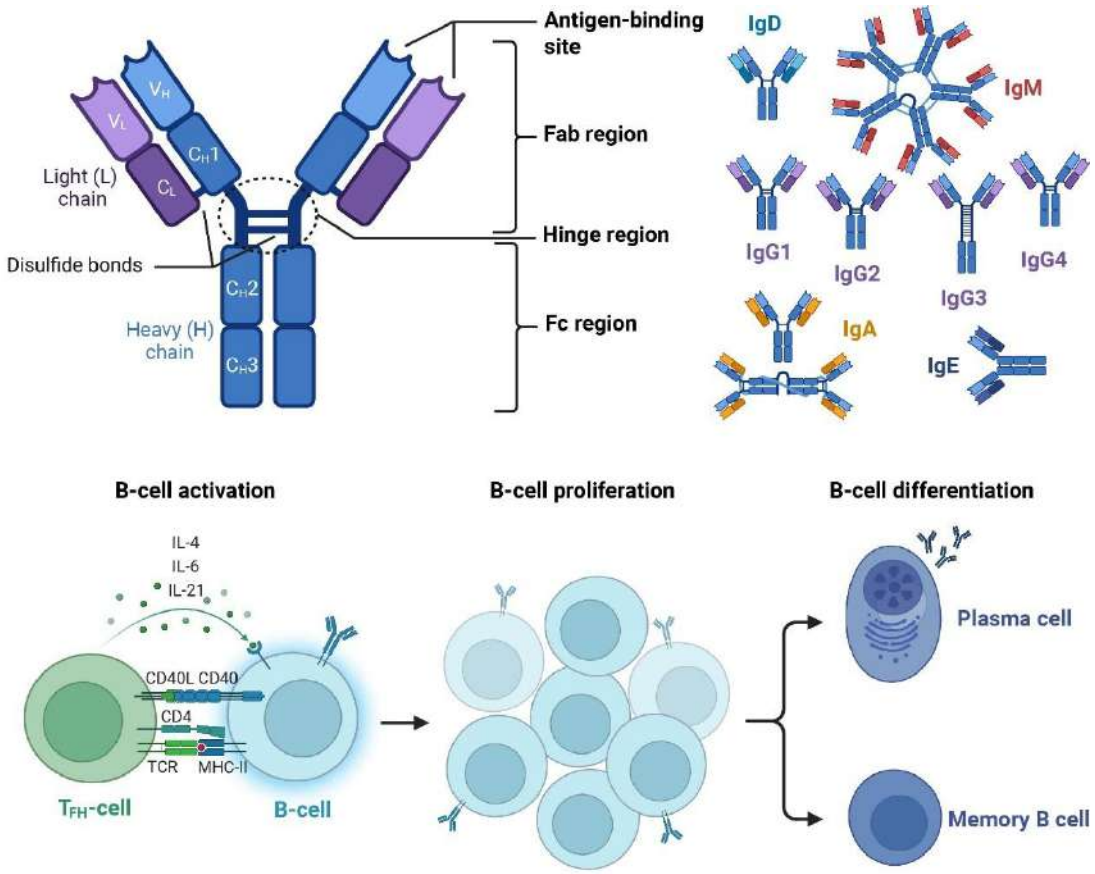

Figure 12 | Antibodies and B-cell differentiation. The upper panel shows the structure of an antibody and the different isotypes. The lower panel shows the B-cell differentiation into plasma and memory B cells. *Created in BioRender.com.* V_L: variable light; V_H: variable heavy, C_L: constant light; C_H: constant heavy; Fab: antigen binding fragment; Fc: crystallizable fragment. IL: Interleukin, MHC: Major histocompatibility complex; TCR: T-cell receptor; T_{FH}: T follicular helper.

on phagocytic cells such as monocytes, macrophages, and neutrophils, a process called antibody-dependent cellular phagocytosis (ADCP). In addition, antibodies bound to antigens on the surface of the body's cells or in the pathogen can initiate antibody-dependent cellular cytotoxicity (ADCC), activating cytotoxic cells such as NKs and granulocytes to induce targeted cell death. Finally, antibodies can also activate the complement system by antibody-dependent complement deposition (ADCD), leading to phagocytosis or direct lysis of the pathogen [324]. Local cytokines modulate these Fc-effector functions by, for instance, upregulating Fc-receptor expression or amplifying complement activation, thereby fine-tuning the immune response to the specific pathogen context [324].

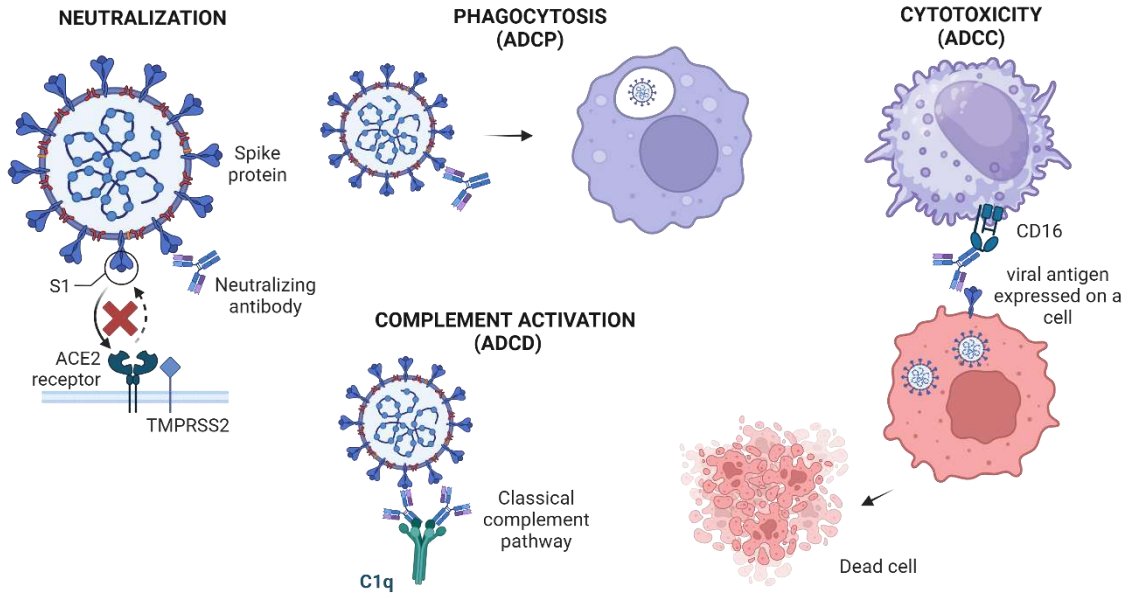

Figure 13 | Antibody functions. *Created in BioRender.com.* ADCP: antibody-dependent cellular phagocytosis; ADCC: antibody-dependent cellular cytotoxicity; ADCD: antibody-dependent complement deposition. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane serine protease 2.

But how does the immune system produce these antibodies and establish immune memory? B cells differentiate into plasma and memory cells (**Figure 12**) [324,343]. Plasma cells are antibody factories, secreting ~2,000 and up to 5,000-10,000 antibody molecules per cell and per second [357]. The BCR on the surface of B cells is a transmembrane Ig (non-secreted). Thus, the antigen-binding specificity of secreted antibodies is the same as the BCR on the parental B lymphocyte [324].

During a primary immune response, $CD4^+$ T_{FH} interact with antigen-specific naïve B cells within lymph nodes to promote their differentiation in specialized structures known as GCs (**Figure 14**). In GCs, antigen-specific B cells undergo extensive proliferation —clonal expansion— and differentiate into ASCs, plasmablasts that differentiate into plasma cells, and MBCs [324,343]. Some B lymphocytes undergo somatic hypermutation (SHM) [358] —an extremely high mutation rate in the Ig variable region— in the GCs to improve antibody affinity. Before the GC formation or within GCs, B cells experience class-switch recombination (CSR) [359] —replacement of the Ig constant region from the initial μ (IgM) to other constant region segments (γ, α, ε) orchestrated by local cytokines— to express alternative antibody isotypes (e.g., IgG, IgA, and IgE) that differ in effector functions

without altering the specificity for the antigen. After these processes, they differentiate into high-affinity ASCs and MBCs, which will proliferate faster than their low-affinity counterparts and dominate the population. This refinement occurs through iterative rounds, producing the later high-affinity antibodies of the immune response. Low-affinity ASCs and MBCs are also generated before the GCs are formed, resulting in the early low-affinity and short-lived antibody responses [324,343]. In addition, specific antigens such as polysaccharides or repetitive non-protein structures (e.g., lipopolysaccharide from gram-negative bacteria) can activate B cells without T-cell involvement (T-independent). However, they produce low-affinity (without undergoing SHM) and with limited memory and isotype switching [324]. The precise signals inducing the fate of B-cell differentiation remain unknown, although several factors, including BCR affinity, duration of antigen in GCs, T-cell help, and inflammatory signals, are critical in its development [360].

Once the pathogen is cleared, short-lived plasma cells (~3-5 days) undergo apoptosis — contraction phase— (**Figure 14**). However, some can transfer to tissue niches, mainly the bone marrow, gut, and spleen, and differentiate into long-lived plasma cells (LLPCs) that continue producing antibodies long after (several months) pathogen clearance [324,343]. For instance, the measles vaccine generates antibodies with an estimated half-life of more than 3,000 years, and tetanus and diphtheria vaccines induce antibodies that last approximately one to two decades [361]. At the same time, high-affinity MBCs remain primed for rapid response upon pathogen re-exposure, where they will expand faster and differentiate into APCs and MBCs again. ASCs and MBCs circulate in the bloodstream, whereas LLPCs predominantly reside in the bone marrow and intestines [324,343]. Since most antibody production requires both T- and B-cell responses, antibodies are often used as a surrogate for the generation of adaptive immune memory.

As previously said, there are different antibody isotypes with different functions and properties, detailed in **Table 5**:

IgD: IgD and IgM are present on the surface of naïve B cells. IgD is barely secreted into circulation, and its function remains elusive. However, it is the second least abundant Ig in plasma (<0.5%) after IgE, with a short half-life (~3 days) [362].

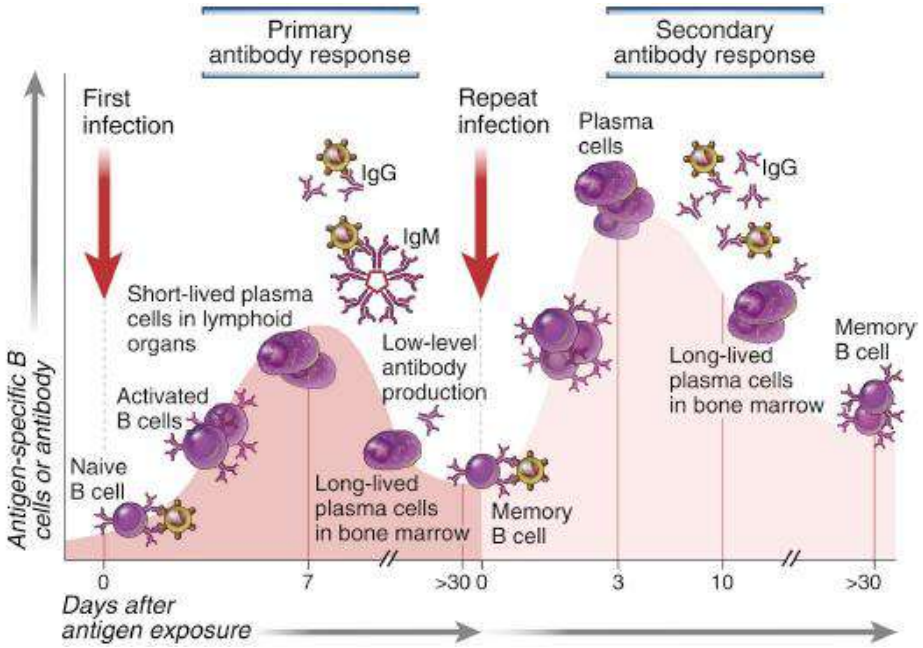

Figure 14 | B-cell and antibody kinetics after primary and secondary responses. *Reproduced with permission from Abbas et al.* [363]

IgM: is the first isotype secreted after B-cell activation. It accounts for less than 10% of plasma Igs with a half-life of about five days [362]. IgM is produced in a pentamer functional form with 10 binding sites, increasing its avidity given that they do not undergo much affinity maturation compared to other isotypes. A consequence of this reduced affinity presents some polyreactivity, allowing it to respond quickly to various antigens in the early stages of the immune response [364]. However, over time, cells producing higher-affinity antibodies and those that undergo class switching become more prevalent. IgM primary functions include opsonization and complement activation [324].

IgG: is the most abundant Ig in plasma (75%) with four subclasses IgG1-4 [362]. Typically, IgG1 and IgG3 are potent triggers of Fc-effector mechanisms, whereas IgG2 and IgG4 induce more subtle responses [362,364]. IgG is the isotype transferred across the placenta, contributing to neonatal immunity. The overall predominance of IgG is partly due to its long half-life in plasma of approximately 21 days [362,364]. IgG molecules are present as monomers, although IgG2 has been observed to form covalent dimers [324,362,364].

IgA: is the second most abundant Ig after IgG in plasma (15%) [362]. However, in mucosal surfaces and secretions (tears, breast milk, saliva, and mucus), IgA is the most abundant isotype [365]. It has two subclasses, IgA1-2: IgA1 predominates in plasma and the upper respiratory tract, accounting for roughly 90% of the total plasma IgA, while IgA2 accounts for the remaining 10% [366]. IgA1 also prevails in the saliva and human milk (60-75%). On the contrary, IgA2 is predominantly found on the mucosal surface of the gut and in urogenital secretions [366]. The half-life of IgA is approximately six days since it has a faster catabolism than IgG [362]. IgA can be found as a monomer, more common in plasma, and as a dimer in secretions [324,362]. IgA plays a critical role in mucosal immunity by neutralizing pathogens, opsonizing, and mediating cell cytotoxicity [362].

IgE: is the least abundant Ig (<0.01%) in plasma among healthy individuals [362]. IgE is associated with helminth infections and allergies [324,362,364]. It binds strongly to receptors on mast cells, basophils, and activated eosinophils. While bound IgE to cells can persist for the lifespan of these cells, free IgE in plasma has a very short half-life of ~3 days [362,367].

Table 5 | Summary of antibody properties.

	IgD	IgM	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Heavy chain gene	δ	μ	γ1	γ2	γ3	γ4	α	ε
Relative abundance (%)	<0.5	10	48	19	4	3	15 (13 IgA1 2 IgA2)	<0.01
Half-life (days)	3	5	23	23	8	23	6	3
Functional form	Mon.	Pen.	Mon.	Mon./ Dim.	Mon.	Mon.	Mon./ Dim.	Mon.
Placental transfer	-	-	+	+/-	+/-	+	-	-
Neutralization	-	++	+++	+++	+++	+++	+++	+
Opsonization	-	+	+++	+/-	++	+	+	-
Complement activation	-	++	+	+/-	++	-	-	-
Granulocyte degranulation	+	-	-	-	-	-	-	+
Fc receptor (FcR)	FcδR	FcμR, Fcα/μr, PolyIgR	FcγR I, II, III FcRn	FcγR II FcRn	FcγR I, II, III FcRn	FcγR I, II FcRn	FcαR, Fcα/μR, PolyIgR	FcεR I, II

Dimeric (Dim.), Monomeric (Mon.), Pentameric (Pen.)

Adaptive immunity to SARS-CoV-2

Our understanding of SARS-CoV-2 and COVID-19 has been evolving at an extraordinary rate since 2020, particularly regarding adaptive immune responses, given their central role in viral control and foundational in effective vaccine development. Longitudinal studies are essential, providing valuable insights into the development of immune memory, persistence, and evolution over time.

Current evidence indicates that $CD4^+$ T cells, $CD8^+$ T cells, and nAbs all contribute to the control of SARS-CoV-2 (**Figure 15**) [335]. T-cell immunity plays a crucial role in protecting against severe disease, while humoral immunity is associated with protection against viral infection [368].

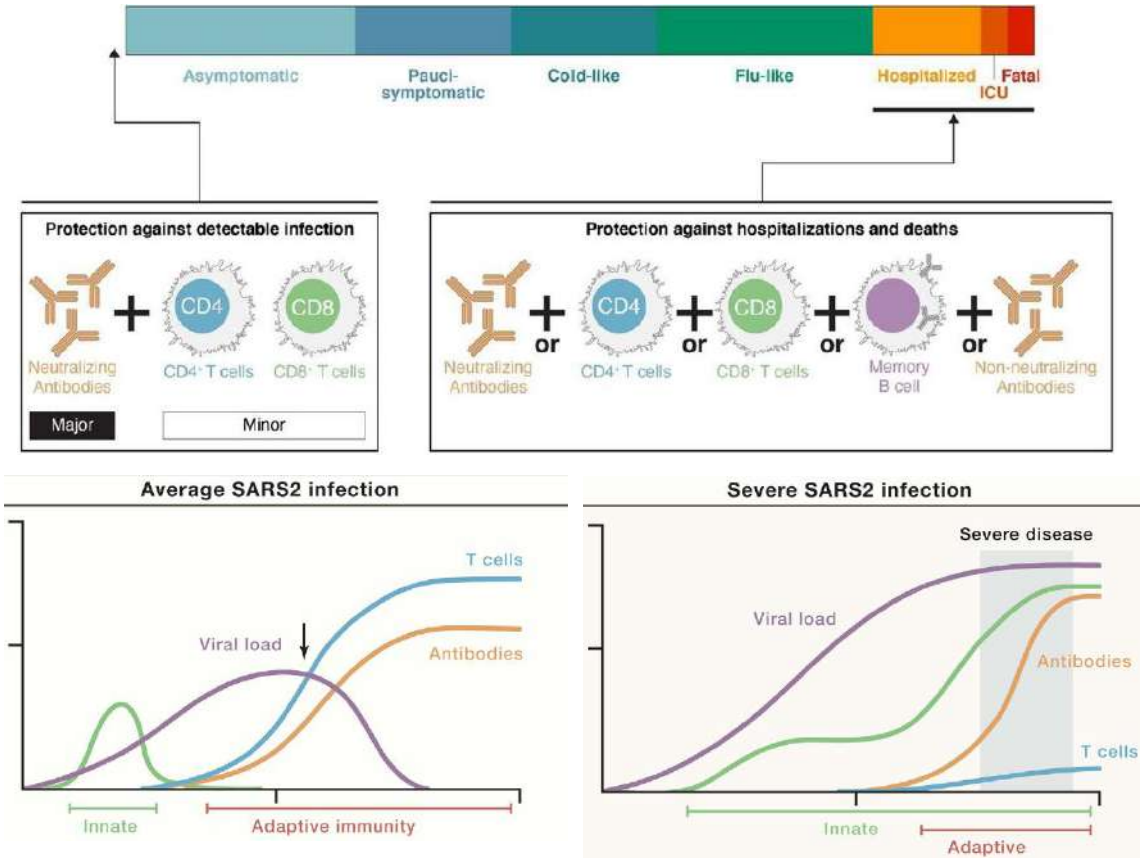

Figure 15 | SARS-CoV-2 adaptive immune responses. The upper panel shows the protective role of different players of adaptive immunity in the course of SARS-CoV-2 infection. *Reproduced with permission from Goldblatt et al.* [368]. The lower panel illustrates the immune responses trajectories in average and severe SARS-CoV-2 infections. *Reproduced with permission from Sette et al.* [335]

A key unsolved question is the duration of acquired immunity. Insights from studies on other seasonal HCoVs, SARS-CoV-1 and MERS provide hints about SARS-CoV-2. For instance, a study monitoring seasonal coronavirus in healthy individuals for more than 35 years determined that reinfection with the same coronavirus frequently occurred at 12 months after infection, suggesting that protecting immunity from natural infection is short-lasting [369]. In contrast, adaptive immune responses to SARS-CoV-1 and MERS are more durable, although particularly following severe cases, with antibody responses persisting for up to 2 years, and T-cell responses lasting 7-11 years [370–373]. Consequently, understanding the longevity of SARS-CoV-2 immunity induced by natural infection and vaccination is critical to reach herd immunity.

Population immunity to SARS-CoV-2 has become complex and heterogeneous, shaped by individual exposure to different SARS-CoV-2 variants and diverse vaccine types and regimens. Individual immune responses are further influenced by host factors such as age, sex, comorbidities, medication, and previous SARS-CoV-2 infections [374]. As of early 2024, about 64% of the global population has completed a primary COVID-19 vaccine series, with Europe showing some of the highest coverage rates [6]. The emergence of the highly transmissible Omicron variant led to a surge in reinfections and BTIs, and it is now suggested that most of the world's population has experienced SARS-CoV-2 infection at least once [375]. Consequently, hybrid immunity, the combination of natural and vaccine-induced immunity regardless of exposure order, is increasingly common [376]. Compounding this complexity, many infections are asymptomatic, making differentiating immune responses originating solely from infection versus vaccination challenging [59]. Given these complexities, longitudinal studies on diverse immune repertoires in the real-world population are essential for understanding how immune memory evolves to guide future control strategies.

For pathogens like SARS-CoV-2, which enter the host through the respiratory mucosa, antibody and cellular immune components in the upper and lower airways are essential for limiting initial infection and controlling viral spread and disease progression [377]. The following sections will focus on the adaptive immune response at a systemic level in healthy adults, which remains the most extensively studied. However, it is important to

note that mucosal immunity, particularly in respiratory tissues, is of great relevance in SARS-CoV-2 infection [378].

I want to emphasize that the knowledge presented in the following sections has largely been uncovered over the past four years. Our understanding of COVID-19 immunology was far more limited when this thesis began in July 2021. Critical knowledge gaps included the specificities, kinetics, and durability of humoral and T-cell responses following SARS-CoV-2 infection and vaccination, the extent to which T cells and antibodies cross-recognize emerging variants, and the determinants influencing these immune responses and their protective roles against reinfection. Addressing these gaps is pivotal for advancing vaccine design and developing improved immunotherapeutic strategies for SARS-CoV-2 and potential future coronaviruses.

T cell-mediated immunity to SARS-CoV-2 (focused on $CD4^+$ and $CD8^+$ T cells)

Targets of T-cell responses

T-cell responses recognize multiple SARS-CoV-2 epitopes, with the S protein being the main target for both $CD4^+$ and $CD8^+$ T-cell responses, followed by M and N proteins, and various non-structural proteins (**Figure 16**) [379–382]. The reason why S glycoprotein is the predominant target for SARS-CoV-2-speicifc T cells is not fully understood, but its large size (encompassing many epitopes) and high expression are likely contributing factors [383]. Targeting multiple viral epitopes enhances the likelihood of recognizing infected cells despite variations in viral protein expression, minimizes the risk of immune escape at a single epitope, and increases the chances of T-cell memory recognizing future infections. Thus, all T-cell epitopes (TCEs) are of great interest for designing pan-coronavirus vaccines [384].

The Immune Epitope Database records over 4,000 SARS-CoV-2 TCEs [385]. SARS-CoV-2 infection induces $CD4^+$ and $CD8^+$ T-cell responses, each recognizing up to 40 epitopes in an individual. The recognized epitopes vary significantly among individuals, reflecting the diversity of human leukocyte antigen (HLA) [386]. During both natural infection and vaccination, a median of 10-11 epitopes specific to the S protein are recognized [387,388]. Notably, one study suggested that mRNA vaccination may elicit broader S-specific T-cell

response compared to infection, underscoring the potential advantage of vaccination in boosting the breadth of S-specific T-cell responses in convalescent individuals [389].

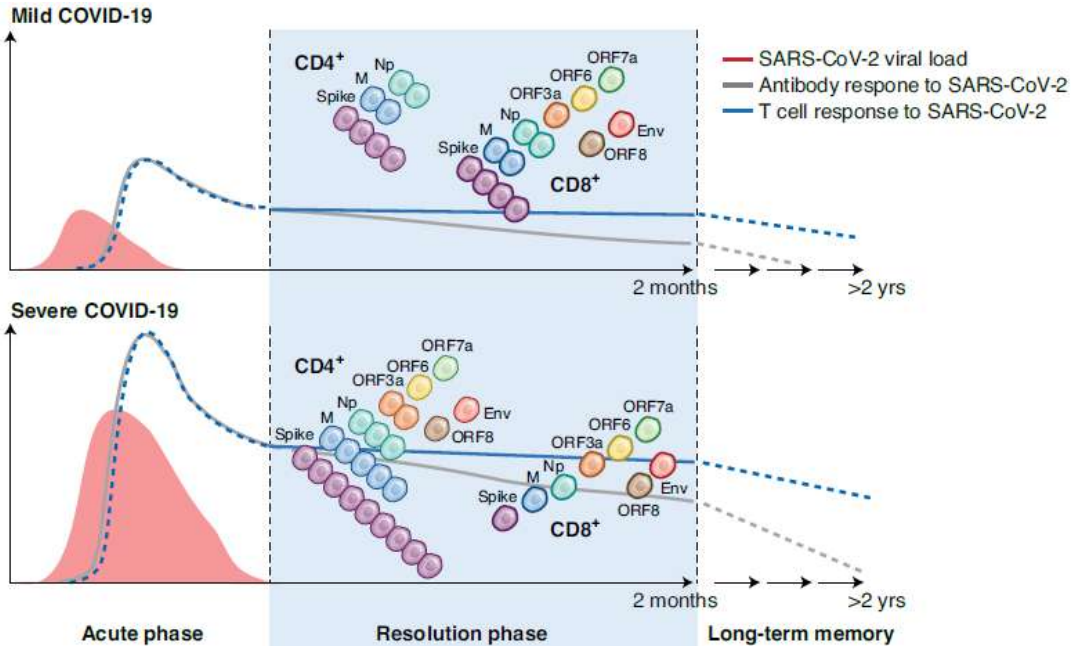

Figure 16 | T-cell response to SARS-CoV-2. Cell colors and numbers represent relative frequencies of indicated protein specificities in the resolution phase of mild and severe COVID-19. *Reproduced with permission from Swadling and Maini [382], based on Peng et al.* [381]. Env.: Envelope; M: Membrane, Np: Nucleocapsid; ORF: Open reading frame.

T-cell responses to infection

Few studies have evaluated T-cell responses prior to symptom onset or a positive PCR test to precisely determine when T cells are engaged following exposure to SARS-CoV-2 and how their early kinetics influence the infection outcome [390]. In a controlled human SARS-CoV-2 challenge, a marked expansion of activated $CD4^+$ and $CD8^+$ T cells was observed, peaking in both blood and nasopharynx 10 days post-inoculation, followed by a modest contraction within the next 28 days [391]. $CD4^+$ and $CD8^+$ T cells to SARS-CoV-2 are detected approximately seven days after symptom onset, with a typical peak at 14 days [392–394]. Grifoni et al. identified SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T-cells in ~100% and ~70% of COVID-19 convalescent patients, respectively, one month after diagnosis. The expansion of $CD4^+$ and $CD8^+$ T cells was observed during the first month after infection, followed by a gradual, slight decline over subsequent months, with half-lives of ~200 days [394]. Notably, the magnitude of SARS-CoV-2-specific $CD4^+$ T-cell responses

was higher than that of $CD8^+$ T cells, which is somewhat unusual for viral infections [379,394–396]. One potential explanation for this phenomenon is the greater propensity of $CD4^+$ T-cell responses to be cross-reactive due to the increased flexibility of peptide binding to MHC-II, accommodating longer peptides. Another possible explanation is the ability of SARS-CoV-2 to downregulate MHC-I expression, which may limit $CD8^+$ T-cell priming and expansion, leading to a relative increase in SARS-CoV-2-specific $CD4^+$ T cells [390,397,398].

SARS-CoV-2-specific $CD4^+$ T-cell responses were predominantly T_H1, with generally undetectable T_H2 and T_H17 cells [379]. T_H1-like effector functions dominated, with IFN-γ and IL-2 being most associated with viral control, but with an interesting potential role for IL-10 co-production in limiting pathology [390]. Robust production of IFN-γ and IL-10 has been observed in mild COVID-19 cases [399], whereas severe COVID-19 often features prolonged IFN-γ production without concurrent IL-10 [400]. One study reported that severe disease was marked by poor polyfunctionality of $CD4^+$ T cells, particularly with limited IFN-γ production and low proliferative capacity, rather than differences in the overall frequency of antigen-specific T cells [401]. SARS-CoV-2-specific $CD8^+$ T cells also demonstrated significant polyfunctionality, secreting IFN-γ, TNF-α, IL-2, granzyme B, and perforin [394].

The induction of T_{FH} by SARS-CoV-2 infection has also been noted, with higher proportions of virus-specific circulating T_{FH} and T_H1 cells associated with mild COVID-19. These proportions correlate with sustained anti-S antibody responses following viral clearance [402–404]. Some severe or fatal COVID-19 cases showed a loss of T_{FH} cells in GCs, impacting B-cell development [405]. Moreover, SARS-CoV-2-specific $CD4^+$ T-cell responses have been shown to predict the magnitude, breadth, and duration of subsequent nAbs responses, highlighting the relevance of coordinated cellular and humoral immunity for long-term protective immunity [406].

Memory $CD4^+$ T cells predominately exhibited T_{CM} and T_{EM} phenotypes, displaying consistency across individuals and stability over eight months after symptom onset. In contrast, memory $CD8^+$ T cells initially displayed a T_{EM} phenotype, which contracted over time, around 7-8 months after symptom onset, as the T_{EMRA} phenotype became more prevalent [394]. Evidence from several studies estimates that memory T-cell responses

following infection can persist for many years and may be independent of disease severity, although is estimated that ~30% of individuals may not develop a robust memory $CD8^+$ T-cell response [394,407–412].

T-cell responses to vaccination

Primary vaccination and booster doses with mRNA vaccines and adenoviral vector vaccines elicited potent T_H1 polyfunctional S-specific $CD4^+$ and $CD8^+$ T-cell responses that persisted for up to six to eight months [413–415]. These responses mirrored the memory phenotypes induced by natural SARS-CoV-2 infection [416,417]. The recombinant S protein NVX-CoV2373-Novavax vaccine induced robust S-specific $CD4^+$ T-cell responses, and although there is limited information, $CD8^+$ T-cell responses seemed to be poor [418,419]. In contrast, inactivated vaccines induced robust $CD4^+$ T-cell responses against all antigens but did not elicit $CD8^+$ T-cell responses [416,420–422]. This limited $CD8^+$ T-cell response was expected for inactivated vaccines, as they contain non-infectious viral particles that do not replicate within host cells. Consequently, antigens from these vaccines are processed through the exogenous pathway and presented by MHC-II, which are recognized by $CD4^+$ T cells. Furthermore, all COVID-19 vaccines induced T_{FH}, which strongly correlated with the development of nAbs [404,416,423–426].

One study compared four different COVID-19 vaccines (BNT162b2, mRNA-1273, Ad26.COV2.S, and NVX-CoV2373) and found that mRNA vaccines elicited the most robust $CD4^+$ T-cell responses. Regarding $CD8^+$ T-cell responses, mRNA vaccines also demonstrated stronger responses than the adenoviral vaccine. Overall, they observed that $CD4^+$ T, $CD8^+$ T, and circulating T_{FH} cell responses induced by these vaccines were equivalent or superior to those observed following infection [416]. In addition, some studies have shown that CoronaVac and BBIBP-CorV inactivated vaccines induced weaker $CD4^+$ T-cell responses than mRNA vaccines [427,428]. This suggests that the mRNA platform generates the most robust SARS-CoV-2 T-cell responses. Additionally, heterologous immunization has been found to enhance cellular immune responses more effectively than homologous regimens, but the extent of this improvement is highly dependent on the specific types of vaccines included in the regimens [383,429].

Although research on updated vaccines is still limited, some studies have reported that bivalent or monovalent with S from BA.5 or XBB.1.5 variant boost T-cell responses but

do not elicit stronger responses to the latest variant included in the vaccine [430,431]. The underlying reasons for this observation remain unclear.

T-cell responses when hybrid immunity

The effect of hybrid immunity on T-cell responses is a subject of debate. While some studies have reported enhanced S-specific T-cell responses in hybrid immunity [432,433], many others have found no significant differences [423,433–436]. This variability can be attributed to several factors, including the timing of sample collection, the methodologies used to measure T-cell responses, and individual differences in immunity (infecting viral variant, viral load, vaccine type and regimen, number of exposures, and the order in which these exposures occurred). Notably, hybrid immunity extends beyond S-specific responses, recognizing a broader range of epitopes, including non-S antigens, as evidenced by different TCR repertoires [437,438]. Furthermore, hybrid immunity elicits qualitative differences in T-cell responses, such as the induction of tissue-localized T cells, which are likely important for protective immunity and are primarily induced by infection or mucosal antigen exposure [434,439–441]. In addition, secondary exposure induces a rapid and extensive recall of memory T-cell populations. One study observed that a BTI with the Delta or Omicron variant in vaccinated individuals triggered a rapid recall of S-specific $CD4^+$ and $CD8^+$ T cells with cellular proliferation and extensive activation evident at day three post-symptom onset, peaking by day six. $CD4^+$ T cells exhibited T_{EM} and T_{CM} phenotypes, whereas $CD8^+$ T cells displayed T_{EM} and T_{EMRA} phenotypes [442].

T-cell cross-reactivity with common cold coronaviruses

An important aspect of SARS-CoV-2 T-cell immunity is cross-reactivity. Multiple studies have demonstrated T-cell cross-reactivity between seasonal coronaviruses and SARS-CoV-2, particularly with the N protein [379,443–445]. Notably, one study observed SARS-CoV-1-specific memory T cells reactive 17 years after infection, with robust cross-reactivity to the SARS-CoV-2 N protein [443]. Given that most individuals have encountered a coronavirus infection at some point in their lives, pre-existing immunity may facilitate a faster and stronger immune response to SARS-CoV-2, potentially mitigating disease severity [446–448]. Although pre-existing SARS-CoV-2-reactive T cells have been detected *ex vivo* in a large proportion of unexposed samples (pre-pandemic, pre-August 2019: ~10–

50% depending on assay and viral targets included), evidence suggests that pre-existing seasonal coronavirus-specific T cells are not a major contributor to the development of SARS-CoV-2 immunity following infection or vaccination [379,390,395,444,449,450].

In addition, it has been observed that antigens unrelated to coronaviruses including cytomegalovirus, influenza A, and commensal bacteria induce SARS-CoV-2 cross-recognition by T cells in samples from unexposed individuals *in vitro* [451–453].

T-cell responses to SARS-CoV-2 variants

Mutations in SARS-CoV-2 variants can potentially lead to decreased T-cell recognition. Nonetheless, studies have shown that individuals previously infected or vaccinated elicit robust cross-reactive cellular responses against a range of SARS-CoV-2 variants, from Alpha to Omicron BA.1 [387,388,454–456]. Furthermore, bioinformatic analyses predict that SARS-CoV-2 evolution is likely to have only a modest impact on T-cell recognition due to the high conservation of TCEs; only a small percentage of these epitopes are affected by mutations [387,454]. At the population level, approximately 80% of the conservation of T-cell reactivity has been observed, with most cases showing no significant reduction in response. However, notable decreases in T-cell reactivity have been reported for specific combinations of individual subjects and viral variants [454,457–461]. Although memory T cells can persist over time and maintain cross-reactivity, the emergence of new and highly mutated variants, such as BA.2.86 and its descendant JN.1, presents a potential challenge to T-cell-mediated cross-reactivity and may contribute to reinfections.

Determinants of T-cell immune response

The magnitude and nature of T-cell responses can be influenced by many factors, such as HLA diversity, age, and sex, which remain incompletely understood [383]. HLA genes are highly polymorphic and directly shape the TCR repertoire, determining which antigens are presented to T cells. Multiple studies have demonstrated associations between specific HLA alleles and differential SARS-CoV-2 immune responses [462–464]. Age has been identified as a critical determinant due to immunosenescence [465,466] —a gradual deterioration of the immune system function with aging, affecting T-cell mediated immunity. It is characterized by a decline in naïve T-cell production, which impacts the generation of new antigen-specific T cells, accumulation of senescent memory T cells

with limited proliferative capacity and altered functions, and a general increase in systemic inflammation. Sex-related differences are controversial. While some studies have found no differences between sexes [394,467], others have reported higher T-cell responses in females than males [468]. Finally, for example, one study observed that pre-existent coronavirus-specific or cross-reactive T cells shape the development of cellular and humoral immune responses after COVID-19 mRNA booster vaccination [469]. In conclusion, many determinants may influence SARS-CoV-2 T-cell responses. Deciphering these factors is essential to improve future control strategies.

T-cell responses in the mucosa

Assessing local airway immune responses in humans is challenging, and only a limited number of studies examined T-cell responses in the airways following SARS-CoV-2 infection or COVID-19 vaccination. Notably, $CD4^+$ and $CD8^+$ T-cell responses are detectable early in the upper respiratory tract (in nasal and BAL samples) after SARS-CoV-2 infection or BTI and can persist for up to seven months [441,470,471]. Interestingly, the existence of T-cell responses has been observed in the lungs after SARS-CoV-2 infection, even in cases where virus-specific T cells were absent in the periphery [472,473]. In contrast, intramuscular vaccination, particularly with mRNA vaccines, has been associated with weaker or minimal T-cell immunity in the lung mucosa [441,471,474]. Findings from animal models further highlight that airway mucosal vaccination strategies can induce both mucosal and systemic immunity [474–476]. These observations underscore the critical need to prioritize the development of COVID-19 mucosal vaccines.

Role of T-cell responses in protection

The initial focus on immune protection against SARS-CoV-2 was on humoral immunity because antibody responses are the primary target of most successful preventive vaccines, and they can be measured relatively easily in large populations. However, accumulating evidence indicates that T cells also play critical roles in controlling established infection and halting viral replication at its earliest stages [390]. SARS-CoV-2-specific $CD4^+$, and to a lesser extent $CD8^+$, T-cell responses have been associated with reduced severity and asymptomatic infection [383,399,450,477,478]. Robust early T-cell responses correlate with a lower peak viral load, rapid viral clearance, and better clinical outcomes [379,392,393,396,477,479,480].

Higher cellular responses to SARS-CoV-2 have been observed in exposed seronegative individuals compared to unexposed individuals, although these responses were lower than those seen in exposed seropositive individuals [396,481,482]. Furthermore, patients with X-linked agammaglobulinemia —primary immunodeficiency characterized by the lack of mature B cells and therefore severe reduction or absence of Igs— have been reported to recover and have SARS-CoV-2 mild disease, suggesting that cell-mediated immunity is vital when a proper humoral response is absent [483–485]. While early treatment of mAbs has demonstrated significant efficacy in preventing infection, its impact in hospitalized patients has shown limited benefit, with only modest effects on viral load [486–488]. All this indicates that, although antibodies contribute to viral clearance, T cells control viral replication after infection [368].

Humoral immunity to SARS-CoV-2 (focused on antibodies)

Seroconversion and seroprevalence

SARS-CoV-2 infected individuals exhibit a high percentage of seroconversion (~90%) —change from a seronegative to a seropositive— for IgM, IgG, and IgA antibodies within 5-15 days after symptoms onset. Specifically, seroconversion to the SARS-CoV-2 S protein occurs in 91-99% of cases [489–492]. The S glycoprotein, particularly the RBD, is the primary target of nAbs elicited by SARS-CoV-2 infection [493–496]. Other viral proteins, such as the N, are also recognized by antibodies after SARS-CoV-2 infection [490,493,494]. Our previous studies demonstrated that seropositivity to SARS-CoV-2 S and RBD proteins exceeded 90% for up to 10-12 months after infection [497,498]. Although complete seroreversion —change from seropositive to seronegative— is rare in healthy adults, antibody levels decline significantly within six months post-infection [376]. Our findings revealed that seroreversion occurred in 35% of non-vaccinated individuals one year after infection [499].

During the early phase of the COVID-19 pandemic (March–April 2020) in Spain, our group reported a low seroprevalence (9.3%) of SARS-CoV-2 antibodies among HCWs in Catalonia [500]. Similarly, a study in Madrid recorded a seroprevalence of 5% during the same period, highlighting low population immunity [154]. Our seroprevalence estimates increased to ~16% by six to nine months post-infection, reflecting the gradual accumulation of immunity through infections [501,502]. With subsequent waves of

infections and the introduction of the mass vaccination campaign, the estimated seroprevalence of RBD protein in Spain had risen to ~90% by 2022 [503].

Humoral responses to infection

The antibody response, including nAbs, peaks between the third and fifth week after SARS-CoV-2 infection and is characterized by the presence of IgM, IgG, and IgA isotypes in the plasma of most infected individuals [504–508]. Subsequently, IgM levels decline within the next month after symptom onset. IgG and IgA antibody levels decline, eventually stabilizing into a plateau phase between 4-6 months post-infection. Despite this decline, IgG antibodies remain detectable in over 80% of convalescent individuals one year after infection [394,498,509–514]. In addition, a recent study in infected-unvaccinated individuals showed 89% of IgG seropositivity up to two years after infection [515]. Notably, higher antibody levels and nAbs were found in individuals with severe COVID-19 than those with mild or asymptomatic disease [516]. Previous research in our group showed that during the first three months after SARS-CoV-2 infection, the prevailing IgG subclass against RBD protein in plasma was IgG1, followed by IgG2 and IgG3 [517]. Similarly, some studies observed that anti-S and anti-N IgG1 and IgG3 subclasses predominate over IgG2 and IgG4 in SARS-CoV-2-infected patients and were detected up to six months post-infection [518–522]. However, due to the relatively recent emergence of SARS-CoV-2, the widespread coverage of vaccines and frequent reinfections, the accurate duration of SARS-CoV-2 antibodies produced by natural infection is still unknown.

SARS-CoV-2 infection has been shown to elicit circulating MBCs and LLPCs [472,510,523–526]. SARS-CoV-2-specific MBCs are detected within two weeks after symptom onset, and their frequencies significantly increased between 3-6 months post-infection, suggesting ongoing GC reactions [394,409], remaining detectable for over one year [515,527,528]. Importantly, mAbs derived from MBCs in convalescent individuals demonstrate increased SHM, binding affinity, and neutralization potency over time, evidencing prolonged antibody affinity maturation [527,529].

The development of Fc-mediated responses has been observed in COVID-19 patients [530–534]. One study showed that ADCC was elicited 10 days post-infection, peaked by 11-20 days, and remained detectable for up to 12 months post-infection [530]. Notably, patients who recovered from severe disease exhibited higher ADCC activity compared to those

who succumbed to severe disease [530]. One study reported that patients with more severe COVID-19 had higher ADCD activity but lower ADCP than patients with milder disease [531]. These functions are complex and depend on many factors, including the specific FcR engaged by antibodies, the cell type expressing these FcRs, the antigen or epitopes targeted, and the antibody-FcR clustering requirement [535]. Fc-effector functions are overlooked in many infectious diseases and vaccines due to the complexity of the assays to measure them. Further investigation into these mechanisms is needed to decipher their role in COVID-19.

Humoral responses to vaccination

All authorized COVID-19 vaccines have shown high seroconversion rates within 15 days after primary vaccination [536,537]. mRNA vaccines elicited strong antibody responses within 1-2 weeks after dose administration. In contrast, adenoviral and inactivated vaccines induce antibody responses that peak later, resembling the kinetics observed during natural infection. For adenoviral vaccines, antibody peak against S protein is seen by day 28-57 post-vaccination, and for inactivated vaccines 2-4 weeks after the second dose [536,538]. Generally, antibodies generated by all COVID-19 vaccines wane substantially within 6-8 months after primary vaccination [539,540]. Notably, a booster dose restores the magnitude of the humoral response, extends antibody durability, and enhances neutralizing activity, often surpassing the initial response [541–546].

Several studies have compared antibody responses elicited by different COVID-19 vaccines. In healthy adults, primary vaccination with mRNA-1273-Moderna induced the most potent S-specific nAb responses, which were slightly higher than those induced by BNT162b2-Pfizer/BioNTech, probably due to the higher amount of mRNA in the former [416,547,548]. Humoral responses generated by these mRNA vaccines were greater than those induced by adenoviral vector vaccines (two doses AZD1222-Oxford/AstraZeneca and one dose Ad26.COV2.S-Janssen/J&J) and had a slower decay [499,549–554]. Inactivated vaccines induced virtually no anti-N antibody response and lower levels of anti-S antibodies that declined faster than mRNA vaccines [555,556]. The recombinant S protein NVX-CoV2373-Novavax induced lower anti-S IgG levels than mRNA vaccines but demonstrated a comparable antibody decay rate [416,557]. These observations suggest that the mRNA vaccines generate the most robust antibody responses. However, antibodies

elicited by mRNA vaccines waned much faster than those induced by natural infection [558]. Additionally, heterologous vaccination regimens resulted in a more robust humoral response than the homologous ones, though the extent of this enhancement depended significantly on the specific combinations [427,429,559–562].

The fourth booster dose of an mRNA vaccine boosts immunity, achieving similar or potentially higher peak responses than those obtained with the third dose [563–565]. A study has demonstrated higher effectiveness of updated bivalent booster than ancestral monovalent booster against hospitalizations and deaths caused by new Omicron subvariants [286]. However, BA.5 bivalent mRNA vaccines have been shown to enhance antibody responses against the ancestral virus but do not significantly improve nAbs against newer Omicron subvariants [566–569]. Similarly, the XBB.1.5 monovalent vaccine increases nAbs against Omicron subvariants up to XBB.1.5 but does not substantially enhance nAbs against BA.2.86 while showing a skewed response towards Wu-1 [570–572]. These observations underscore the phenomenon of immune imprinting, which will be explained later in this section.

S- and RBD-specific MBCs are also efficiently primed by COVID-19 vaccines [416,423,573–575]. However, the affinity maturation after primary vaccination with mRNA vaccines appears to be qualitatively poor compared to that induced by SARS-CoV-2 infection [407,576]. This discrepancy may be attributed, at least in part, to the timing between the two doses since a longer time than the usual schedule results in a greater magnitude and quality of the neutralizing response [577,578]. In addition, one study detected the presence of anti-S IgG-secreting LLPCs up to six months post-mRNA vaccination, with an increase in SHM frequencies over time [574]. Nevertheless, robust evidence on the induction of LLPCs by COVID-19 vaccination remains limited and has not been thoroughly investigated across all COVID-19 vaccine platforms.

Recent investigations have found increased anti-S IgG4 levels following repeated mRNA vaccination, a phenomenon not observed with adenoviral vector or recombinant COVID-19 vaccines [579–581]. Interestingly, vaccines for other infectious diseases, such as HIV, malaria, and pertussis, have also been reported to induce higher-than-normal IgG4 synthesis [582–585]. Whether this IgG4 increase in the context of COVID-19 confers protection or represents an immune tolerance mechanism and its implications remain

unclear. Elevated IgG4 levels, however, are known to reduce Fc-effector functions [586,587]. Such functions are important aspects of immune defense and have been elicited by COVID-19 vaccines [588–591]. Notably, differences in Fc-effector functions have been observed between COVID-19 vaccine platforms, even among mRNA vaccines. For instance, antibodies induced by mRNA-1273-Moderna showed higher ADCP and ADCC than those elicited by BNT162b2-Pfizer/BioNTech [589]. Further research is needed to understand the biological significance of these observations and the potential impact on vaccine efficacy and durability.

Humoral responses when hybrid immunity

In contrast to the T-cell response, growing evidence, including our previous research, suggests that hybrid immunity enhances SARS-CoV-2 humoral responses, particularly of nAbs [423,433,434,499,538,592–594]. Vaccination boosted RBD-specific MBCs in individuals previously infected with SARS-CoV-2. These MBCs exhibited greater SHM and affinity maturation than those induced by vaccination alone, which is also reflected in antibodies with greater potency and breadth of neutralization [423,527,594–596]. Likewise, SARS-CoV-2 BTIs in previously vaccinated individuals induced more potent, broad, and durable nAb responses than naïve-individuals [567,595]. Hybrid humoral immunity appears to be influenced by several factors, including the timing and course of infection, the number and nature of exposures, and the infecting variant [597–600]. Moreover, secondary exposure induces a rapid and extensive recall of humoral responses. For instance, one study observed that a BTI with the Delta or Omicron variant in vaccinated individuals triggered a rapid increase in antibody levels against the infecting variant as early as day 4 after symptom onset, peaking around day 14 [442].

Humoral cross-reactivity with common cold coronaviruses

Antibodies capable of cross-reacting between SARS-CoV-2 and SARS-CoV-1 or seasonal HCoVs have been observed [228,601,602]. Previous investigations by our team showed cross-reactivity between the N protein from SARS-CoV-2 and other HCoVs, with stronger reactivity observed for *Alpha*- than *Beta*-coronaviruses, despite the former having a lower sequence identity [170]. Cross-reactivity with the S glycoprotein has also been reported [603,604]. High levels of pre-existing cross-reactive antibodies have been

associated with milder clinical manifestations of SARS-CoV-2 infection in some studies, including our previous findings [502,605–609], but not in others [610–613].

Interestingly, we observed an increase in antibody levels to the C-terminal region of the N protein following S-based immunization, suggesting bystander activation [614]. Additionally, our group identified cross-reactivity between SARS-CoV-2 and antigens from malaria, helminths, and protozoa [615]. In addition, other studies have reported cross-reactivity with the dengue virus and the diphtheria-tetanus-pertussis (DTP) vaccine [616,617].

Humoral responses to SARS-CoV-2 variants

The emergence of SARS-CoV-2 variants harboring mutations in the S protein has raised concerns within the scientific community regarding its potential to evade pre-existing humoral immunity established through natural infection or vaccination. It is now well-established that SARS-CoV-2 variants have altered the neutralizing capacity of antibodies generated by infection with the ancestral strain or vaccination. This effect has been particularly pronounced in the most recently emerged variants, reducing vaccine effectiveness. Studies have shown that nAbs in plasma from COVID-19 convalescent or vaccinated individuals exhibit diminished recognition of Alpha, Beta, Gamma, Epsilon, Kappa, and Delta variants compared to Wu-1 [459,618–621], and it is lost to the Omicron subvariants [515,622–624]. In addition, Fc-mediated functionality generated from the ancestral virus or vaccination was also lower against Beta, Delta, and Omicron variants compared to the Wu-1 [459,535,625–627]. Sequential booster doses, heterologous regimens, and hybrid immunity have been shown to reduce susceptibility to Omicron infection while enhancing and broadening cross-reactivity and Fc-effector functions up to the Omicron BA.1 variant [515,628–633]. Additionally, prior infection with earlier Omicron subvariants has provided some protection against newly emerged ones. For example, infection with the BA.1 or BA.2 variants conferred modest protection against BA.4/5 [634]. The emergence of highly mutated variants such as BA.2.86 and its descendant JN.1 poses a significant challenge to humoral responses and may contribute to an increased risk of reinfections and BTIs.

Immune imprinting

An intriguing recent observation is that mRNA-vaccinated individuals who had subsequent Omicron BA.1 BTI experienced an increase in antibody responses to the Wu-1 and lower relative responses to the BA.1 [635]. Likewise, Omicron BA.5 BTI back-boosted anti-Wu-1 responses while eliciting low neutralization of the latest sublineages, particularly BA.2.75 and BQ.1.1 [636,637]. In addition, as noted before, efforts to update booster vaccines with S protein from new variants have been hindered by pre-existing immunity. These findings are reminiscent of a phenomenon observed in influenza [638], known as immune imprinting —also called original antigenic sin (OAS); upon exposure to a variant of a previously encountered antigen, the immune response is skewed towards the original antigen rather than to the new one, often resulting in a suboptimal response against the new antigen—. It is thought to occur because MBCs generated by the original antigen are the primary source of antibodies when a new variant is encountered, potentially inhibiting the generation of new clones of ASCs and MBCs specific to the variant [343]. Long-lasting GCs may mitigate the effect of immune imprinting, as the persistence of GCs correlates with the extent of SHM of MBCs and ASCs, which increases clonal diversity and, thus, the potential to respond by boosting immunogens [343]. Indeed, of late, one study has shown that repeated Omicron exposures and prolonged Omicron-specific B-cell maturation alleviates immune imprinting induced by ancestral SARS-CoV-2 [639,640].

Determinants of humoral immune response

As for T-cell responses, the magnitude and durability of humoral responses to SARS-CoV-2 infection or vaccination are influenced by various factors are not yet fully elucidated [343]. Immunosenescence impacts humoral responses, leading to reduced production of naïve B cells and a decline in GC function, which affects affinity maturation and class switching [641]. In a previous collaboration with Karachaliou et al., we observed that individuals over 60 exhibited lower SARS-CoV-2 antibody responses after infection than those aged 60 or younger [501]. Additionally, the same study found that smokers had lower anti-SARS-CoV-2 antibodies vs. non-smokers, whereas overweight participants had higher antibody levels than those normal-weight [501]. Sex-related differences in humoral responses remain a subject of debate. Some studies report no significant differences

between sexes [642], while others indicate higher humoral responses in females than males [642–644] or the opposite [629,645]. These discrepancies may arise due to variations in disease severity, the course of infection, the type of vaccine administered, and other contextual factors. Prior research in our group identified baseline factors associated with higher SARS-CoV-2 antibody levels nine months after infection during the first and second pandemic waves. These factors included hospitalization, the presence of fever, anosmia and/or hypogeusia, a history of allergies, and occupation. Conversely, smoking was negatively associated with antibody levels [498]. In summary, numerous factors can shape SARS-CoV-2 humoral responses, and understanding these determinants is crucial for enhancing future control strategies.

Humoral responses in the mucosa

Secretory IgA (sIgA) in the respiratory tract may play a critical role in preventing transmission of SARS-CoV-2 through the airway. This protection is based on tissue localization and the more potent neutralizing activity of dimeric sIgA than monomeric serum IgA or antibodies of other isotypes [516,646]. Several studies, including some conducted by our group, have detected anti-SARS-CoV-2 antibodies in saliva after infection [167,647–649]. Circulating IgA and IgG levels in the blood correlated with their corresponding isotype in saliva, with a steeper decline of antibodies in saliva than in blood [649,650]. In BAL samples from COVID-19 patients, anti-S and anti-N antibodies were detected, with IgG being the most predominant Ig [629,651]. Indeed, IgA and IgM levels in saliva peaked by day 20 after symptom onset, whereas IgG peaked between 31–45 days. Although IgA and IgM appeared earlier, they declined faster than IgG [649]. Airway IgG and IgA levels dropped significantly within three months but remained detectable for up to nine months post-infection [652,653]. As in the systemic humoral immune response, SARS-CoV-2-specific antibody levels in mucosal tissues were higher in moderate/severe than in asymptomatic/mild disease [654–656]. Our previous study observed that asymptomatic children had higher levels of IgM, IgG, and IgA in saliva compared to symptomatic cases, indicating a strong neutralizing response at the mucosal level that could prevent symptomatic infection [647]. Supporting this, mucosal neutralization has been associated with nasal SARS-CoV-2-specific IgA [657]. In contrast, intramuscular vaccines induce weaker humoral responses in mucosal tissues than natural infection [474,652,658]. BAL

samples from donors with hybrid immunity had more airway mucosa SARS-CoV-2 antibodies and MBCs than those from only vaccinated [471]. These observations underscore the critical need to prioritize the development of COVID-19 mucosal vaccines.

Role of humoral responses in protection

Studies have demonstrated that passive transfer of nAbs protects animal models when challenged with SARS-CoV-2 [659,660]. In humans, binding antibody levels, nAbs, Fc-effector functions, and MBCs have been associated with protection against SARS-CoV-2 infection and severe disease [368,661–663]. Mucosal humoral responses are inversely correlated with the viral load in nasopharyngeal swabs, indicating that a solid early nasal antibody response may play a key role in limiting disease by initiating or facilitating early viral clearance [629]. One study observed that anti-S antibodies mediating ADCP were linked to survival [533], and another suggested that Fc-dependent antibody profiles could predict the clinical trajectory of COVID-19 [532]. In addition, anti-S nAbs have been associated with protection against the ancestral and the Alpha, Beta, and Delta variants [368,664–669]. However, this protective strong correlation disappears for Omicron subvariants [670,671]. Significantly, while reinfections and BTIs with SARS-CoV-2 variants occur even in the presence of immune memory, prior infection or vaccination is associated with over 80% reduced risk of infection in the following seven months [672]. Moreover, individuals with hybrid immunity exhibit a lower risk of reinfection compared to those only infected or only vaccinated [576,673,674].

Hypotheses and Objectives

Hypotheses

1. Antibody responses to SARS-CoV-2 persist for at least 12 months after infection and are longer-lasting than those induced by COVID-19 vaccination. These responses exhibit considerable heterogeneity, influenced by factors such as age, sex, comorbidities, and prior SARS-CoV-2 exposures.

2. The nature of the first antigen encounter with SARS-CoV-2 (through infection or vaccination) significantly shapes the quality, magnitude, and durability of adaptive immune responses.

3. The emergence of new SARS-CoV-2 variants poses a significant threat to pre-existing immunity elicited by earlier infections or COVID-19 vaccination due to immune evasion mechanisms.

Objectives

The aim of this thesis is to characterize the adaptive immune responses, specifically antibody and T-cell responses, elicited by SARS-CoV-2 infection, COVID-19 vaccination, or a combination of both. This study focuses on responses to the ancestral virus and its major emerging variants over three years, aiming to determine their duration, the factors influencing them, and the immune determinants of protective immunity.

The specific objectives are:

1. To investigate the kinetics, maintenance, and determinants of antibody responses to SARS-CoV-2 over time, induced by infection, vaccination, or a combination of both.

2. To assess whether the nature of first antigen SARS-CoV-2 exposure (through infection or vaccination) impacts adaptive responses to SARS-CoV-2 over time.

3. To evaluate the cross-recognition of adaptive responses elicited by SARS-CoV-2 infection and COVID-19 vaccination against newly emerged viral variants.

Materials, Methods and Results

Chapter 1

Sustained seropositivity up to 20.5 months after COVID-19

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Sustained seropositivity up to 20.5 months after COVID-19

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Abstract

This study evaluated the persistence of IgM, IgA, and IgG to SARS-CoV-2 spike and nucleocapsid antigens up to 616 days since the onset of symptoms in a longitudinal cohort of 247 primary health care workers from Barcelona, Spain, followed up since the start of the pandemic. The study also assesses factors affecting antibody levels, including comorbidities and the responses to variants of concern as well as the frequency of reinfections. Despite a gradual and significant decline in antibody levels with time, seropositivity to five SARS-CoV-2 antigens combined was always higher than 90% over the whole study period. In a subset of 23 participants who had not yet been vaccinated by November 2021, seropositivity remained at 95.65% (47.83% IgM, 95.65% IgA, 95.65% IgG). IgG seropositivity against Alpha and Delta predominant variants was comparable to that against the Wuhan variant, while it was lower for Gamma and Beta (minority) variants and for IgA and IgM. Antibody levels at the time point closest to infection were associated with age, smoking, obesity, hospitalization, fever, anosmia/hypogeusia, chest pain, and hypertension in multivariable regression models. Up to 1 year later, just before the massive roll out of vaccination, antibody levels were associated with age, occupation, hospitalization, duration of symptoms, anosmia/hypogeusia, fever, and headache. In addition, tachycardia and cutaneous symptoms associated with slower antibody decay, and oxygen supply with faster antibody decay. Eight reinfections (3.23%) were detected in low responders, which is consistent with a sustained protective role for anti-spike naturally acquired antibodies. Stable persistence of IgG and IgA responses and cross-recognition of the predominant variants circulating in the 2020–2021 period indicate long-lasting and largely variant-transcending humoral immunity in the initial 20.5 months of the pandemic, in the absence of vaccination.

Keywords: COVID-19, SARS-CoV-2, Antibody, Seroprevalence, Kinetics, IgM, IgG, IgA, Health care workers, Duration

Introduction

The maintenance and effectiveness of adaptive immunity directed against SARS-CoV-2 after primary infection are key questions in understanding and controlling the COVID-19 pandemic and any future emerging new coronavirus threat. Despite the global start of vaccination campaigns by the end of 2020, a substantial percentage of the world's population remains unvaccinated, and their capacity to resist infections relies only on naturally acquired immunity. We have previously shown that 90% of those infected with SARS-CoV-2 remain seropositive 1 year after discharge [1, 2]. To our knowledge, the duration of antibody responses following natural infection has not been assessed beyond 13-20 months to date [3–10].

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SARS-CoV-2 elicits robust humoral immune responses, including production of virus-specific immunoglobulin M (IgM), IgA, and IgG. IgM and IgA isotypes dominate the early effector antibody response to SARS-CoV-2, and IgA greatly contributes to virus neutralization at mucosal sites [11, 12]. In serum, the three isotypes display neutralizing activity, with IgM and IgG1 (predominant subclass of IgG) being the most important contributors [13].

Reinfection and COVID-19 disease rates, including severe cases, may increase if immunity wanes in those who do not get vaccinated. The emergence of SARS-CoV-2 variants of concern (VoC) with high transmissibility and potentially lower susceptibility to antibodies has raised the question of whether antibodies induced by the original Wuhan strain will still protect against reinfections or only against severe COVID-19 [14]. Therefore, data on the long-term persistence and efficacy of the immune response is of vital importance to foresee the evolution of the COVID-19 pandemic especially with more contagious emerging variants like Delta and Omicron [15–18]. Data could also be useful to infer the potential duration of vaccine-elicited immunity, which started to be studied a year after the onset of the pandemic.

There is a wide heterogeneity in how individuals respond to SARS-CoV-2 infection in terms of type and potency of immune responses, resulting in diverse viral and clinical presentations and susceptibilities. Systematic reviews and meta-analyses have concluded that men, those over 65 years of age, smokers, and patients with comorbidities such as hypertension, diabetes, cardiovascular disease, cerebrovascular disease, chronic obstructive pulmonary disease (COPD), chronic kidney disease, and cancer, contribute significantly to disease severity and COVID-19 prognostic [19–27]. However, thus far, very few studies have assessed the effect of comorbidities on SARS-CoV-2 immune responses, including antibodies that mediate neutralizing protective effector functions [28, 29]. Furthermore, it is also likely that individuals also vary in their capacity to maintain protective antibody responses in time, and the factors determining humoral immune memory are not known.

The main objectives of this study were to evaluate the kinetics of anti-SARS-CoV-2 antibodies over a period of 20.5 months in convalescent unvaccinated individuals from a well-characterized longitudinal cohort of health care workers (HCW) (CoviCatCentral), to assess the effect of clinical and demographic variables on the antibody levels, and to estimate the prevalence of reinfections.

Methods

Study design and subjects

Two hundred forty-seven HCW presenting with COVID-19 in three primary care counties in Barcelona, Spain, were recruited in a prospective cohort from March 2020 [1] and followed up during 2021, with sample collection performed at different time points (T) per individual: T0, July–August 2020; T1, September 2020; T2, October 2020; T3, November 2020; T4, January-February 2021; T5, March–April 2021; T6, May–June 2021; T7, July 2021; and T8, November 2021. Infections were detected by antigen rapid diagnostic tests (RDTs) and quantitative reverse transcription polymerase chain reaction (RT-qPCR) performed on participants with symptoms of COVID-19 or who had been in close contact with someone with SARS-CoV-2 infection. Overall, primary infections occurred between pre-T0 and T4. The effect of baseline characteristics on the anti-SARS-CoV-2 antibody response 1 year after the onset of the pandemic has already been reported [1] except for comorbidities and other risk factors that are addressed here: chronic kidney disease, COPD, asthma, cardiovascular disease, neurological diseases, digestive diseases, autoimmune diseases, cancer, immunosuppression (disease or drug-related), obesity, pregnancy, diabetes mellitus, dyslipidemia, hypertension, depression and/or anxiety, and hypothyroidism. Anti-SARS-CoV-2 serologic testing was performed at nine cross-sectional visits, and data on those not being vaccinated by mid-November 2021 is analyzed here. The later visits included in this analysis were T6 (May-June 2021), T7 (July 2021), and T8 (November 2021). The baseline (T0, July–August 2020) sample was obtained from the SeroCatCentral/VisCat study. T6 ($N=72$) included 22 physicians or dentists, 35 nurses, and 15 with other job categories like customer and social services staff, with median (IQR) age of 45 (13) years and 86.3% being women; T7 ($N=39$) included 11 physicians/dentists, 21 nurses, and 7 others, with median (IQR) age of 48 (13) years and 87.2% women; T8 ($N=23$) included 4 physicians/dentists, 13 nurses, and 6 others, with median (IQR) age of 49 (13) years and 87% women.

The study protocols were approved by the IRB *Comitè Ètic d'Investigació Clínica IDIAP Jordi Gol* (codes 20/186-PCV, 20/094-PCV and 20/162-PCV), and written informed consent was obtained from participants.

SARS-CoV-2 antibody measurements

Naturally acquired IgM, IgA, and IgG responses to SARS-CoV-2 were quantified by Luminex. The antigen panel included five proteins: the spike full length protein (S) (aa 1-1213 expressed in Expi293 and His tag-purified) produced at the Center for Genomic Regulation (CRG, Barcelona), and its subregion S2 (purchased from Sino-Biological), the receptor-binding domain (RBD) kindly donated by the Krammer lab (Mount Sinai, New York), the nucleocapsid (N) full length (FL) protein, and the

specific C-terminal (CT) region (both expressed in-house in ISGlobal in *E. coli* and His tag-purified). In addition, the RBD proteins of four VoC (Alpha, Beta, Gamma and Delta, produced at CRG) were tested in the first and last three visits. Coupling of SARS-CoV-2 proteins to MagPlex® polystyrene 6.5 µm COOH-microspheres (Luminex Corp, Austin, TX, USA) was done as described [1, 30, 31]. Antigen-coupled microspheres were added to a 384-well Clear® flat bottom plate (Greiner Bio-One, Frickenhausen, Germany) in multiplex (2000 microspheres per analyte per well) in a volume of 90 µL of Luminex Buffer (1% BSA, 0.05% Tween 20, 0.05% sodium azide in PBS) using 384 channels Integra Viaflo semi-automatic device (96/384, 384 channel pipette). Two hyperimmune pools (one for IgG, and another one for IgA and IgM) were used as positive controls in each assay plate for QA/QC purposes and were prepared at 2-fold, 8 serial dilutions from 1:12.5. Pre-pandemic samples were used as negative controls to estimate the cutoff of seropositivity. Ten microliters of each dilution of the positive control, negative controls, and test samples (prediluted 1:50 in 96 round-bottom well plates) was added to a 384-well plate using Assist Plus Integra device with 12 channels Voyager pipette. Plasma samples had been previously assessed for optimal sample dilution to avoid saturated responses, tested here at 1:500. To quantify IgM and IgA responses, test samples and controls were pre-treated with anti-human IgG (Gullsorb) at 1:10 dilution, to avoid IgG interferences. Technical blanks consisting of Luminex Buffer and microspheres without samples were added in 4 wells to detect and adjust for non-specific microsphere signals. Plates were incubated for 1 h at room temperature in agitation (Titramax 1000) at 900 rpm and protected from light. Then, the plates were washed three times with 200 µL/well of PBS-T (0.05% Tween 20 in PBS), using BioTek 405 TS (384-well format). Twenty-five microliters of goat anti-human IgG phycoerythrin (PE) (GTIG-001, Moss Bio) diluted 1:400, goat anti-human IgA-PE (GTIA-001, Moss Bio) 1:200, or goat anti-human IgM-PE (GTIM-001, Moss Bio) 1:200 in Luminex Buffer was added to each well and incubated for 30 min. Plates were washed and microspheres resuspended with 80 µL of Luminex Buffer, covered with an adhesive film, and sonicated 20 s on sonicator bath platform, before acquisition on the Flexmap 3D® reader. At least 50 microspheres per analyte per well were acquired, and median fluorescence intensity (MFI) was reported for each analyte. Assay positivity cut-offs specific for each isotype and antigen were calculated as 10 to the mean plus 3 standard deviations of $\log_{10}$-transformed MFI values of 128 pre-pandemic controls (Additional file 1: Fig. S1). Positive serology was defined by being positive for IgG, IgA and/or IgM to any of the SARS-CoV-2 wild type of the antigens tested (NFL, NCT, S, RBD, S2).

Data analysis

We modeled antibody level trajectories over time with linear mixed models (LMM) using linear and quadratic fix effect terms for the time since infection and a random effect intercept to account for the dependency of longitudinal observations coming from the same individual. We repeatedly fitted LMMs changing our outcome of interest, which were the $\log_{10}$(MFI) for the different antigen and antibody isotype pairs. Considering that we modeled the $\log_{10}$(MFI) and that MFI signal is supposed to be relatively linear with antibody levels, negative (or positive) linear trends imply a constant negative (or positive) exponential antibody levels decay (or growth), whereas deviations from a linear trend for the $\log_{10}$(MFI) imply an acceleration or deceleration of the exponential antibody change. Estimated fixed effect regression coefficients and their standard deviations were used for prediction of temporal curves of antibody population averages and their 95% confidence intervals (CI). The associations between baseline determinants, clinical presentations, comorbidities and levels of antibodies were assessed at the time point closest to infection (between 5 and 9 months) and, at a later time point just prior to vaccination, about a year after infection (T4). Both univariable linear regression and stepwise regression models were fit to determine the effects of baseline variables on antibody levels ($\log_{10}$MFI). Multivariable models were selected based on the Akaike and Bayesian information criteria and adjusted r-square parameter. Finally, the formulas of the models were selected specifically at the antibody isotype level. For an easier interpretation of the results, a transformed beta value (%) of the log-linear model was calculated with the formula: ([10^beta]-1)*100, giving the difference (in percentage) in antibody levels when comparing to the reference group for categorical variables or for a one-unit increase for continuous variables. Likewise, a transformed beta value (%) of the log-log model was calculated with the formula: ([10^(beta* $\log_{10}$(1.1))]-1)*100, giving the difference (in percentage) in antibody levels for a 10% increase of the predictor variable, for continuous variables. Finally, we also assessed the association of the same baseline variables with differences in the rate of antibody changes as were estimated in our LMM fits of each antibody isotype kinetics. This association was estimated as a fix effect interaction with the time since symptom onset and was repeatedly estimated for all variables while controlling for a false discovery rate of 5%. Reinfected individuals were not excluded from the analysis of antibody kinetics or from the models to assess the associations of variables with antibody levels

or decay. *p*-values were considered statistically significant at the 5% level. All data collected were managed and analyzed using the R software version 4.1.2.

Results and discussion

Of the total 247 HCW with past COVID-19 disease included in the cohort, Table 1 shows the number of non-vaccinated participants tested serologically per visit (T0–T8), involving 809 plasma samples and 15,267 antibody-antigen pair measurements overall. Among them, SARS-CoV-2 seropositivity combining all Ig isotypes and antigens was >95% up to November 2021 ($N=23$). The highest seropositivity was for IgG (~96%), especially for anti-S and anti-RBD responses, and IgA (~96%), mainly for anti-S responses. Seropositivity for IgM was ~48%, mainly for anti-RBD responses. Compared to July 2021, IgG levels remained stable and IgA and IgM seropositivity was increased in November 2021, probably due to an increase in asymptomatic infections, coinciding with the start of the sixth wave in Catalonia. This increase can be observed in the trajectory plots between T7 and T8 in Fig. 1, and it is especially evident for IgM to RBD and IgA to RBD and NFL. The kinetics of antibody levels up to 616 days since symptoms onset are shown in Fig. 1. The decay was more pronounced for anti-N than anti-S IgGs, with a remarkable sustain of S and S2 antibodies, less so for RBD. Overall, there was a slight but significant increase in IgA levels to S and S2 with time as observed by the predicted positive change in levels (Fig. 1), in contrast to the gradual decrease in antibody levels to the other antigens. Consistently, multivariable models at T4 had negative beta coefficients for all except IgA to S antigens that did not significantly wane with days since symptoms onset (Additional file 1: Table S1). Anti-S IgA unexpected rise might be related to sub-patent re-exposures resolved at the mucosal compartment. Thus, antibody kinetics after natural infection appeared to be more stably sustained than that after COVID-19 vaccination, which has been reported by vaccine manufacturers to decline more pronouncedly by 6–9 months [32–34].

We repeated the longitudinal analysis excluding post-reinfection samples from the 8 participants for which we had RT-qPCR diagnosed reinfections and obtained nearly identical results. Some quadratic models gave a positive slope at long times since infection, which could be due to asymptomatic reinfections by Delta and/or Omicron variants, and/or poor goodness of fit for antibody levels owing to the sparsity of data at this interval of time and to the relative simplicity of the model we chose (quadratic) to avoid overfitting.

There was substantially lower binding of circulating antibodies to RBD Beta, followed by Gamma and Delta variants, compared to the wild type, and less difference for Alpha, with an increase in seroprevalence at the later time points (Additional file 1: Table S2). Alpha (B.1.1.7) was first detected in the study area in the summer of 2020 (when B.1.177 was the predominant lineage) [35] and prevailed from February (> 50%) till June 2021 (80–99% cases). Delta (B.1.617.2) was first detected in May 2021, raising to 10% in June, and predominating since July (> 50%) till November 2021 (80–100% cases). Omicron (B.1.529) was first detected early December and was already majoritarian (> 56%) in January 2022. Beta and Gamma frequencies were negligible. Thus, the raise in seropositivity against Delta by T8 could be a mixture of cross-recognition and undetected asymptomatic reinfections at the fifth Spanish pandemic wave (summer–fall 2020).

According to the USA Centers for Disease Control and Prevention [36], reinfection is defined as occurring ≥90 days after initial positive testing or ≥45 days with background information supporting contact with confirmed cases or the reappearance of COVID-19–like symptoms. In our high-risk population (frontline unvaccinated HCW), there were 8/247 reinfections (incidence of 3.23%), with a mean time between first and second infection of 279 days (range 58–586). In a meta-analysis of 19 studies [37], the incidence of reinfection in recovered COVID-19 patients ranged from 0 to 20%. The pooled reinfection rate was 0.65% (95% CI 0.39–0.98%), with high heterogeneity ($I^2=99\%$). One of the studies showing a higher incidence of reinfection (15%) was in HCW from a hospital in Barcelona [38]. In our cohort, the mean age of reinfected individuals was 43.9 ± 9.5 years, 7 were female, and 62.5% had a comorbidity. The comorbidities, clinical presentations, dates of infections, and serology are presented in Additional file 1: Table S3. Seven of the reinfections were symptomatic, 85.7% had similar clinical symptoms in both episodes, and 14.3% had a milder form of disease in the second episode. In no case was the second infection more severe than the first, in contrast to another study where 27.8% of reinfected patients had more severe symptoms in the second episode [39].

Before the second positive RT-qPCR diagnosis, five reinfection cases had negative serology, one was undetermined, and two had positive serology. Among the latter, one (asymptomatic) had a weak antibody positive response, and the other (reporting a close positive contact) had a strong serological response (RBD IgG 10 times above the cutoff, S IgG 8 times above the cutoff). In this second case, the reinfection was with Delta. According to the Public Health England report, Delta increased the chances of reinfection by up to 46% compared to Alpha [40]. Overall, serology data suggest that

Table 1 SARS-CoV-2 seropositivity (overall, by isotype, and by isotype-antigen pair) in a cohort of pre-exposed non-vaccinated health care workers over 2020 and 2021

	T0 ($n=127$)		T1 ($n=122$)		T2 ($n=118$)		T3 ($n=52$)		T4 ($n=151$)		T5 ($n=105$)		T6 ($n=72$)		T7 ($n=39$)		T8 ($n=23$)	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Overall seropositivity	118	92.91%	111	90.98%	109	92.37%	49	94.23%	145	96.03%	105	100%	71	98.61%	38	97.44%	22	95.65%
IgM seropositivity	81	63.78%	65	53.28%	54	45.76%	22	42.31%	76	50%	48	45.71%	11	15.28%	4	10.26%	11	47.83%
IgM N CT	5	3.94%	3	2.46%	3	2.54%	3	5.77%	6	3.97%	2	1.90%	1	1.39%	0	0%	1	4.35%
IgM N FL	5	3.94%	2	1.64%	1	0.85%	0	0%	2	1.32%	1	0.95%	1	1.39%	0	0%	1	4.35%
IgM RBD	70	55.12%	54	44.26%	44	37.29%	16	30.77%	57	37.75%	41	39.05%	11	15.28%	3	7.69%	11	47.83%
IgM S	35	27.56%	38	31.15%	32	27.12%	16	30.77%	45	29.80%	28	26.67%	3	4.17%	1	2.56%	2	8.70%
IgM S2	22	17.32%	21	17.21%	20	16.95%	10	19.23%	34	22.52%	17	16.19%	1	1.39%	1	2.56%	0	0%
IgA seropositivity	112	88.19%	100	81.97%	90	76.27%	43	82.69%	134	88.74%	94	89.52%	57	79.17%	30	76.92%	22	95.65%
IgA N CT	12	9.45%	29	23.77%	24	20.34%	19	36.54%	56	37.09%	12	11.43%	2	2.78%	1	2.56%	1	4.35%
IgA N FL	33	25.98%	18	14.75%	23	19.49%	12	23.08%	37	25%	20	19.05%	4	5.56%	4	10.26%	8	34.78%
IgA RBD	82	64.57%	86	70.49%	75	63.56%	40	76.92%	114	75.50%	84	80.00%	32	44.44%	14	35.90%	17	73.91%
IgA S	105	82.68%	91	74.59%	80	67.80%	42	80.77%	115	76.16%	82	78.10%	52	72.22%	28	71.79%	22	95.65%
IgA S2	97	76.38%	73	59.84%	72	61.02%	40	76.92%	112	74.17%	78	74.29%	40	55.56%	19	48.72%	21	91.30%
IgG seropositivity	118	92.91%	110	90.16%	108	91.53%	47	90.38%	143	94.70%	104	99.05%	70	97.22%	38	97.44%	22	95.65%
IgG N CT	14	11.02%	100	81.97%	83	70.34%	29	55.77%	97	64.24%	25	23.81%	3	4.17%	2	5.13%	0	0%
IgG N FL	110	86.61%	98	80.33%	83	70.34%	23	44.23%	76	50.33%	49	46.67%	8	11.11%	5	12.82%	9	39.13%
IgG RBD	118	92.91%	110	90.16%	108	91.53%	47	90.38%	142	94.04%	104	99.05%	64	88.89%	35	89.74%	22	95.65%
IgG S	118	92.91%	109	89.34%	106	89.83%	47	90.38%	142	94.04%	102	97.14%	70	97.22%	38	97.44%	22	95.65%
IgG S2	118	92.91%	108	88.52%	104	88.14%	44	84.62%	141	93.38%	102	97.14%	66	91.67%	38	97.44%	22	95.65%

T, time point; T0, July–August 2020; T1, September 2020; T2, October 2020; T3, November 2020; T4, January–February 2021; T5, March–April 2021; T6, May–June 2021; T7, July 2021; T8, November 2021. Numbers in parentheses refer to samples tested

n, number of individuals who donated samples per time point (first row) and were positive by serology for each antibody/antigen pair (subsequent rows). Those who just received a first vaccine dose in the prior 6 days were included. The total number of pre-exposed individuals in the study cohort who were not vaccinated in 2021 was 161 at T4, 109 at T5, 77 at T6, 44 at T7, and 30 at T8. *N* nucleocapsid, *FL* full length, *CT* C-terminus, *S* spike, *RBD* receptor-binding domain

most of the reinfections were due to insufficient natural immunity [36, 41], and the last case was probably due to immune escape, i.e., naturally acquired immunity to the original variant was not effective against Delta [42, 43]. In this subset of individuals with reinfections, there were significant increases in the slope for IgG (RBD, $p=0.027$; S, $p=0.008$; S2, $p=0.014$) and IgA (S, $p=0.023$; S2, $p=0.014$) levels, all with rho > 0.21.

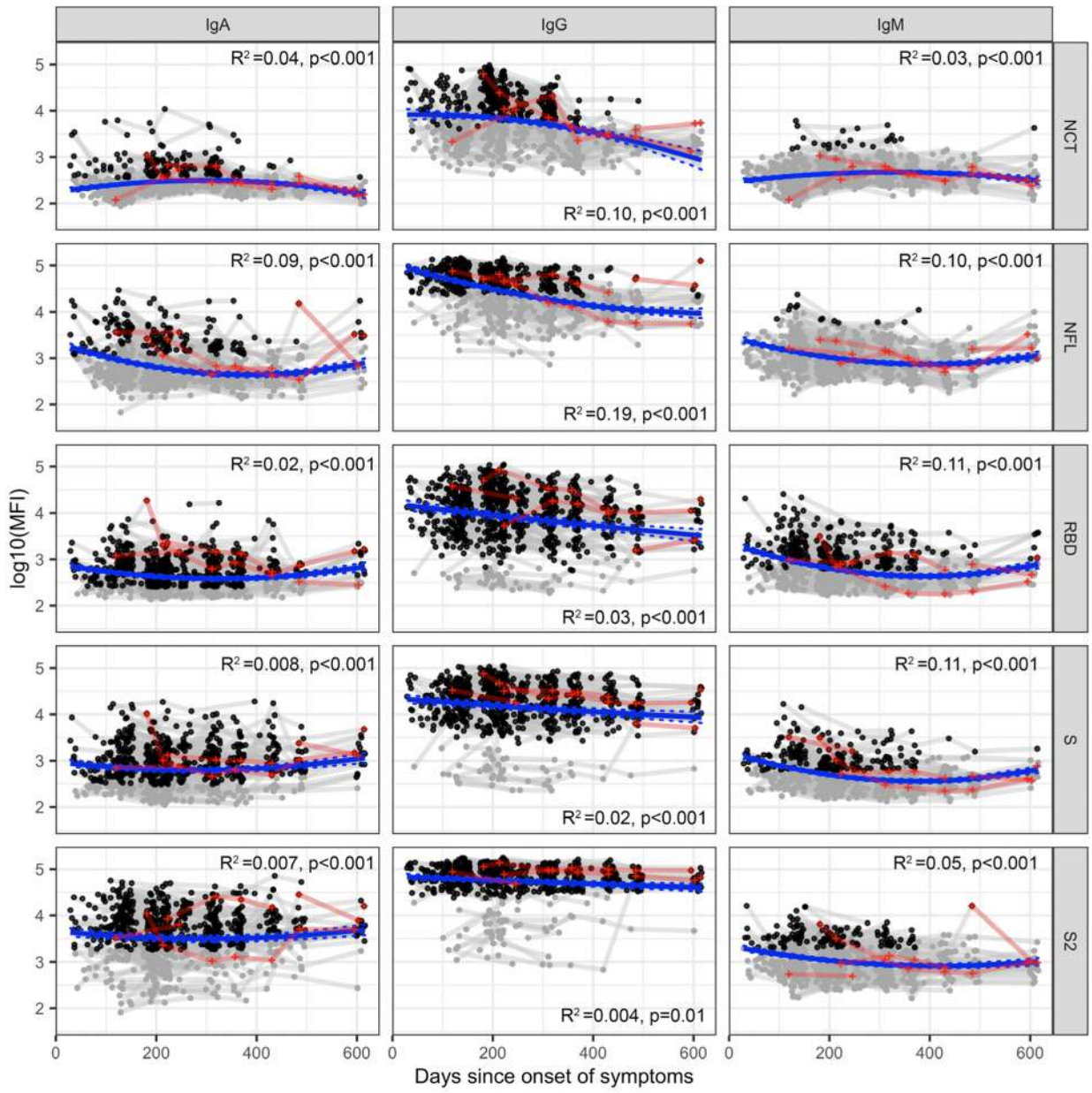
Two hundred twenty-three patients (90%) had at least one comorbidity. The most frequent was depression/anxiety (19.3%), followed by having had previous allergies (15.7%) and dyslipidemia (14.8%). We assessed baseline factors and comorbidities associated to antibody levels measured in the first sample post-infection available from each participant (from 5 to 9 months post infection) by multivariable stepwise regression models adjusting by time since infection (Table 2). Baseline variables most consistently and significantly associated with higher antibody levels 5–9 months after infection were age, obesity ($n=24$), hypertension ($n=18$), and variables related to the initial COVID-19 episode: hospitalization ($n=25$), fever ($n=163$), anosmia and/or hypogeusia ($n=133$), chest pain ($n=41$), and duration of symptoms (Table 2). Specifically, age was positively associated with anti-N IgA and IgG responses, having 2–2.5% higher levels with each year older. Hypertensive individuals had 57% higher N FL IgA levels, and obesity was associated with 25% lower N FL IgM levels. HCW who had anosmia/hypogeusia or fever had significantly higher IgG levels to all antigens than those without these conditions. Chest pain was associated with 20% higher N CT IgM levels. Higher IgA was positively associated with symptoms duration (median 22 days, IQR 12–34; N FL, rho = 0.116, $p=0.083$; RBD, rho = 0.238, $p<0.001$; S, rho = 0.244, $p<0.001$). Hospitalized patients had 79% times higher RBD IgA levels than those non-hospitalized. Baseline factors associated with lower IgG levels included smoking, with 44% less IgG to N CT, 36% less to N FL and 51% less to RBD than non-smokers (Table 2). Variables significantly associated with antibody levels ~1 year after infection and just before most HCW received the first vaccine dose (T4), are shown in Additional file 1: Table S1. Additional factors significantly associated with lower IgA and IgG levels later on were being physician or nurse compared to other occupations in the primary care health sector and headache symptoms during the initial COVID-19 episode. All other variables, symptoms, or sequelae were either not statistically significantly associated with antibody levels or weakly associated in univariable models. Apart from the reported associations with antibody levels at the time closest to and farthest from infection, we also assessed a potential association of the same variables with differences in the rate of antibody changes as estimated in Fig. 1. The most consistent significant variables were tachycardia and cutaneous symptoms, associated with slower antibody decay, and oxygen supply, with faster antibody decay (Additional file 1: Table S4).

Previous acute phase studies have shown that COVID-19 severity is associated with higher antibody responses. Here, hospitalization was associated with higher Ig levels many months after convalescence, suggesting that severity does not affect the stability of memory B cells and antibody-producing plasma cells [44–47]. Common symptoms such as fever and very specific symptoms such as altered smell and taste were also associated with higher antibody levels. Interestingly, hypertension was also positively associated with higher antibodies levels, consistent with some studies [29, 48] but contrary to others [49, 50]. We found that obesity was negatively related to IgM levels, similarly to post-vaccination studies in Italian HCW [50]. Smoking has been previously associated with lower antibody responses [28, 50–52], and we showed that this effect persists after several months, mainly affecting IgG. Finally, lower antibody levels in physicians and nurses in later time points could be due to work-related stress or burn out, which might affect immune memory fitness [53–55].

Limitations of this study include the lack of cellular or neutralizing antibody data, the specific focus on symptomatic HCW, and the limited sample size at later visits due to high vaccination coverage. Because of the screening of only those HCW with symptoms or contact with infected cases, we may have missed several reinfections. Another limitation is that we did not sequence the

(See figure on next page.)

Fig. 1 SARS-CoV-2 seropositivity in a cohort of pre-exposed non-vaccinated health care workers over 2020 and 2021. SARS-CoV-2 IgA, IgG, and IgM antibody (Ab) levels (log_{10} median fluorescence intensity, MFI) by days since COVID-19 symptoms onset. Black dots represent seropositive and gray ones represent seronegative responses. Samples from the same participant are joined by gray lines. Highlighted in red are samples from individuals after a documented reinfection by RT-qPCR. The blue solid line represents the predicted population average calculated using linear mixed models with linear and quadratic fix effect terms for the dependency on time since symptoms onset. Dashed lines correspond to 95% confidence interval. Predicted antibody level changes relative to levels at the onset of symptoms are reported in the table below at 300 and 600 days after it. Reported marginal R^2 gives a measure of the goodness of fit and corresponds to the ratio of variance explained by time since infection over the total variance of the outcome, including the modeled random intercept. Significance of fits departing from that of lack of antibody change (null hypothesis) were assessed using a log-likelihood ratio test comparing a full model containing a linear and quadratic term for time since infection and a reduced model containing none of them. Ab, antibody



Predicted Ab concentration change relative to symptoms onset (% MFI with 95 % CI)

	IgA		IgG		IgM	
	300 days	600 days	300 days	600 days	300 days	600 days
NCT	82.8 (53.6; 117.5)	-2.6 (-23.7; 23.3)	-35.3 (-55.5;-6.0)	-86.1 (-91.9; -76.3))	71.6 (45.6; 102.2)	12.3 (-9.9; 39.9)
NFL	-78.6 (-83.0; -73.0)	-62.6 (-72.1; -49.8)	-81.8 (-85.8; -76.8)	-89.0 (-92.0; -84.9)	-72.2 (-77.2; -66.1)	-63.7 (-71.9; -53.1)
RBD	-48.1 (-57.9; -36.1)	-14.4 (-34.9; 12.5)	-49.3 (-64.0; -28.8)	-71.2 (-81.4; -55.6)	-77.3 (-81.5; -72.2)	-68.4 (-75.5; -59.3)
S	-29.1 (-44.6; -9.4)	19.5 (-12.5; 63.2)	-32.4 (-49.9; -8.8)	-48.5 (-64.7; -25.0)	-73.4 (-77.7; -68.2)	-60.9 (-68.7; -51.3)
S2	-28.1 (-46.8; -2.7)	31.1 (-10.1; 91.2)	-21.8 (-37.6; -2.0)	-23.8 (-42.9; 1.7)	-56.3 (-64.2; -46.7)	-53.7 (-64.1; -40.3)

Fig. 1 (See legend on previous page.)

virus genome of the first infection and only some of the second infections; therefore, we cannot confirm reinfection with another SARS-CoV-2 variant. However, reinfections described occurred > 45 days after the first infection, and all of them had a negative RT-qPCR after the first infection and an increase in antibody levels after the 2nd infection. Future investigations should elucidate what threshold of antibodies correlate with protection

Table 2 Factors affecting Ig levels (log_{10} median fluorescent intensity) 5–9 months after COVID-19 by multivariable stepwise regression models

		N CT	N FL	RBD	S	S2
		Beta (%) 95% CI	**Beta (%) 95% CI**	**Beta (%) 95% CI**	**Beta (%) 95% CI**	**Beta (%) 95% CI**
IgA	Age	**0.9 (0.15; 1.66)**	**1.9 (0.77; 3.04)**	0.48 (− 0.46; 1.43)	0.98 (− 0.23; 2.22)	1.02 (− 0.54; 2.61)
	Shivers	7.38 (− 8.68; 26.27)	17.13 (− 7.94; 49.02)	18 (− 3.79; 44.74)	18.04 (− 9.27; 53.59)	9.08 (− 22.22; 52.98)
	Symptoms duration	0.21 (− 0.09; 0.52)	**0.46 (0.01; 0.92)**	**0.48 (0.1; 0.87)**	**0.57 (0.07; 1.06)**	0.35 (− 0.29; 0.99)
	Sputum	− 0.53 (− 27.1; 35.73)	21.77 (− 23.28; 93.26)	26.52 (− 14.49; 87.19)	4.24 (− 37.08; 72.7)	− 12.9 (− 54.47; 66.64)
	Fever	− 5.07 (− 21; 14.07)	9.16 (− 16.92; 43.43)	13.39 (− 10.05; 42.93)	28 (− 5.02; 72.5)	26.53 (− 13.77; 85.65)
	Anosmia/hypogeusia	7.96 (− 7.85; 26.48)	− 1.43 (− 22.1; 24.72)	3.22 (− 15.45; 26.03)	24.88 (− 3.44; 61.51)	**49.2 (7.2; 107.64)**
	Hospitalization	14.35 (− 23.18; 70.2)	− 8.98 (− 49.6; 64.39)	**79.25 (8.58; 195.91)**	47.25 (− 22.82; 180.94)	85.13 (− 19.29; 324.64)
	Hypertension	18.89 (− 11.13; 59.07)	**56.55 (1.57; 141.29)**	8.63 (− 24.73; 56.77)	− 12.94 (− 45.74; 39.69)	12.92 (− 38.5; 107.32)
	Dizziness	− 4.2 (− 21.91; 17.53)	− 1.46 (− 27.28; 33.52)	18.82 (− 8.17; 53.73)	20.71 (− 13.39; 68.25)	35.75 (− 11.4; 107.98)
	Oxygen	17.09 (− 27.3; 88.57)	35.15 (− 33.44; 174.42)	− 18.38 (− 55.23; 48.81)	− 13.7 (− 60.2; 87.14)	− 34.56 (− 75.8; 76.93)
	Cough	9.41 (− 7.11; 28.87)	4.24 (− 18.27; 32.95)	6.83 (− 13.09; 31.31)	14.26 (− 12.42; 49.07)	3.92 (− 26.15; 46.25)
IgG	Age	**2.71 (1.37; 4.08)**	**2.23 (1.2; 3.27)**	**2.3 (0.66; 3.97)**	1.31 (− 0.2; 2.84)	0.69 (− 0.4; 1.79)
	Shivers	27.7 (− 5.25; 72.12)	12.11 (− 10.82; 40.94)	27.37 (− 11.63; 83.59)	17.65 (− 16.21; 65.21)	6.24 (− 16.88; 35.79)
	Dyspnea	8.58 (− 20.4; 48.09)	1.35 (− 20.11; 28.57)	− 0.57 (− 32.02; 45.42)	3.96 (− 26.96; 47.97)	− 4.24 (− 25.81; 23.59)
	Fever	**107.38 (49.19; 188.25)**	**89.46 (47.19; 143.88)**	**192.5 (95.41; 337.82)**	**152.84 (73.85; 267.7)**	**82.01 (38.84; 138.59)**
	Anosmia/hypogeusia	**50.43 (13.84; 98.8)**	**58.13 (27.7; 95.8)**	**90.52 (35.41; 168.06)**	**104.14 (48.67; 180.3)**	**73.92 (38.3; 118.71)**
	Hospitalization	36.15 (− 31.97; 172.47)	7.73 (− 36.71; 83.38)	52.13 (− 34.96; 255.88)	38.26 (− 37.19; 204.38)	29.59 (− 26.74; 129.24)
	Dizziness	4.2 (− 27.37; 49.48)	12.64 (− 14.58; 48.54)	17.46 (− 24.5; 82.75)	6.62 (− 29.27; 60.73)	3.68 (− 22.94; 39.49)
	Myalgia/arthralgia	− 0.88 (− 26.47; 33.62)	− 5.9 (− 25.16; 18.31)	− 16.5 (− 42.08; 20.39)	− 9.84 (− 35.81; 26.64)	− 8.22 (− 28.2; 17.32)
	Oxygen	13.09 (− 51.02; 161.11)	14.62 (− 39.65; 117.71)	38.62 (− 50.26; 286.33)	27.63 (− 50.73; 230.58)	6.18 (− 46.63; 111.25)
	Cough	23.2 (− 7.49; 64.07)	21 (− 2.86; 50.73)	29.93 (− 8.52; 84.56)	23.34 (− 10.96; 70.86)	21.7 (− 3.84; 54.03)
	Ex-smoker	0.69 (− 27.97; 40.75)	− 4.64 (− 26.23; 23.28)	4.65 (− 30.57; 57.72)	5.92 (− 27.63; 55.04)	2.3 (− 22.32; 34.72)
	Smoking	**− 43.78*(− 66.34; − 6.09)**	**− 36.12 (− 56.9; − 5.34)**	**− 51.11 (− 73.92; − 8.34)**	− 44.12 (− 68.82; 0.16)	− 32.05 (− 55.43; 3.61)
IgM	Shivers	− 6.18 (− 18.05; 7.4)	0.41 (− 15.79; 19.72)	14.29 (− 9.05; 43.62)	15.08 (− 5.92; 40.77)	7.81 (− 12.62; 33.01)
	Pain chest	**19.63 (0.22; 42.79)**	6.45 (− 15.45; 34.01)	2.84 (− 23.74; 38.68)	8.62 (− 16.56; 41.39)	20.01 (− 8.85; 57.99)
	Sputum	3.67 (− 20.83; 35.77)	7.94 (− 24.01; 53.31)	45.59 (− 7.68; 129.61)	24.46 (− 16.72; 86.02)	0.26 (− 34.07; 52.45)
	Anosmia/hypogeusia	− 1.56 (− 14.2; 12.94)	− 2.61 (− 18.55; 16.45)	3.32 (− 18.08; 30.31)	4.06 (− 15.21; 27.7)	22.74 (− 0.86; 51.96)
	Hospitalization	4.63 (− 25.82; 47.59)	8.15 (− 30.87; 69.19)	38.26 (− 22.67; 147.19)	31.32 (− 21.34; 119.23)	33.55 (− 21.74; 127.92)
	Oxygen	− 6.06 (− 37.76; 41.79)	24.27 (− 27.26; 112.31)	40.07 (− 30.12; 180.75)	22.2 (− 33.82; 125.64)	− 19.77 (− 57.68; 52.09)
	Cough	7.06 (− 7.33; 23.46)	3.06 (− 13.72; 25.31)	16.5 (− 8.05; 49.27)	14.89 (− 6.65; 43.13)	20.07 (− 3.52; 50.67)
	Obesity	− 15.41 (− 31.54; 4.53)	**− 25.48 (− 43.42; − 1.86)**	− 19.05 (− 43.38; 15.73)	− 19.68 (− 41.4; 10.09)	− 13.48 (− 37.72; 20.21)

Statistically significant variables indicated in bold font. *SARS-CoV-2 N* nucleocapsid, *FL* full length, *CT* C-terminus, *S* spike, *RBD* receptor-binding domain

against infection and disease, the determinants of antibody longevity, and what features of naturally-acquired antibody kinetics may predict that of vaccine-elicited responses.

In conclusion, our study demonstrates a robust persistence of SARS-CoV-2 antibodies after ~1.7 years, with seropositivity greater than 90% in unvaccinated individuals up to 20.5 months after COVID-19 symptoms onset. The maintenance of anti-S IgG, whose levels highly correlate with neutralizing antibodies [31], appears to be clinically relevant in protecting individuals particularly against the wild type and Alpha variants, despite lack of vaccination, consistent with having symptomatic reinfections in low responders and those reinfected with the more transmissible Delta variant. Antibody kinetics after natural infection appear to be stably sustained, more so than after vaccination, which has led to the implementation of booster immunizations, particularly in face of more contagious VoCs like Omicron. However, previously infected individuals also benefit from vaccination, as hybrid immunity seems to confer the greatest protection against SARS-CoV-2 infections and their symptoms [56].

Abbreviations

S: Spike; RBD: Receptor-binding domain; HCW: Health care workers; BSA: Bovine serum albumin; COPD: Chronic obstructive pulmonary disease; FL: Full length; PBS: Phosphate buffered saline; MFI: Median fluorescence intensity; PE: Phycoerythrin; RDTs: Rapid diagnostic tests; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; T: Timepoint; VoC: Variants of concern.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12916-022-02570-3.

Additional file 1: Table S1. Association of baseline variables and comorbidities with levels of SARS-CoV-2 antibodies at time point 4 (January-February 2021) prior to the massive rollout of vaccination. **Table S2.** Seropositivity against the SARS-CoV-2 receptor-binding domain antigen from variants of concern. **Table S3.** Reinfections. **Table S4.** Significant associations of baseline variables with differential antibody rate of change. **Fig. S1.** Overall distribution of antibody responses for each isotype and antigen pair of the 128 pre-pandemic samples (negative controls) along with the cutoffs.

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Authors' contributions
Designed the study: CD, GM, JVA, ARC, ARM. Recruited and followed up patients and collected clinical data: ARM, JVA, ARC, AFC. Performed the laboratory analysis: SA, RR, MV, EPT, DB, AJ. Performed the statistical analysis: GRO, SA, RR, DM, QMC. Produced the proteins for immunoassays: NRM, CC, LI. Coordinated or managed the study and/or laboratory work: JZ, RA, GM, CD. Wrote the first draft: CD. All authors read and approved the final manuscript.

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Availability of data and materials
Data and materials are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
The study protocols were approved by the IRB ***Comitè Ètic d'Investigació Clínica IDIAP Jordi Gol*** (codes 20/186-PCV, 20/094-PCV and 20/162-PCV), and written informed consent was obtained from participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

SUPPLEMENTARY MATERIAL

SUSTAINED SEROPOSITIVITY UP TO 20.5 MONTHS AFTER COVID-19

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ADDITIONAL FILE 1

Table S1. Association of baseline variables and comorbidities with levels of SARS-CoV-2 antibodies at time point 4 (January-February 2021) prior to the massive rollout of vaccination. Only those variables that were selected in the stepwise procedure and were statistically significant in the multivariable regression models are shown.

	IgA										IgG									
	N CT		N FL		RBD		S		S2		N CT		N FL		RBD		S		S2	
	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value
Age	0.90	0.043	2.11	0.001							1.68	0.010	2.01	0.001						
Occupation (ref: Other[1])																				
Nurse or auxiliary nurse	-30.58	0.009	-42.15	0.006	-43.90	0.001	-49.43	0.001	-45.93	0.015	-47.63	0.001	-47.32	0.0004	-49.95	0.009	-38.53	0.039		
Physician or dentist	-27.92	0.027			-40.59	0.006	-46.80	0.005	-41.40	0.047	-37.05	0.029			-45.10	0.032	-39.46	0.045		
Smoking (ref: no)																				
Smoker							106.86	0.024												
Days since COVID-19 onset symptoms			-0.31	<0.0001							-0.43	<0.0001	-0.46	<0.0001	-0.34	0.001	-0.19	0.026	-0.17	0.009
Duration symptoms (days)							0.66	0.013												
Hospitalization first COVID-19 episode															147.13	0.006	108.29	0.013		
Symptoms first COVID-19 episode																				
Fever											60.02	0.007	54.21	0.006	117.39	0.001	116.50	0.000	65.96	0.002
Anosmia/hypogeusia																	72.20	0.002	53.13	0.002
Headache	-20.67	0.037							-38.76	0.015	-34.39	0.009					-33.90	0.029		

[1] Social worker, customer service, technician, driver, maintenance worker, IT worker, X-ray technician, others.
N, nucleocapsid; FL, full length; CT, C-terminus; S, spike; RBD, receptor-binding domain.
For IgM, the only significant variable was days since onset of COVID-19 symptoms, with the following transformed beta coefficients: RBD -0.31%, p<0.0001; S -0.26%, p<0.0001; S2 -0.25%, p<0.0001.

Table S2. Seropositivity against the SARS-CoV-2 receptor binding domain antigen from the different variants.

		T0 (n=126)		T6 (n= 72)		T7 (n=39)		T8 (n=23)	
	Positive	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
IgA	**Wuhan**	82	64.57%	32	44.44%	14	35.90%	17	73.91%
	Alpha	85	67.46%	35	48.61%	14	35.90%	18	78.26%
	Beta	5	3.97%	5	6.94%	1	2.56%	2	8.70%
	Gamma	36	28.57%	7	9.72%	3	7.69%	10	43.48%
	Delta	88	69.84%	29	40.28%	9	23.08%	18	78.26%
IgG	**Wuhan**	118	92.91%	64	88.89%	35	89.74%	22	95.65%
	Alpha	118	93.65%	58	80.56%	31	79.49%	22	95.65%
	Beta	84	66.67%	27	37.50%	12	30.77%	12	52.17%
	Gamma	111	88.10%	46	63.89%	25	64.10%	19	82.61%
	Delta	118	93.65%	50	69.44%	28	71.79%	22	95.65%
IgM	**Wuhan**	70	55.12%	11	15.28%	3	7.69%	11	47.83%
	Alpha	70	55.56%	7	9.72%	3	7.69%	12	52.17%
	Beta	5	3.97%	1	1.39%	1	2.56%	1	4.35%
	Gamma	13	10.32%	1	1.39%	1	2.56%	2	8.70%
	Delta	75	59.52%	10	13.89%	4	10.26%	15	65.22%

T, timepoint. T0, July-August 2020. During early 2021 and before T6 (May-June 2021), Alpha was the predominant variant in Catalonia. Delta appeared in May in the study area and rose steadily till predominating (56-77%) in July 2021. During T7 (July 2021) and T8 (November 2021), Delta predominated (80-100%).

Table S3. Reinfections.

		1st episode		2nd episode			
Socio-demographic variables	**Comorbidities**	**Symptoms period (level)**	**RT-qPCR results**	**Symptoms period (level)**	**RT-qPCR/RDT results**	**Period between infections**	**Serology**
Female, 59 yr, administrative	Smoking, hypertension, neurological disorder, depression, autoimmune disease	23/03/20-25/03/20 (mild)	23/03/20 (+) 06/04/20 (-)	20/05/20-22/05/20 First wave (mild)	20/05/20 (+), 25/06/20 (-)	1 month, 24 days (58 days)	Not known 04/20 (+): 09/20
Female, 42 yr, physician	None	24/03/20-25/05/20 (moderate)	27/03/20 (+) 24/04/20 (-)	08/20-10/20 Second wave (mild -moderate)	25/08/20 (+) 09/10/20 (-)	4 months, 4 days (130 days)	(-): 05 & 08/20 (+): 09/20
Female, 30 yr, nurse	Obesity	15/03/20-14/05/20 (mild)	02/04/20 (+) 22/04/20 (-)	13/10/20-23/12/20 Second wave (mild)	13/10/20 (+)	6 months, 25 days (212 days)	(-): 09/20 (+): 10/20
Female, 45 yr, physician	Autoimmune disease	20/03/20-03/04/20 (mild)	25/03/20 (+), 06/04/20 (-)	No symptoms	19/11/20 (+)	7 months, 21 days (239 days)	(+ weak): 09/20
Male, 41 yr, nurse	None	16/03/20-19/03/20 (mild)	19/03/20 (+), 30/03/20 (-)	07/12/20-09/12/20 Third wave (mild)	07/12/20 (RDT+) 22/12/20 (+)	8 months, 18 days (266 days)	(-): 09/20 (+): 07/21
Female, 37 yr, nurse	Neurological disorder, obesity	09/04/20-04/05/20 (mild)	09/04/20 (+), 27/04/20 (-)	09/01/21-22/01/21 Third wave (mild)	11/01/21 (RDT+), 21/01/21 (+)	9 months, (275 days)	(-): 10/20 (+): 07/21
Female, 44 yr, nurse	None	25/03/20-22/04/20 (mild)	25/03/20 (+), 15/04/20 (-)	09/07/21-22/07/21 Fifth wave (mild)	12/07/21 (+) Delta B.1.617.2	1 year, 3 months, 11 days (471 days)	(-): 03/21 (+): 07/21
Female, 49 yr, nurse	None	14/03/20-24/04/20 (mild)	17/03/20 (+), 07/04/20 (-)	21/10/21-05/11/21 Fifth wave (mild)	21/10/21 (+) Delta B.1.617.2	1 year, 7 months, 7 days (586 days)	(+): 07/21 (+): 10/21

Table S4. Significant associations of baseline variables with differential antibody rate of change. Transformed time-interaction beta values were obtained from regression coefficient estimates of interaction terms between baseline variables of interest and time since the onset of symptoms in linear mixed models as described in the Data Analysis section. We converted time since infection from days to months for an easier interpretation. Thus, transformed betas can be directly understood as antibody MFI percentual monthly increases (or decreases) over those antibody MFI values that would have been measured had no differential rate of change existed. P-values for each interaction term were obtained from t-statistics using Satterthwaite's method for denominator degrees of freedom [1]. Only significant interaction terms were reported in the table, out of 533 tests conducted for each baseline variable separately. Reported adjusted p-values were corrected for a false discovery rate of 5% following the Benjamini-Hochberg procedure [2].

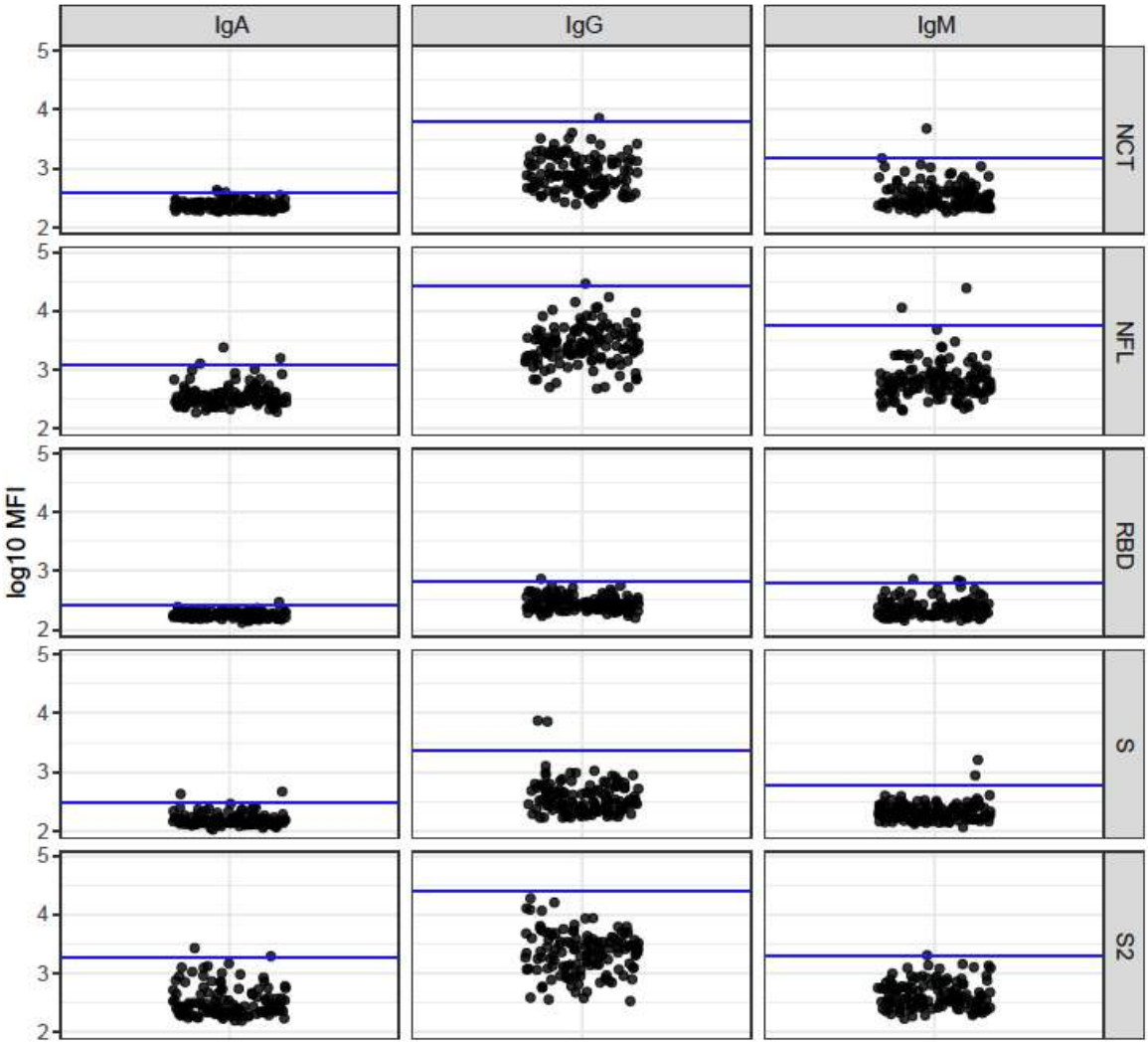
	IgA								IgM						IgG							
	N FL		RBD		S		S2		N CT		RBD		S		N FL		RBD		S		S2	
	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value	%Beta	p-value
Age							-0.213	0.017			-0.1602	0.005										
Duration symptoms (days)																			0.1064	0.037		
Post COVID condition									-3.176	0.005												
Hospitalization first COVID-19 episode											-5.274	0.004	-4.071	0.012								
Oxygen first COVID-19 episode			-6.510	0.024			-8.140	0.027			-6.181	0.009										
Symptoms first COVID-19 episode:																						
Dyslipidemia											-5.384	0.028										
Asthenia																	-6.378	0.036				
Dyspnea	-3.906	0.005																				
Sputum					-4.941	0.028	-5.882	0.028														
Ear															-3.499	0.040						
Tachycardia							7.362	0.001							6.481	0.001	8.517	0.001	7.850	0.001	8.571	0.001
Cutaneous	4.776	0.028													6.290	0.009					4.766	0.000
Headache							4.327	0.048														
Fever													-3.146	0.028								

N, nucleocapsid; FL, full length; CT, C-terminus; S, spike; RBD, receptor-binding domain.

1. Alexandra Kuznetsova, Per B. Brockhoff and Rune H. B. Christensen (2017) lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, 82(13), 1–26. doi:10.18637/jss.v082.i13

2. Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B*, **57**, 289–300. http://www.jstor.org/stable/2346101.

Figure S1. Overall distribution of antibody responses for each isotype and antigen pair of the 128 pre-pandemic samples (negative controls) along with the seropositivity cutoffs.



Chapter 2

Eleven-month longitudinal study of antibodies in SARS-CoV-2 exposed and naïve primary health care workers upon COVID-19 vaccination

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Eleven-month longitudinal study of antibodies in SARS-CoV-2 exposed and naïve primary health care workers upon COVID-19 vaccination

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Abstract

We evaluated the kinetics of antibody responses to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike (S) and nucleocapsid (N) antigens over five cross-sectional visits (January–November 2021), and the determinants of pre-booster immunoglobulin levels, in a prospective cohort of vaccinated primary health care workers in Catalonia, Spain. Antibodies against S antigens after a full primary vaccination course, mostly with BNT162b2, decreased steadily over time and were higher in pre-exposed (n = 247) than naïve (n = 200) individuals, but seropositivity was maintained at 100% (100%IgG, 95.5% IgA, 30.6% IgM) up to 319 days after the first dose. Antibody binding to variants of concern was highly maintained for IgG compared to wild type but significantly reduced for IgA and IgM, particularly for Beta and Gamma. Factors significantly associated with longer-term antibodies included age, sex, occupation, smoking, adverse reaction to vaccination, levels of pre-vaccination SARS-CoV-2 antibodies, interval between disease onset and vaccination,

Carlota Dobaño and Anna Ramírez Morros shared first authorship, Gemma Moncunill and Anna Ruiz Comellas shared last authorship.

hospitalization, oxygen supply, post COVID and symptomatology. Earlier morning vaccination hours were associated with higher IgG responses in pre-exposed participants. Symptomatic breakthroughs occurred in 9/447 (2.01%) individuals, all among naïve (9/200, 4.5%) and generally boosted antibody responses. Additionally, an increase in IgA and/or IgM seropositivity to variants, and N seroconversion at later time points (6.54%), indicated asymptomatic breakthrough infections, even among pre-exposed. Seropositivity remained highly stable over almost a year after vaccination. However, gradually waning of anti-S IgGs that correlate with neutralizing activity, coupled to evidence of an increase in breakthrough infections during the Delta and Omicron predominance, provides a rationale for booster immunization.

KEYWORDS
antibodies, baseline determinants, cohort, COVID-19, duration, health care workers, kinetics, SARS-CoV-2, spike, vaccine, vaccine breakthroughs, variants

INTRODUCTION

Two years into the COVID-19 pandemic and 1 year after the start of vaccination rollout, the world faced a peak of cases associated with the highly contagious Omicron variant of concern (VoC) of SARS-CoV-2. Immunity achieved through natural infection and vaccination has had a large impact in containing disease severity and deaths, but transmission has not been interrupted and breakthrough infections are common, though often asymptomatic or mild [1]. Key questions remain regarding the correlates of protection [2–4], durability of immunity and evasion capacity of emerging VoCs, which prevent a rational prioritization of second-generation vaccines and the design of booster immunization policies. The mRNA-1273 (Moderna) [5] and BNT162b2 (Pfizer/BioNTech) [6] vaccines remain 93% and 84% effective, respectively, 6 and 4 months after a second dose, but an apparent decline of protection in subsequent months led to the implementation of 3rd doses, even when a substantial number of individuals may still have high antibody and cellular immune responses. Most vaccines maintain binding and functional antibodies against many SARS-CoV-2 variants, with Beta (B.1.351) and Omicron (B.1.1.529) having the lowest antibody recognition [7, 8]. However, data on primary vaccination effectiveness are lacking beyond 9 months, even though booster administrations (3rd and beyond) were implemented in many countries [9–13].

There is significant individual heterogeneity in the immune response to natural infection and to partial immunization, less so at the peak response after a full primary vaccination course [14], and immunity differs by vaccine [15]. A more potent response, so-called hybrid immunity, is achieved following SARS-CoV-2 infection (more so if symptomatic) and vaccination, even with only one dose [15–17]. Other factors affecting primary vaccine responses include interval between doses [18], comorbidities and smoking [15]. However, it is less clear what determinants affect the maintenance of immune responses as time progresses and thus who should be revaccinated and when. Moreover, it is likely that, as antibodies decline months after vaccination, there is variability also in their decay rate. Therefore, it is important to identify the determinants of sustained immunity to move towards more personalized evidence-based vaccine strategies if and when protective immunity wanes.

To better understand determinants of durability of vaccine antibody responses, we performed a longitudinal cohort study between January and November 2021 in 447 health care workers (HCW) with and without prior COVID-19, and assessed demographic, clinical (symptoms, comorbidities) and epidemiological factors affecting the levels of antibodies almost a year after vaccination, just before the implementation of the 3rd booster and the onset of the Omicron wave (sixth) in Spain. Such data are relevant to establish what factors impact resistance to breakthrough infections, and rationally define when revaccination may be warranted.

MATERIALS AND METHODS

Study subjects

The CoviCatCentral cohort is composed of two groups of primary HCW, recruited in three primary care counties in Barcelona, Spain, who were offered COVID-19

vaccination starting December 2020. The first group was composed of individuals recruited since the first wave of the COVID-19 pandemic (March–April 2020, $n = 247$) with symptomatic SARS-CoV-2 infection confirmed by reverse transcriptase polymerase chain reaction and/or antigen rapid diagnostic test (RDT); all HCW with COVID-19 were invited to participate. HCW were subsequently visited at seven cross-sectional surveys up to end November 2021, with venous blood collection since September 2020. The second group was composed of naïve HCW recruited since March–April 2021 after full primary vaccination ($n = 200$), having similar characteristics (age, sex, professional category, smoking habits) to the pre-exposed group; participants were visited at four cross-sectional surveys, with venous blood collection up to end November 2021. Demographic and clinical data were collected at baseline and during follow-up visits through telephone interviews and questionnaires by study physicians and nurses. Recorded baseline information included history of previous environmental allergies, smoking habits, and symptoms in the SARS-CoV-2 infected ones (fever, shivers, headache, asthenia, myalgia, arthralgia, dyspnoea, chest pain, cough, sputum production, anosmia, hypogeusia, odynophagia, tachycardia, dizziness and thrombosis). For the multivariable regression analysis, symptoms were grouped into categories: digestive, otolaryngology, neurological, ophthalmology (conjunctival hyperemia, tearing, dry eyes, blurred vision) and skin disorders. Follow-up questionnaires registered comorbidities, including chronic kidney disease, chronic obstructive pulmonary disease, asthma, cardiovascular disease, neurological diseases, digestive diseases, autoimmune diseases, cancer, immunosuppression (disease or drug-related), diabetes mellitus, dyslipidemia, hypertension, depression and/or anxiety and hypothyroidism, as well as pregnancy status, obesity and other cardiovascular risk factors (smoking habits), and new or persistent symptoms, including sequelae post COVID-19 condition (occurrence of symptoms 3 months after COVID-19, with symptoms and effects lasting for at least 2 months). Reinfections and vaccine breakthroughs were captured by passive case detection.

The study protocol was approved by the IRB *Comitè Ètic d'Investigació Clínica IDIAP Jordi Gol* (codes 20/094-PCV and 20/162-PCV) and written informed consent was obtained from participants.

Antibody measurements

Levels of IgM, IgA and IgG were quantified in plasma by Luminex. The performance of these Luminex assays to detect seropositivity has been previously reported being of a 100% specificity and 95.78% sensitivity at ≥14 days, and 95.65% sensitivity at ≥21 days since the onset of symptoms, with areas under the curve of 0.977 and 0.999, respectively [19]. Recombinant proteins included the nucleocapsid (N) full length (FL) protein and the C-terminal fragment (N CT), both produced at ISGlobal, the spike (S) FL protein produced at the Centre for Genomic Regulation (CRG), its subregion S2 (SinoBiological), the receptor-binding domain (RBD) donated by the Krammer lab, and the RBD proteins of four VoCs produced at CRG: Alpha, Beta, Delta and Gamma. Antigen-coupled microspheres were added in multiplex to a 384-well μClear® flat bottom plate (Greiner Bio-One) in 90 μl of Luminex Buffer (1% BSA, 0.05% Tween 20, 0.05% sodium azide in PBS) using an Integra Viaflo semi-automatic device. Positive control pools were added to each assay plate as serially diluted titration curves for QA/QC purposes. Pre-pandemic samples ($n = 128$) were used as negative controls. Test and control plasma samples were added to the 384-well plate using an Assist Plus Integra device. All samples were tested at 1:500 dilution, and additionally at 1:5000 dilution for anti-S IgG antibodies to avoid saturation upon vaccination. For IgM and IgA, samples were pre-treated with anti-Human IgG (Gullsorb) at 1:10 dilution, to avoid IgG interferences. Technical blanks (Luminex Buffer and microspheres without samples) were added to control for non-specific signals. Plates were incubated for 1 h at room temperature in agitation at 900 rpm and protected from light. Then, plates were washed three times with 200 μl/well of PBS-T (0.05% Tween 20 in PBS), using a BioTek 405 TS. Twenty-five microlitres of goat anti-human IgG-phycoerythrin (PE) (GTIG-001, Moss Bio) at 1:400, goat anti-human IgA-PE (GTIA-001, Moss Bio) at 1:200, or goat anti-human IgM-PE (GTIM-001, Moss Bio) at 1:200 in Luminex buffer, were added to each well and incubated for 30 min. Plates were washed and microspheres resuspended with 80 μl of Luminex Buffer and acquired on a Flexmap 3D® reader (at least 50 microspheres per analyte per well), and median fluorescence intensity (MFI) was reported for each analyte. The cutoff for seropositivity was calculated with pre-pandemic plasma samples as 10 to the mean + 3 standard deviations of $\log_{10}$-transformed MFI values.

Data analysis

The percentage decrease in seropositivity for RBD VoCs compared to the RBD wild type (WT) Wuhan was calculated as: ([seropositivity RBD WT − seropositivity RBD VoC]/seropositivity RBD WT) × 100. The changes in

TABLE 1 Population characteristics of the entire study cohort according to COVID-19 pre-exposure (up to timepoint 8), and when antibodies were assessed (11 months after vaccination)

	Full study cohort			Timepoint 8		
	All $N = 447$	Pre-exposed $N = 247$ (55.3%)	Naïve $N = 200$ (44.7%)	All $N = 382$	Pre-exposed $N = 194$ (50.8%)	Naïve $N = 188$ (49.2%)
Age[a]	47 (17)	47 (17)	46 (17)	48 (17)	48.5 (17.8)	47 (16.3)
Sex (female)	375 (83.9%)	205 (83.0%)	170 (85.0%)	320 (83.8%)	160 (82.5%)	160 (85.1%)
Occupation						
Physician or dentist	146 (32.7%)	86 (34.8%)	60 (30.0%)	126 (33.0%)	70 (36.1%)	56 (29.8%)
Nurse or auxiliary nurse	195 (43.6%)	111 (44.9%)	84 (42.0%)	161 (42.1%)	82 (42.3%)	79 (42.0%)
Other[b]	106 (23.7%)	50 (20.2%)	56 (28.0%)	95 (24.9%)	42 (21.6%)	53 (28.2%)
Site						
Bages	227 (50.8%)	122 (49.4%)	105 (52.5%)	191 (50.0%)	92 (47.4%)	99 (52.7%)
Osona	109 (24.4%)	72 (29.1%)	37 (18.5%)	100 (26.2%)	65 (33.5%)	35 (18.6%)
Anoia	111 (24.8%)	53 (21.5%)	58 (29.0%)	91 (23.8%)	37 (19.1%)	54 (28.7%)
Type vaccine (Dose 1–Dose 2)[c]						
Moderna alone or combined	2 (0.4%)	2 (0.8%)	0 (0.0%)	2 (0.5%)	2 (1.0%)	0 (0.0%)
Pfizer alone	407 (91.1%)	207 (83.8%)	200 (100.0%)	373 (97.6%)	185 (95.4%)	188 (100.0%)
Astrazeneca or Janssen	6 (1.4%)	6 (2.4%)	0 (0.0%)	6 (1.6%)	6 (3.1%)	0 (0.0%)
Astrazeneca and Pfizer combined	2 (0.4%)	2 (0.8%)	0 (0.0%)	1 (0.3%)	1 (0.5%)	0 (0.0%)
Number doses[c]						
One	76 (17.0%)	76 (30.8%)	0 (0%)	71 (18.6%)	71 (36.6%)	0 (0.0%)
Two	341 (76.3%)	141 (57.1%)	200 (100%)	311 (81.4%)	123 (63.4%)	188 (100%)
Days since 1st vaccination[a]	na	na	na	304 (30.8)	289.5 (105.2)	307 (11)
Days since 2nd vaccination[a,d]	na	na	na	285 (13.5)	281 (25)	286 (11)
Hour vaccine Dose 1[a,e]	17 (4)	16 (4)	17 (3)	17 (4)	16 (4)	18 (3.3)
Hour vaccine Dose 2[a,f]	17 (3)	16 (3)	17 (2)	17 (3)	17 (3)	17 (2)
Adverse events Dose 1[g]						
None	187 (41.8%)	65 (26.3%)	122 (61.0%)	173 (45.3%)	58 (29.9%)	115 (61.2%)
Local	89 (19.9%)	53 (21.5%)	36 (18.0%)	80 (20.9%)	48 (24.7%)	32 (17.0%)
Systemic	141 (31.5%)	99 (40.1%)	42 (21.0%)	129 (33.8%)	88 (45.4%)	41 (21.8%)
Adverse events Dose 2[h]						
None	138 (30.9%)	55 (22.3%)	83 (41.5%)	123 (32.2%)	44 (22.7%)	79 (42.0%)
Local	29 (6.5%)	10 (4.0%)	19 (9.5%)	29 (7.6%)	10 (5.2%)	19 (10.1%)
Systemic	174 (38.9%)	76 (30.8%)	98 (49%)	159 (41.6%)	69 (35.6%)	90 (47.9%)
Smoking						
No	292 (65.3%)	175 (70.9%)	117 (58.5%)	241 (63.1%)	132 (68.0%)	109 (58.0%)
Ex-smoker	84 (18.8%)	54 (21.9%)	30 (15.0%)	79 (20.7%)	50 (25.8%)	29 (15.4%)
Yes	71 (15.9%)	18 (7.3%)	53 (26.5%)	62 (16.2%)	12 (6.2%)	50 (26.6%)
Baseline comorbidities	268 (60%)	148 (59.9%)	120 (60%)	236 (61.8%)	124 (63.9%)	112 (59.6%)
Days between onset symptoms and vaccination[a,i]	na	304 (69)	na	na	303.5 (69.8)	na
Hospitalization first COVID-19 episode	na	25 (10.1%)	na	na	21 (10.8%)	na

(Continues)

TABLE 1 (Continued)

	Full study cohort			Timepoint 8		
	All $N = 447$	**Pre-exposed** $N = 247$ (55.3%)	**Naïve** $N = 200$ (44.7%)	**All** $N = 382$	**Pre-exposed** $N = 194$ (50.8%)	**Naïve** $N = 188$ (49.2%)
Intensive care unit first COVID-19 episode	na	1 (0.4%)	na	na	1 (0.5%)	na
Oxygen first COVID-19 episode	na	16 (6.5%)	na	na	15 (7.7%)	na
Any symptoms first COVID-19 episode	na	247 (100%)	na	na	194 (100%)	na
Duration symptoms (days)[a]	na	22 (21.5)	na	na	20.5 (19.8)	na
Post COVID condition	na	117 (47.4%)	na	na	95 (49%)	na

Note: See Table S1 for detailed list of comorbidities and symptoms.
Abbreviations: NA, missing data; na, not applicable.
[a]Median (IQR).
[b]Social worker, customer service, technician, driver, maintenance worker, IT worker, X-ray technician, others.
[c]na (no doses received): in the full study cohort, 30 (6.7%) among all, 30 (12.1%) among exposed.
[d]na (without Dose 2): 71 (all), 71 (pre-exposed).
[e]Full study cohort: na (without Dose 1): 30 (all), 30 (pre-exposed). NA (missing data from Dose 1): 22 (all), 17 (pre-exposed), 5 (naïve) T8 only: NA (missing data from Dose 1): 18 (all), 14 (pre-exposed), 4 (naïve).
[f]Full study cohort: na (without Dose 2): 106 (all), 106 (pre-exposed). NA (missing data from Dose 2): 10 (all), 3 (pre-exposed), 7 (naïve) T8 only: na (without Dose 2): 71 (all), 71 (pre-exposed). NA (missing data from Dose 2): 8 (all), 2 (pre-exposed), 6 (naïve).
[g]Full study cohort: na (without Dose 1): all 30 (6.7%), pre-exposed 30 (12.1%).
[h]Full study cohort: na (without Dose 2): All 106 (23.7%), pre-exposed 106 (42.9%); T8 only: All 71 (18.6%), pre-exposed 71 (36.6%).
[i]Full study cohort: na (without Dose 1): 30.

post-vaccination levels in relation to days since first dose were expressed as Spearman coefficient (ρ) with p-values. Univariable and multivariable stepwise linear regression models were fit to identify the variables affecting the antibody levels ($\log_{10}$ MFI) up to 11 months after vaccination. Multivariable models were selected based on the Akaike information criterion, Bayesian information criterion and adjusted r-square parameters. For an easier interpretation of the results, a transformed beta value (%) of the log-linear model was calculated with the formula: $([10^{\text{beta}}] - 1) \times 100$, giving the difference (in percentage) in antibody levels when comparing to the reference group for categorical variables or for a one-unit increase for continuous variables. Likewise, a transformed beta value (%) of the log–log model was calculated with the formula: $([10^{(\text{beta} \times \log10(1.1))}] - 1) \times 100$, giving the difference (in percentage) in antibody levels for a 10% increase of the predictor variable, for continuous variables. p-values were considered statistically significant at the 5% level. All data collected were managed and analysed using the R software version 4.1.2.

RESULTS

The characteristics of the study population at baseline and at the later timepoint 8 (T8) visit, when the determinants of long-lasting antibody levels were evaluated, are included in Table 1 and Table S1. Most HCW (97.6%) were vaccinated with BNT162b2 Comirnaty (Pfizer/BioNTech), and a minority received mRNA-1273 Spikevax (Moderna), ChAdOx1 nCov-19 Vaxzebria (AstraZeneca) or Ad26.COV2.S (Janssen, Johnson and Johnson), or combinations.

SARS-CoV-2 seropositivity over time

Seropositivity rates over five cross-sectional visits (T4-T8) following COVID-19 vaccination for each antibody isotype/antigen pair are shown in Table S2, and stratified by prior COVID-19 disease in Table 2. All participants had seroconverted for at least one immunoglobulin/S antigen pair after vaccination, and this was maintained up to 11 months, with no seroreversions. IgG responsiveness was the highest, reaching 100% in all timepoints except the first visit (T4) when some exposed participants had only received one vaccine dose. For IgA and IgM, the percentage of responders was higher in the vaccinated pre-exposed compared to the naïve individuals, reflecting hybrid immunity. Thus, non-responders or hypo-responders were not identified in these HCW cohorts. The most immunogenic antigen was the full-length S, followed by RBD and by S2, which were shown to highly correlate with neutralizing antibodies

TABLE 2 Percentage (%) seropositivity after vaccination stratified by prior COVID-19 status

	T4	T5		T6		T7		T8	
	Exposed ($n = 79$)	Naïve ($n = 200$)	Exposed ($n = 133$)	Naïve ($n = 198$)	Exposed ($n = 165$)	Naïve ($n = 188$)	Exposed ($n = 187$)	Naïve ($n = 188$)	Exposed ($n = 194$)
Positive	97.47%	100%	100%	100%	100%	100%	100%	100%	100%
IgA	96.20%	98%	96.99%	89.90%	96.97%	81.38%	95.72%	93.09%	97.94%
IgA N CT	84.81%	6.50%	44.36%	2.02%	15.76%	2.66%	12.83%	4.79%	11.34%
IgA N FL	50.63%	3%	20.30%	2.02%	9.09%	3.72%	6.95%	10.11%	19.07%
IgA RBD	94.94%	87%	93.98%	39.90%	86.67%	31.91%	86.63%	62.23%	95.36%
IgA S	96.20%	97.50%	96.99%	88.89%	96.97%	80.32%	95.72%	93.09%	97.94%
IgA S2	93.67%	75%	93.23%	26.77%	80.61%	17.55%	78.61%	43.62%	93.30%
IgG	97.47%	100%	100%	100%	100%	100%	100%	100%	100%
IgG N CT	88.61%	10%	67.67%	0%	19.39%	2.13%	12.30%	0.53%	0.52%
IgG N FL	59.49%	1%	36.84%	1.01%	12.73%	1.06%	4.28%	6.91%	27.32%
IgG RBD	96.20%	100%	100%	100%	100%	100%	100%	100%	100%
IgG S	97.47%	100%	100%	100%	100%	100%	100%	100%	100%
IgG S2	96.20%	99.50%	100%	92.93%	98.18%	73.94%	96.26%	67.02%	96.39%
IgM	68.35%	42.50%	65.41%	9.60%	17.58%	5.85%	12.30%	22.34%	38.66%
IgM N CT	1.27%	2.50%	2.26%	1.01%	1.21%	0%	1.07%	3.19%	2.06%
IgM N FL	1.27%	1%	0%	1.52%	0%	0.53%	0%	2.66%	1.03%
IgM RBD	48.10%	17%	45.86%	1.52%	11.52%	1.60%	8.02%	7.98%	30.93%
IgM S	58.23%	38.50%	48.12%	7.58%	6.67%	3.72%	3.21%	11.17%	13.92%
IgM S2	40.51%	7%	20.30%	0%	2.42%	0%	2.14%	1.06%	8.25%

Note: T4 – January/February 2021, T5 – March/April 2021, T6 – May/June 2021, T7 – July 2021, T8 – November 2021. Participants with diagnosis of COVID-19 before vaccination were classified as exposed, and those without prior COVID-19 diagnosis (recruited at T5) as naïve. The colour visualized as a heatmap whereby the intensity of the green color is proportional to the magnitude of the % value

Abbreviations: *n*, number of individuals who donated samples per timepoint; RBD, receptor-binding domain.

in our prior studies [15, 20]. The inclusion of N antigens also allowed assessing the evolution of seropositivity over time in those with prior COVID-19 history, which generally decreased over time. Furthermore, N serology allowed to identify about 1% (for IgG and IgM) to 3% (for IgA) prior unnoticed SARS-CoV-2 exposures, according to N FL seropositivity, in the infection-naïve cohort where individuals did not have prior documented COVID-19 diagnosis. Seropositivity to N CT was excluded from this analysis as we have previously identified a potential antibody cross-reactivity with S following vaccination [21]; indeed seropositivities in naïve individuals post vaccination (T5) were higher for N CT than for N FL (2.5% for IgM, 6.5% for IgA and 10% for IgG) but decreased over time, thus suggesting a transient nature. Finally, N serology also allowed to monitor potential breakthrough infections (see below), with an increase in N FL seroprevalence noted from T7 to T8 for both naïve and pre-exposed individuals (up to 1.8% IgM, 14.6% IgA and 17.3% IgG). The increase in N FL seroprevalence was accompanied by an increase in IgM and IgA to S antigens, coinciding with the onset of the sixth wave in Spain [22], in face of waning antibody levels (see kinetics below).

Antibody recognition of SARS-CoV-2 variants

Seropositivity rates to SARS-CoV-2 RBD from VoCs in comparison to WT RBD over three cross-sectional visits (T6–T8) after COVID-19 vaccination, are shown in Table 3 and Table S3. There was little variation in IgG seropositivity against RBD from VoCs, with an overall decrease between 4.7% and 5.8% against Beta (9.0%–11.7% among COVID-19 pre-exposed) and negligible (up to 1%) against Alpha, Delta, or Gamma. However, there was less cross-variant antibody recognition for IgA, with a greater percentage decrease in seropositivity against RBD from Beta (∼50%) > Gamma (∼30%) > Delta (∼5%) > Alpha (∼1%), being about two-fold higher or more in pre-exposed. There was higher IgA

TABLE 3 Percentage (%) change in seropositivity to receptor binding domain (RBD) after vaccination for variants of concern (VoC) compared to Wuhan, stratified by COVID-19 status. Data are visualized as a heatmap whereby the intensity of the color is proportional to the magnitude of the % value; green indicate decrease and red increase.

	T6		T7		T8	
	Naïve ($n = 198$)	Exposed ($n = 165$)	Naïve ($n = 188$)	Exposed ($n = 187$)	Naïve ($n = 188$)	Exposed ($n = 194$)
IgA RBD Alpha	−2.53[a]	−0.69	3.32	0.62	−3.42	0.53
IgA RBD Beta	87.34	42.66	78.35	47.54	72.65	35.68
IgA RBD Gamma	62.03	19.58	58.32	24.08	34.18	11.89
IgA RBD Delta	5.06	0.7	21.65	1.24	−15.39	0
IgG RBD Alpha	0	0	1.06	0	0	0
IgG RBD Beta	9.6	1.21	11.7	3.21	9.04	0.52
IgG RBD Gamma	0	0	1.6	1.07	1.06	0
IgG RBD Delta	0	0	1.06	1.07	0	0
IgM RBD Alpha	−165.79	26.39	−66.25	33.29	−19.92	−1.75
IgM RBD Beta	33.55	89.5	100	100	73.31	91.65
IgM RBD Gamma	66.45	89.5	66.88	93.39	53.38	76.63
IgM RBD Delta	−199.34	36.89	−99.38	19.95	−79.2	−33.46

Note: Calculated as (seropositivity RBD Wuhan − seropositivity RBD VoC)/seropositivity RBD Wuhan × 100.
[a]Negative percentage values indicate increase in seropositivity.

seropositivity to the Alpha variant compared to the WT at T6 when Alpha was the predominant VoC circulating, and higher IgA seropositivity to the Delta variant compared to the WT at T8 when Delta became the predominant VoC (Table 3 and Table S3). A similar gradient of VoC cross-reactivity with WT was seen for IgM among pre-exposed individuals, with lower variant-transcending recognition compared to IgG (particularly for Beta and Gamma, with ∼80% decrease in seropositivity), but seroprevalences were much lower (Table S3). However, among vaccinated naïve individuals, IgM seropositivity rates increased for Alpha (predominant at T6) and Delta (predominant at T7 and T8) RBD, with a general most prominent increase at T8 when IgG antibodies were the lowest, coinciding with the onset of the sixth wave in Spain (just pre-Omicron), possibly indicative of subclinical incident infection breakthroughs.

Antibody kinetics following vaccination

The evolution of SARS-CoV-2 antibody levels ($\log_{10}$ MFI) over up to 319 days for each immunoglobulin isotype/antigen pair, including VoC RBDs, is shown in Figure 1. The correlation between antibody levels with days since the first vaccine dose resulted in negative and significant Rho Spearman correlation coefficients for IgG and IgA, indicative of waning. IgA appeared to have somewhat slower antibody decay than IgG, and the slope of IgG decay seemed more pronounced post-vaccination ($\rho \cong -0.5$) compared to post-natural infection ($\rho \cong -0.25$) in a prior analysis of this cohort over a similar follow-up time [23]. In multivariable regression models, time since vaccine dose 1 was negatively and significantly associated with IgG to S and RBD antigens at T8 (Table 4). Regarding IgM, even though most participants were seronegative at T8, the slopes of the correlation lines were more stable and, for some antigens (N FL, RBD), ρ values were positive due to increases in levels in T8, as seen with seropositivity. Stratifying by COVID-19 status pre-vaccination, IgA and IgG levels were higher for individuals with prior symptomatic diagnosis of SARS-CoV-2 but the slopes of the waning curves did not seem to diverge substantially from those of naïve individuals (Figure 2). Consistently, the strongest determinant for T8 antibody levels in multivariable regression models was having had COVID-19 before vaccination (Table 4). At the individual level, a 10% increase in pre-vaccination IgG levels significantly increased T8 post-vaccination levels by 3.85% (95% CI 1.03–3.05) against S, 4.16% (95% CI 1.03–3.25) against RBD and 6.02% (95% CI 1.05–4.92) against S2 (all $p < 0.0001$) antigens.

The pattern for anti-N antibodies differed between groups, as expected, with waning in levels for pre-exposed individuals while naïve had flat negative values, with an increase also noted at T8 for N FL IgM and IgA (Figure 2).

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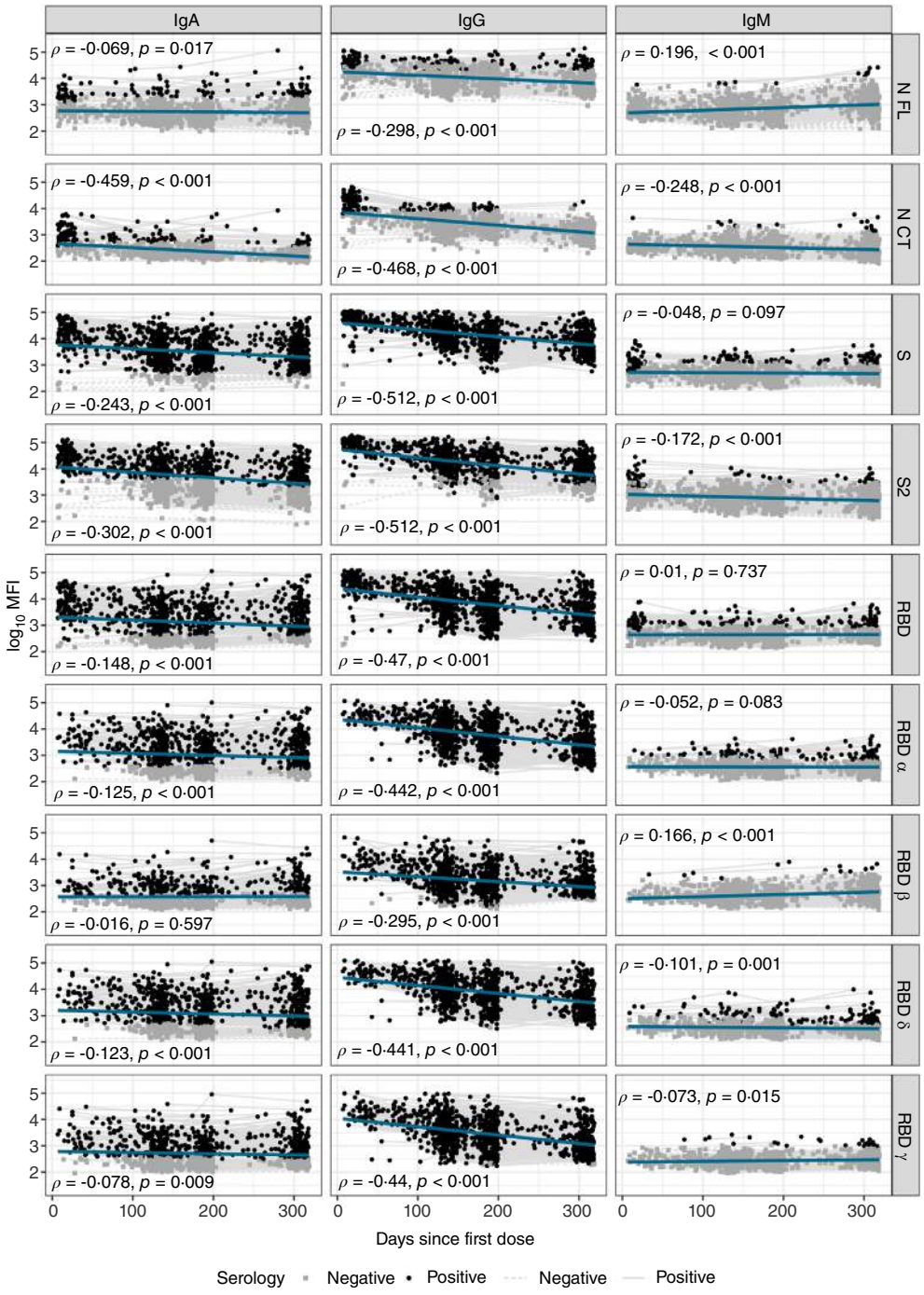

FIGURE 1 SARS-CoV-2 antibody levels by days since first dose of COVID-19 vaccination. IgA (1/500 dilution), IgG-N FL and N CT (1/500 dilution), IgG-S, S2, RBD, RBD α, RBD β, RBD δ and RBD γ (1/5000 dilution) and IgM (1/500 dilution) antibody levels (log_{10} median fluorescence intensity). Black dots represent seropositive and grey squares seronegative individuals. Data from the same individual are linked by grey lines (continuous lines in seropositive and dashed in seronegative). The blue solid lines represent the fitted linear regression models. The Spearman correlation coefficients and *p*-values are reported in each plot. Antigens: Nucleocapsid full length (N FL) and C-terminus (N CT), spike full length (S), S2 subunit, and receptor-binding domain for wild type and variants of concern

Other factors affecting late post-vaccination antibody levels

Baseline and follow-up variables significantly associated with lower T8 post-vaccination antibody levels by multivariable regression models included older age, male sex, being a physician or a nurse compared to other occupations, smoking, and not having had adverse events (AEs) during primary vaccination (Table 4).

Among individuals with prior history of COVID-19, variables significantly associated with higher T8 post-vaccination antibody levels by multivariable regression models included longer intervals since disease onset, hospitalization, not having received supplementary oxygen, as well as having had anosmia/ageusia, fever, dyspnoea, or shivers (Table 4). Post COVID condition was associated with lower IgM levels to RBD. In addition, the hour of first dose vaccination had an effect, with significantly lower levels of IgG to S and all RBD variants with increased time, that is, each hour increase in the vaccination time (from 8:30 AM) reduced post-vaccination T8 IgG levels between 3.8% and 5.7%, adjusted by other significant

TABLE 4 Variables significantly associated with antibody levels to spike antigens post vaccination (T8) by multivariable linear regression models

	IgM RBD		IgM S		IgM S2		IgA RBD		IgA S		IgA S2		IgG RBD		IgG S		IgG S2	
	Beta[a]	*p*-value	Beta	*p*-value	Beta	*p*-value	Beta	*p*-value	Beta	*p*-value	Beta	*p*-value	Beta	*p*-value	Beta	*p*-value	Beta	*p*-value
Full cohort																		
Age	−1.06	0.0002	−1.50	<0.0001	−1.89	<0.0001									0.77	0.0427		
Sex (ref: male)			23.95	0.0074														
Cohort (ref: exposed)	−38.29	<0.0001	−13.51	0.03	−34.87	<0.0001	−78.05	<0.0001	−70.47	<0.0001	−78.67	<0.0001	−74.75	<0.0001	−63.99	<0.0001	−74.57	<0.0001
Occupation (ref: other[b])																		
Physician or dentist									−25.80	0.0484	−26.16	0.0492			−21.13	0.032		
Nurse or auxiliary nurse															−22.40	0.0186	−22.42	0.0205
Smoking (ref: no)													−39.97	0.0006	−24.80	0.0164	−21.53	0.0443
Days since 1st vaccination													−0.36	0.0046	−0.28	0.0072	−0.24	0.0226
Adverse events Dose 1 (ref: none)																		
Systemic													32.88	0.0224				
Adverse events Dose 2 (ref: none)[c]																		
Local																	56.68	0.0189
Systemic													43.06	0.0125	42.66	0.0018	45.21	0.0017
Days between onset symptoms and Dose 1[d]							0.38	<0.0001	0.30	<0.0001			0.19	0.0084	0.15	0.0123		
Pre-exposed cohort																		
Age	−1.38	0.0037	−1.51	0.0002	−1.69	0.0036									1.02	0.0371		
Occupation (ref: other)																		
Physician or dentist																		
Days between onset symptoms and Dose 1							0.32	0.0001	0.24	0.0019			0.17	0.0042	0.14	0.0044	0.11	0.045
Adverse events Dose 1 (ref: none)																		
Systemic													34.17	0.042				

(Continues)

TABLE 4 (Continued)

	IgM RBD		IgM S		IgM S2		IgA RBD		IgA S		IgA S2		IgG RBD		IgG S		IgG S2	
	Beta[a]	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**	**Beta**	***p*-value**
Adverse events Dose 2 (ref: none)																		
Local													147.75	0.0043	99.88	0.0112	105.21	0.0226
Systemic													61.03	0.0106	36.93	0.0493	43.87	0.0495
Hospitalization first COVID-19 episode									190.0	0.0242	170.12	0.0418	321.31	0.0001	200.69	0.0003	144.39	0.0067
Oxygen first COVID-19 episode									−66.4	0.041	−71.24	0.0242						
Post COVID condition	−19.41	0.0387																
Symptoms first COVID-19 episode																		
Anosmia/Ageusia													37.66	0.0097	36.31	0.003	47.89	0.0006
Dispnea													42.13	0.0098	28.31	0.0289	35.90	0.0139
Fever													40.29	0.0153	31.52	0.0195	38.09	0.0118
Shivers			18.00	0.0454														
Digestive																		

[a]Beta coefficient has been transformed into percentage for easier interpretation (see Materials and Methods).

[b]Other: social worker, customer service, technician, driver, maintenance worker, IT worker, X-ray technician, others.

[c]71 na (not applicable) values: not vaccinated with Dose 2. Separate model removing the variable number of doses because all individuals included had received two doses.

[d]188 na (not applicable) values: naïve cohort, without onset of symptoms. Separate model removing variable cohort because all individuals included were pre-exposed.

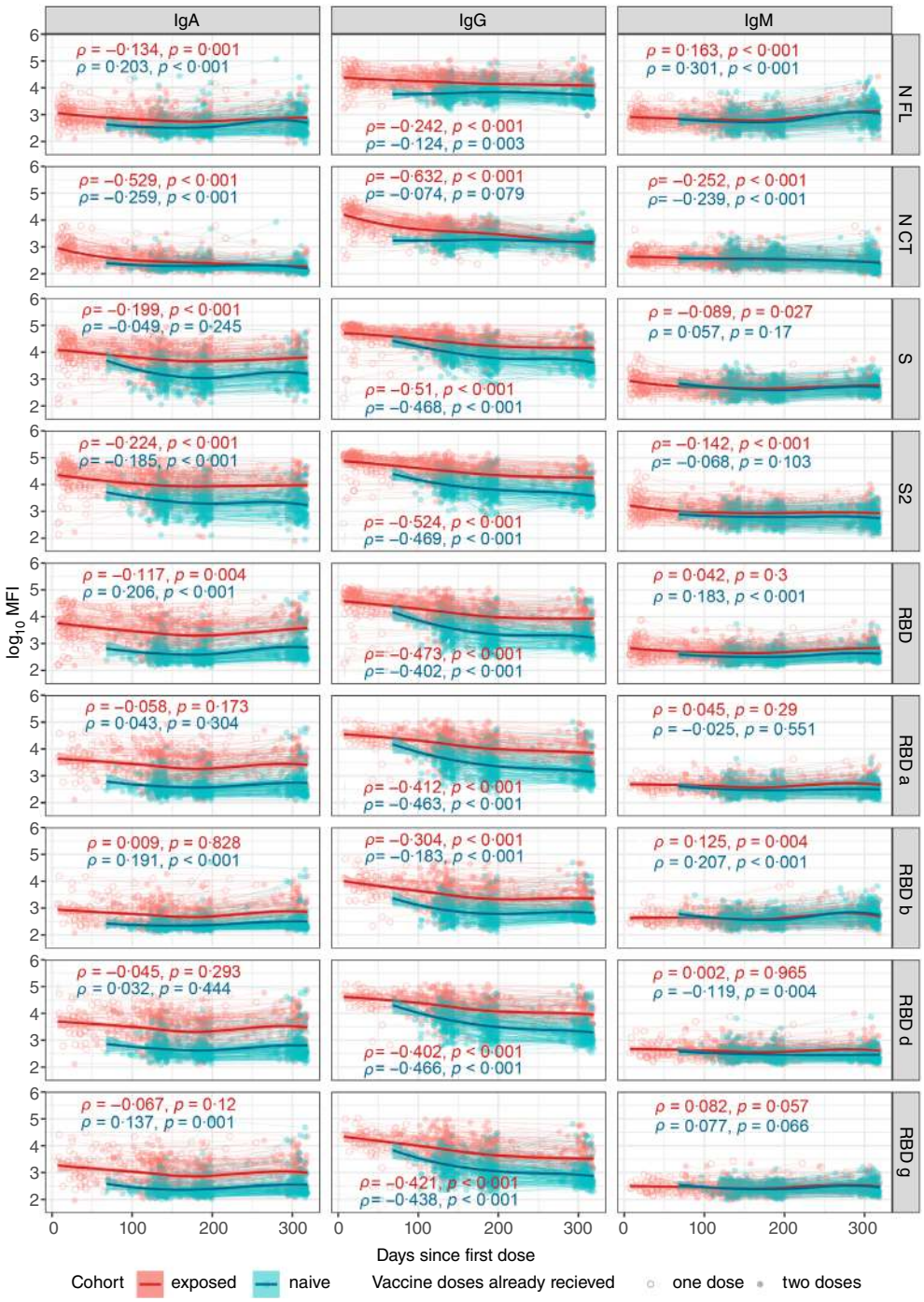

FIGURE 2 SARS-CoV-2 antibody levels by days since first dose of vaccination and by previous COVID-19 status. IgA (1/500 dilution), IgG-N FL and N CT (1/500 dilution), IgG-S, S2, RBD, RBD α, RBD β, RBD δ and RBD γ (1/5000 dilution) and IgM (1/500 dilution) levels ($\log_{10}$ median fluorescence intensity). Red dots are individuals with prior COVID-19 diagnosis and blue dots individuals without (open circles for 1st doses and full circles for 2nd doses). Data from the same individual are linked by coloured lines. The red and blue solid LOESS lines represent the fitted curves of the pre-exposed and naïve individuals, respectively. The Spearman correlation coefficients and *p*-values are reported for pre-exposed and naïve individuals separately. Antigens: Nucleocapsid full length (N FL) and C-terminus (N CT), spike full length (S), S2 subunit, and receptor-binding domain for wild type and variants of concern

covariates (Table 5). The determinants associated with T8 IgG levels to RBD VoCs were very similar to those shown in Table 4 for the RBD WT (data not shown).

Type of vaccine had minor effects because very few HCW received products other than Pfizer/BioNTech. In spite of this, in multivariable models, adenoviral (AstraZeneca and/or Janssen) and Pfizer/BioNTech vaccines induced 56.7% ($p = 0.005$) and 37.5% ($p = 0.015$) lower cross-reactive N CT IgA levels at T8, respectively, than vaccination schedules including Moderna [21]. Among pre-exposed HCW, adenoviral vaccination induced 51.2% ($p = 0.029$) lower cross-reactive N CT IgA levels than Moderna, and Pfizer/BioNTech 74% ($p = 0.028$) higher RBD IgM levels than Moderna.

Breakthrough infections and antibody boosting

There were nine symptomatic vaccine breakthroughs detected, all between HCW with no history of COVID-19, all at the time of Delta predominance during the fifth

TABLE 5 Effect of 1 h increase in the Dose 1 vaccination time (from 8.30 AM) on levels of IgG antibodies up to 11 months later in health care workers with prior history of COVID-19

Antigens	%Beta	95% CI	*p*-value
S	−3.81	−0.0325 to −0.0013	0.034
RBD Wuhan	−5.45	−0.0429 to −0.0057	0.011
RBD Alpha	−5.66	−0.0442 to −0.0064	0.009
RBD Beta	−4.38	−0.0381 to −0.0008	0.041
RBD Delta	−5.00	−0.0406 to −0.0039	0.018
RBD Gamma	−4.95	−0.0418 to −0.0023	0.029
S2	−3.21	−0.032 to 0.0037	0.118

Note: Multivariable models including as regression variables also age, sex, professional category, vaccine adverse events, time between onset of symptoms and Dose 1, hospitalization, oxygen support, symptoms (fever, shivers, dyspnoea, anosmia/ageusia, digestive, dyslipidemia) and their duration. Significant associations or trends are shown. Negative transformed beta coefficient means that there is a decrease in antibodies with increased hours.

wave in Spain (Summer–Autumn 2021). Figure 3 and Figure S1 show the boost in antibodies following those patent infections. An increase in N seropositivity at T8 (Figure 1) was also indicative of asymptomatic breakthroughs, still during the Delta predominance and when the Omicron wave just started. Thus, there were 25/382 seroconverters to N FL (6.54% asymptomatic breakthroughs), of whom 11 (44%) had prior COVID-19 history (i.e., 2.9% asymptomatic breakthroughs among pre-exposed).

DISCUSSION

Our study shows that seropositivity after one or two vaccine doses was maintained in all participants of a prospective HCW cohort up to 11 months after initial immunization, although levels gradually decreased but with high heterogeneity. Infection breakthroughs (2.01% symptomatic and 6.54% asymptomatic) were more frequent towards the end of the follow up and coinciding with predominance of more highly transmissible VoCs. We confirmed that individuals with prior history of COVID-19 still had higher responses almost a year after vaccination, and that post-vaccination levels were positively associated with pre-vaccination levels. Remarkably, compared with our prior analysis post one-year in the same cohort [23], a more steep decay in antibodies was seen after vaccination than following natural exposure, indicating that SARS-CoV-2 infection may induce better memory responses or longer-lived plasma cells.

Although we do not have an absolute correlate of protection to ascertain what circulating levels may suffice to prevent infection, and to rationally indicate when boosters would be beneficial, the occurrence of breakthrough infections suggests that such threshold was likely crossed for a number of individuals, more over in face of a more contagious VoC (Delta) with a high degree of immune escape [24, 25]. Of note, none of the breakthroughs were related to severe disease. In addition to symptomatic cases, seroconversion of N FL antibodies (including IgM) was indicative of asymptomatic infections that appeared to increase further at the later timepoints (T8) when the lowest levels of IgG were attained. Although advances are underway to establish correlates of protection [3, 26–30], not having them also makes it more difficult to link analysis of antibody waning with effectiveness [31], and to predict the impact of VoC like Omicron. In prior studies, 1 month after the second dose of Moderna, 85% of participants had neutralizing activity against Omicron but after 7 months this was reduced to 55% [32].

Here, factors affecting the levels of longer-term antibodies were similar to those involved in early peak responses (age, sex, smoking, time intervals), but with some additional results. We consistently found that physicians and nurses had lower responses than other occupations in the primary care health sector, and this could be due to work-related stress or burn out [33, 34]. The finding that patients who received oxygen supplement also had lower levels is somewhat surprising as more COVID-19-like symptoms (e.g., fever, dyspnoea, shivers) and severe disease are associated with higher antibody levels [35, 36]. Oxygen requirement could be indicative of a status of immune suppression and poorer response to vaccination, or it could be related to a worse recovery, or have a long-term impact that affects vaccine responses months later. As models were controlled for time between infection and vaccination, this factor would not be a confounder. Interestingly, lower RBD IgM levels were associated with post COVID condition, and this could be related to the pathophysiological mechanisms of this heterogeneous syndrome that at the moment remain unknown.

As reported in our prior hospital-based studies [15], having AEs after vaccination was associated with higher antibody levels; consistently with the age pattern of lower responses with age, more frequent AEs were seen in younger than older individuals. Of note, this impact of AEs on antibody levels appears to affect not only the early peak responses but also the long-term steady phases. Interestingly, we found a significant association between earlier vaccination and better immune response, but only in those pre-exposed. Previous studies have

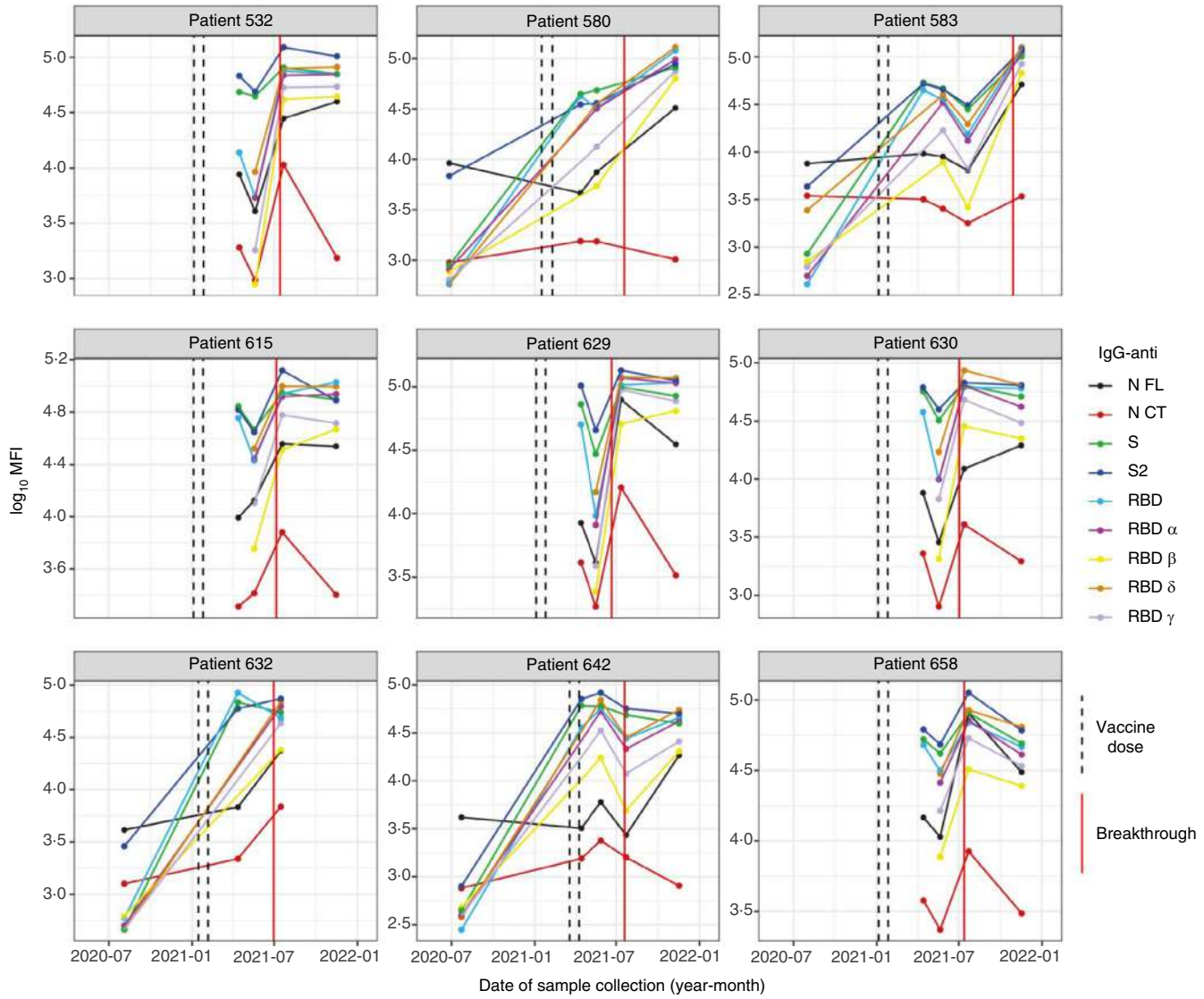

FIGURE 3 Dynamic changes in IgG levels in nine patients with symptomatic breakthroughs. Vaccine doses as dashed lines and infections as red lines. IgG levels as $\log_{10}$ median fluorescence intensity (1/500 dilution). Antigens: Nucleocapsid full length (N FL) and C-terminus (N CT), spike full length (S), S2 subunit, and receptor-binding domain for wild type and variants of concern. IgA and IgM in Figure S1

reported similar results for COVID-19 and other vaccines [37, 38], while one study reports a better response if vaccination is in the afternoon [39]. Our data indicate a benefit of early morning vaccination to attain better immunogenicity and durability.

Information on when antibodies wane below protective levels is needed to avoid too frequent booster immunizations in high-income countries that may not be necessary in younger or healthier populations or even be counter-productive [40], and to direct limiting vaccine supplies for more vulnerable populations (unvaccinated, elderly and immune compromised). Ensuring maximum vaccine coverage worldwide will, in turn, slow down the emergence of VoC that may threaten vaccine effectiveness. In individuals boosted with a 3rd dose, neutralizing titres against Omicron variant were much higher, and remained detectable 6 months after the booster [32]. However, there are also memory B and T lymphocytes that would respond rapidly after an infection and control it to not progress to severe forms, therefore only antibodies may not fully predict protection against severe COVID-19.

The study has some limitations, like the focus on HCW that may not be representative of the overall population, particularly not including the elderly in whom seroreversions in the 11-month period would have been expected. In addition, we do not report neutralizing activity, but our prior studies have shown a very strong

correlation with anti-S IgG and IgA levels. Our investigation precedes the implementation of vaccine boosters and the rise of Omicron, but few studies have performed such long and deep analyses of long-term antibodies after primary vaccination and including all the main VoC up to the Delta waves. Ongoing experiments will address the role of cellular immunity in this population in relation to antibody maintenance and immune escape.

In conclusion, data indicate that older physician or nurse males not having vaccine reactogenicity will be more likely to have lower antibodies within a year timeframe, and thus be prioritized for booster vaccinations. On the other hand, non-smoking younger individuals with hybrid immunity as a result of a prior COVID-19 episode with certain features (anosmia/ageusia, fever, dyspnoea and hospitalization but not oxygen supply) would maintain higher antibody levels and be less likely to need vaccine boosters in the timeframe that many Western countries have adopted them. The study also manifests the benefit of administering the vaccines earlier in the morning and as late as possible after a COVID-19 episode, and confirms that Moderna is superior to Pfizer/BioNTech and AstraZeneca vaccines, despite the asymmetrical distribution. Future biannual longitudinal follow-up studies will address further the breadth and maintenance of immunity and the waning of antibodies following the booster doses, including the impact on emerging variants like Omicron on breakthrough infections.

AUTHOR CONTRIBUTIONS

Concept and design: Carlota Dobaño, Gemma Moncunill, Anna Ramírez-Morros, Josep Vidal-Alaball, Anna Ruiz-Comellas. *Acquisition, analysis, or interpretation of data*: Anna Ramírez-Morros, Selena Alonso, Marta Vidal, Rocío Rubio, Alfons Jiménez, Esther Prados, Carlota Dobaño, Gemma Moncunill, Josep Vidal-Alaball, Anna Ruiz-Comellas. *Produced antigens*: Natalia Rodrigo Melero, Carlo Carolis, Luis Izquierdo. *Sample processing*: Robert A. Mitchell, Chenjerai Jairoce, Diana Barrios, Rocío Rubio, Selena Alonso. *Statistical analysis*: Gemma Ruiz-Olalla, Selena Alonso, Rocío Rubio. *Supervision*: Carlota Dobaño, Jasmina Zanoncello, Gemma Moncunill, Ruth Aguilar, Anna Ruiz-Comellas, Josep Vidal-Alaball. *Obtained funding*: Carlota Dobaño. Had full access to the study data and take responsibility for the integrity and the accuracy of the data and analysis: Carlota Dobaño, Gemma Moncunill, Anna Ruiz-Comellas and Josep Vidal-Alaball. Drafting of the first manuscript: Carlota Dobaño. Critical revision of the manuscript for important intellectual content: Anna Ramírez-Morros, Selena Alonso, Gemma Ruiz-Olalla, Marta Vidal, Rocío Rubio, Esther Prados de la Torre, Chenjerai Jairoce, Robert A. Mitchell, Diana Barrios, Alfons Jiménez, Jasmina Zanoncello, Luis Izquierdo, Natalia Rodrigo Melero, Carlo Carolis, Josep Vidal-Alaball, Ruth Aguilar, Gemma Moncunill, Anna Ruiz-Comellas.

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CONFLICT OF INTEREST

All authors have read the journal's policy on disclosure of potential conflicts of interest. Authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding authors upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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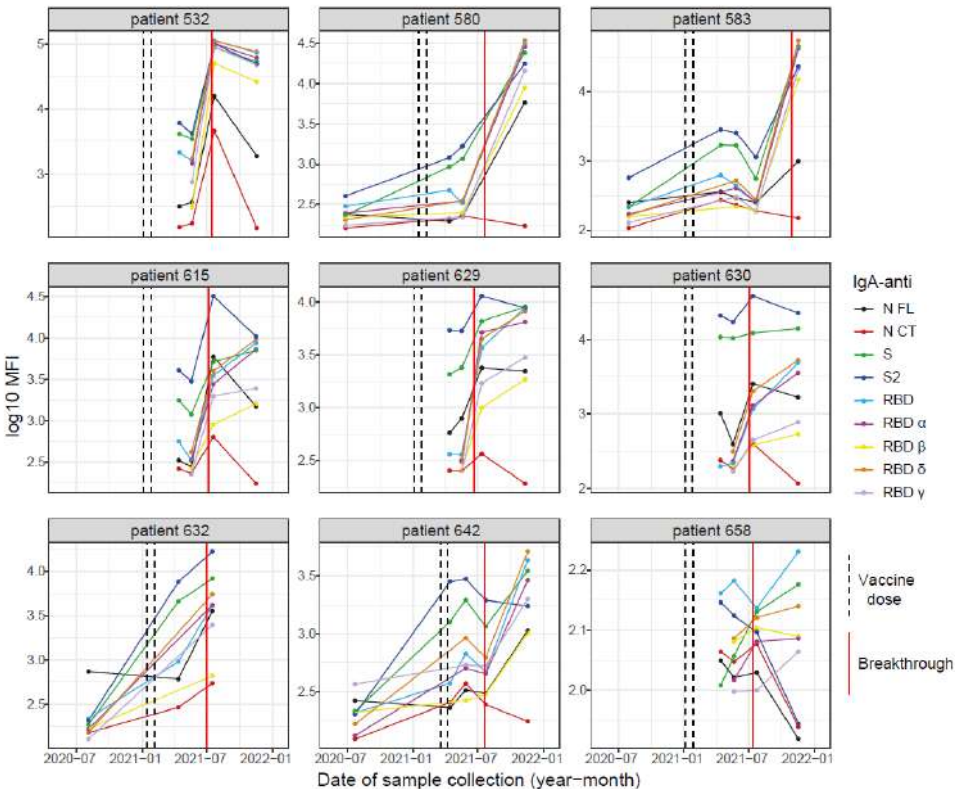
SUPPLEMENTARY MATERIAL

Figure S1. Changes in IgA **(A)** and IgM **(B)** levels (log_{10}MFI) in nine vaccinated patients after symptomatic breakthrough infection. Antigens: nucleocapsid full length (N FL) and C-terminus (N CT), spike full length (S), S2 subunit, and receptor-binding domain (RBD) for wild type and variants of concern.

A



B

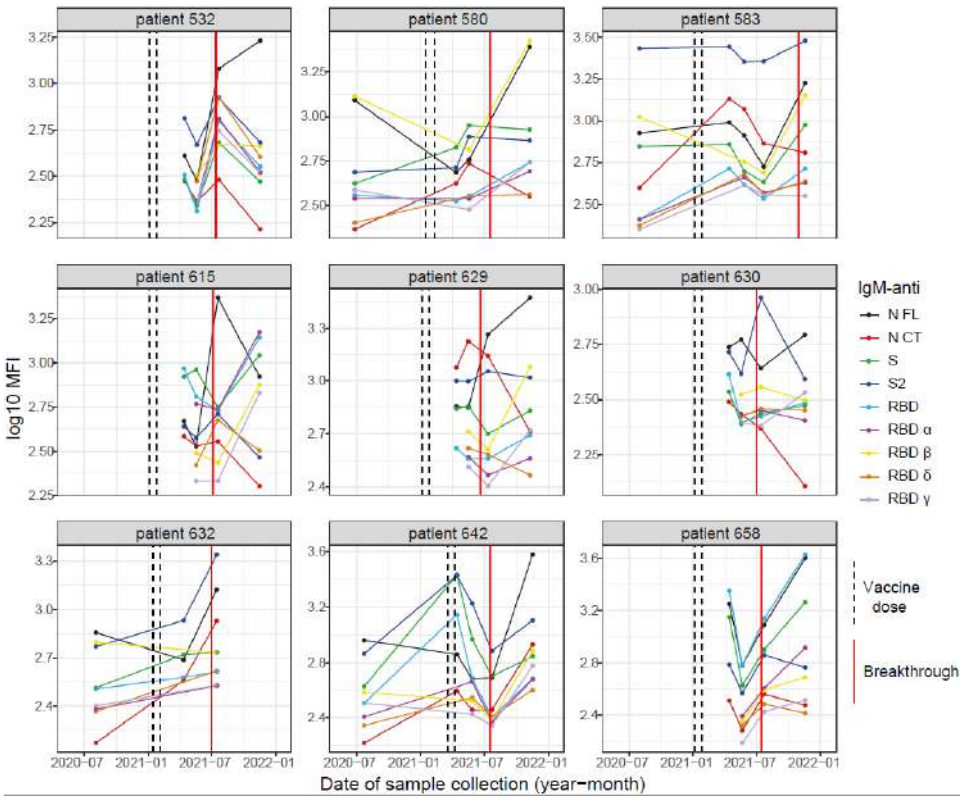


Table S1. Detailed baseline comorbidities and symptoms of the entire study cohort (up to timepoint 8), and when antibodies assessed (11 months after vaccination).

	Full study cohort		Timepoint 8	
	Pre-exposed	**Naive**	**Pre-exposed**	**Naive**
	N=247 (55.3%)	N=200 (44.7%)	N=194 (50.8%)	N=188 (49.2%)
Baseline comorbidities				
Allergy	41 (16.6%)	16 (8.0%)	33 (17.0%)	16 (8.5%)
Asthma	16 (6.5%)	15 (7.5%)	12 (6.2%)	14 (7.4%)
Autoimmune	23 (9.3%)	15 (7.5%)	19 (9.8%)	15 (8.0%)
Cancer	10 (4.0%)	10 (5.0%)	9 (4.6%)	10 (5.3%)
Cardiac disease	4 (1.6%)	3 (1.5%)	2 (1.0%)	3 (1.6%)
Depression	51 (20.6%)	47 (23.5%)	42 (21.6%)	42 (22.3%)
Digestive disease	22 (8.9%)	13 (6.5%)	19 (9.8%)	13 (6.9%)
Dyslipidemia	35 (14.2%)	25 (12.5%)	32 (16.5%)	23 (12.2%)
Diabetes Mellitus	6 (2.4%)	3 (1.5%)	4 (2.1%)	3 (1.6%)
Hypotiroidism	26 (10.5%)	13 (6.5%)	23 (11.9%)	12 (6.4%)
Hypertension	20 (8.1%)	13 (6.5%)	16 (8.2%)	12 (6.4%)
COPD	1 (0.4%)	2 (1.0%)	1 (0.5%)	2 (1.1%)
Chronic kidney disease	2 (0.8%)	0 (0.0%)	1 (0.5%)	0 (0.0%)
Neurological disease	24 (9.7%)	26 (13.0%)	22 (11.3%)	23 (12.2%)
Immunodepression	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Pregnancy	3 (1.2%)	0 (0.0%)	3 (1.5%)	0 (0.0%)
Obesity	26 (10.5%)	16 (8.0%)	22 (11.3%)	16 (8.5%)
Symptoms first COVID-19 episode				
Anosmia/Ageusia	147 (59.5%)	na	116 (59.8%)	na
Fever	178 (72.1%)	na	136 (70.1%)	na
Shivers	121 (49.0%)	na	94 (48.5%)	na
Headache	179 (72.5%)	na	138 (71.1%)	na
Dizziness	44 (17.8%)	na	32 (16.5%)	na
Myalgia/Arthralgia	144 (58.3%)	na	114 (58.8%)	na
Cough	145 (58.7%)	na	118 (60.8%)	na
Dispnea	81 (32.8%)	na	63 (32.5%)	na
Thorax pain	44 (17.8%)	na	29 (14.9%)	na
Digestive	136 (55.1%)	na	102 (52.6%)	na
Asthenia	192 (77.7%)	na	149 (76.8%)	na
Oftalmological	43 (17.4%)	na	31 (16.0%)	na
Cutaneous	35 (14.2%)	na	30 (15.5%)	na
Odynophagia	82 (33.2%)	na	64 (33.0%)	na
Sputum	16 (6.5%)	na	10 (5.2%)	na
Tachycardia	33 (13.4%)	na	24 (12.4%)	na
Neurological	22 (8.9%)	na	15 (7.7%)	na
Otorhino	113 (45.7%)	na	89 (45.9%)	na
Thrombosis	1 (0.4%)	na	1 (0.5%)	na

na = not applicable

Table S2. Seropositivity over time after vaccination.

	T4 (n=79)		T5 (n=333)		T6 (n=363)		T7 (n=375)		T8 (n=382)	
	n	%	n	%	n	%	n	%	n	%
Total	77	97.47%	333	100%	363	100%	375	100%	382	100%
IgA	76	96.20%	325	97.60%	338	93.11%	332	88.53%	365	95.55%
IgA N CT	67	84.81%	72	21.62%	30	8.26%	29	7.73%	31	8.12%
IgA N FL	40	50.63%	33	9.91%	19	5.23%	20	5.33%	56	14.66%
IgA RBD	75	94.94%	299	89.79%	222	61.16%	222	59.20%	302	79.06%
IgA S	76	96.20%	324	97.30%	336	92.56%	330	88.00%	365	95.55%
IgA S2	74	93.67%	274	82.28%	186	51.24%	180	48.00%	263	68.85%
IgG	77	97.47%	333	100%	363	100%	375	100%	382	100%
IgG N CT	70	88.61%	110	33.03%	32	8.82%	27	7.20%	2	0.52%
IgG N FL	47	59.49%	51	15.32%	23	6.34%	10	2.67%	66	17.28%
IgG RBD	76	96.20%	333	100%	363	100%	375	100%	382	100%
IgG S	77	97.47%	333	100%	363	100%	375	100%	382	100%
IgG S2	76	96.20%	332	99.70%	346	95.32%	319	85.07%	313	81.94%
IgM	54	68.35%	172	51.65%	48	13.22%	34	9.07%	117	30.63%
IgM N CT	1	1.27%	8	2.40%	4	1.10%	2	0.53%	10	2.62%
IgM N FL	1	1.27%	2	0.60%	3	0.83%	1	0.27%	7	1.83%
IgM RBD	38	48.10%	95	28.53%	22	6.06%	18	4.80%	75	19.63%
IgM S	46	58.23%	141	42.34%	26	7.16%	13	3.47%	48	12.57%
IgM S2	32	40.51%	41	12.31%	4	1.10%	4	1.07%	18	4.71%

T4 – January/February 2021, T5 – March/April 2021, T6 – May/June 2021, T7 – July 2021, T8 – November 2021.
n, number of individuals who donated samples per timepoint

Table S3. Seropositivity over time after vaccination for variants of concern.

	T6			T7			T8		
	Naive (n=198)	Exposed (n=165)	Total (n=363)	Naive (n=188)	Exposed (n=187)	Total (n=375)	Naive (n=188)	Exposed (n=194)	Total (n=382)
IgA RBD Wuhan	39.9	86.67	61.16	31.91	86.63	59.2	62.23	95.36	79.06
IgA RBD Alpha	40.91	87.27	61.98	30.85	86.09	58.4	64.36	94.85	79.84
IgA RBD Beta	5.05	49.7	25.34	6.91	45.45	26.13	17.02	61.34	39.53
IgA RBD Gamma	15.15	69.7	39.94	13.3	65.77	39.47	40.96	84.02	62.83
IgA RBD Delta	37.88	86.06	59.78	25	85.56	55.2	71.81	95.36	83.77
IgG RBD Wuhan	100	100	100	100	100	100	100	100	100
IgG RBD Alpha	100	100	100	98.94	100	99.47	100	100	100
IgG RBD Beta	90.4	98.79	94.21	88.3	96.79	92.53	90.96	99.48	95.29
IgG RBD Gamma	100	100	100	98.4	98.93	98.67	98.94	100	99.48
IgG RBD Delta	100	100	100	98.94	98.93	98.93	100	100	100
IgM RBD Wuhan	1.52	11.52	6.06	1.6	8.02	4.8	7.98	30.9	19.63
IgM RBD Alpha	4.04	8.48	6.06	2.66	5.35	4	9.57	31.44	20.68
IgM RBD Beta	1.01	1.21	1.1	0	0	0	2.13	2.58	2.36
IgM RBD Gamma	0.51	1.21	0.83	0.53	0.53	0.53	3.72	7.22	5.5
IgM RBD Delta	4.55	7.27	5.79	3.19	6.42	4.8	14.3	41.24	28.01

T6 – May/June 2021, T7 – July 2021, T8 – November 2021.
n, number of individuals who donated samples per timepoint

Chapter 3

High-resolution kinetics and cellular determinants of SARS-CoV-2 antibody response over two years after COVID-19 vaccination

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High-resolution kinetics and cellular determinants of SARS-CoV-2 antibody response over two years after COVID-19 vaccination

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) studies usually rely on cross-sectional data of large cohorts but limited repeated samples, overlooking significant inter-individual antibody kinetic differences. By combining Luminex, activation-induced marker (AIM) and IFN-γ/IL-2 Fluorospot assays, we characterized the IgM, IgA, and IgG antibody kinetics using 610 samples from 31 healthy adults over two years after COVID-19 vaccination, and the T-cell responses six months post-booster. Antibody trajectories varied among isotypes: IgG decayed slowly, IgA exhibited an initial sharp decline, which gradually slowed down and stabilized above the seropositivity threshold. Contrarily, IgM rapidly dropped to undetectable levels after primary vaccination. Importantly, three vaccine doses induced higher and more durable anti-spike IgG and IgA levels compared to two doses, whereas infection led to the highest antibody peak and slowest antibody decay rate compared to vaccination. Comparing with ancestral virus, antibody levels recognizing Omicron subvariants had a faster antibody decay. Finally, polyfunctional T cells were positively associated with subsequent IgA responses. These results revealed distinctive antibody patterns by isotype and highlight the benefits of booster doses in enhancing and sustaining antibody responses.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), causing coronavirus disease 2019 (COVID-19), has resulted in more than 700 million cases and more than 7 million deaths [1]. SARS-CoV-2 continues circulating among humans and evolves to evade previous immunity [2].

Vaccines are one of our most powerful tools in the fight against infectious diseases, as evidenced by their role in curbing the COVID-19 pandemic, offering significant protection against severe disease and death [3]. However, a rise in breakthrough infections (BTIs) has occurred due to waning immunity across different vaccine platforms and the emergence of SARS-CoV-2 Omicron variants of concern (VoCs) capable of escaping existing immunity more efficiently than previous

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VoCs [4].

Population immunity is heterogeneous, as individuals gained immunity from different SARS-CoV-2 variants, and diverse vaccine types and regimens over time [5]. Furthermore, the individual immune response is affected by host factors as age, sex, comorbidities, medication, and previous SARS-CoV-2 infections [6]. Primary vaccination induces a robust immune response, involving neutralizing antibodies and memory B and T cells [7]. Although complete seroreversion in healthy adults is rare, antibodies decline significantly over 6 months after vaccination or infection [7]. B- and T-cell responses appear to be more durable than plasma antibody levels [8]. Recent investigations have shown that antibodies and cellular responses to SARS-CoV-2 are detectable up to 3 years after infection [9], although with decreasing efficacy against the new SARS-CoV-2 VoCs [10], opening up a window of opportunity for BTIs. With the spread of Omicron VoC, the most transmissible variant to date, it has been suggested that most of the world's population has experienced SARS-CoV-2 infection at least once [11]. Thus, the combination of vaccine-induced and naturally immunity, known as hybrid immunity, is increasingly common [7].

The available data on hybrid immunity present conflicting findings. Some studies showed that it offers greater and broader protection, due to higher antibody titers and increased VoCs recognition, reduced risk of SARS-CoV-2 infection, and prevention of hospital admission and severe disease compared to natural or vaccine-induced immunity alone [12–14]. However, other studies showed no differences in the strength of natural, vaccine-induced, or hybrid immunity in terms of neutralizing antibodies or cellular immunity [13]. Although several studies including ours have described the acquisition and evolution of immune responses following vaccination and infection, very few have followed up the same individuals with multiple closely repeated measures over an extended period to define with high precision the kinetics of the antibody response and its heterogeneity. In this regard, a close interrogation of longitudinal cohorts with individual heterogeneity on natural and vaccine exposures will shed light on the duration of the adaptive immune response after vaccination, to guide vaccine schedule strategies and future vaccine development.

This study aimed to investigate the kinetics and duration of IgM, IgA and IgG responses to COVID-19 vaccination and the contribution of hybrid immunity in 31 healthy volunteers with different histories of SARS-CoV-2 exposures. We characterized the antibody kinetics against SARS-CoV-2 nucleocapsid (N) and spike (S) antigens, including Wuhan, Alpha, Delta and several Omicron VoCs, for up to two years with 55 follow-up visits after primary vaccination. Furthermore, we measured SARS-CoV-2-specific T-cell responses 6 months after booster vaccination and evaluated their association with subsequent antibody responses.

2. Material and methods

2.1. Study design

We followed 31 healthy volunteers from Barcelona for two years after vaccination (February 2021–March 2023), with up to 55 visits involving finger prick blood collection for serology. Participants received different vaccine types detailed in Table 1 and Supplementary Table 1. Venous blood was collected from 25 participants 6 months after booster dose (3rd dose) for cellular analysis. At enrollment, sociodemographic and clinical data were collected. Vaccinations, adverse events, infections, and symptoms were tracked at each visit. SARS-CoV-2 BTIs were captured by self-report positive RT-PCR or antigen test. Non-reported infections (asymptomatic or untested) were detected by serology, when an increase $\geq$ 4-fold of anti-N IgG, IgA and/or IgM in between visits. From these data, participants were classified into hybrid immunity (infected and vaccinated regardless of the order in which these occurred) and only vaccinated. The study was approved by Hospital Clínic de Barcelona Clinical Research Ethics Committee (code HCB/2021/0505), and all participants provided written informed consent.

Table 1
Characteristics of the study population.

	Entire cohort *N = 31*	Hybrid immunity *N = 17*	Vaccinated *N = 14*	p-value
	At end of study	At 6 months after booster dose		
Sex n (%)				
Female	19 (61.3 %)	11 (64.7 %)	8 (57.1 %)	0.952[a]
Age mean (s.d.)	42.8 (15.0)	41.6 (18.6)	44.2 (9.28)	0.615[b]
Infected before vaccination n (%)	4 (12.9 %)	4 (12.9 %)		
Breakthrough infections (BTIs) n (%)				
After 1st dose	1 (3.22 %)	1 (5.88 %)	.	
After 2nd dose	5 (16.12 %)	5 (29.41 %)	.	
After 3rd dose	18 (58.06 %)	10 (58.82 %)	.	
After 4th dose	3 (9.68 %)	.	.	
Never infected n (%)	6 (19.35 %)	.	14 (100 %)	
Number of vaccine doses n (%)				0.274[c]
2	3 (9.68 %)	3 (17.6 %)	0 (0 %)	
3	18 (58.1 %)	8 (47.1 %)	10 (71.4 %)	
4	10 (32.3 %)	6 (35.3 %)	4 (28.6 %)	
Days since last infection median [IQR]	184 [121; 256]	156 [113; 348]	.	
Vaccine regimens* n (%)				
2 doses				
mRNA	2 (6.46 %)			
viral vector + mRNA	1 (3.22 %)			
3 doses				
mRNA	14 (45.17 %)			
viral vector/inactivated + mRNA	4 (12.9 %)			
4 doses				
mRNA	6 (19.35 %)			
viral vector + mRNA	4 (12.9 %)			

*mRNA (Spikevax, Comirnaty), viral vector (Jcovden, Vaxzevria), inactivated (CoronaVac). Detailed regimens are shown in Supplementary Table 1.

[a] Chi-square test.

[b] t-test.

[c] Fisher test.

2.2. Antibody assays

Luminex technology was used to measure IgM, IgG, and IgA levels (median-fluorescence-intensity (MFI)) to Wuhan and VoCs antigens from plasma samples. The panel included the N, S, its subregions S1, S2, and receptor-binding-domain (RBD) from Wuhan and RBD from Alpha, Delta and Omicron BA.1, BA.2, BA.4/5, BQ.1.1 and XBB VoCs. Wuhan antigens and RBD from Alpha and Delta were obtained as previously described [15,16]. Codon-optimized nucleotide fragments encoding the RBD Omicron subvariants were cloned into a pcDNA3.1/Zeo$^{(+)}$ expression vector (Thermo Fisher Scientific), recombinant proteins were produced by transient transfection of Freestyle™ 293-F suspension cells (Thermo Fisher Scientific) and subsequently purified from supernatant by high-performance chromatography, as previously described [17]. Samples were tested following the protocol described previously [18] at dilutions 1:500 for IgA and IgM, and 1:5000 for IgG to avoid saturation upon vaccination. For IgM and IgA assays, samples were pretreated with 1:10 anti-Human IgG (GullSORB™), to deplete IgG and avoid its interference. Positive control plasma pools were added as serially diluted for QA/QC purposes. Technical blanks were added to control for non-specific signals. Prepandemic samples (n = 128) were used as negative controls to estimate seropositivity cutoffs. They were calculated as 10 to the mean plus 3 s.d. of $\log_{10}$MFI values. Samples were considered seropositive when they had a positive response to at least one isotype-antigen pair from the Wuhan SARS-CoV-2. Antibody data were $\log_{10}$-transformed to perform statistical analysis and visualization.

2.3. T cellular assays

Process of peripheral blood mononuclear cells (PBMCs) isolation and details of assays are included in Supplementary Materials. Peptide pools used for SARS-CoV-2 stimuli included S, N, and membrane (M) [PepTivator® SARS-CoV-2 Prot_S, Prot_N, Prot_M, Miltenyi] antigens. We combined the stimuli according to vaccine or non-vaccine antigens, S

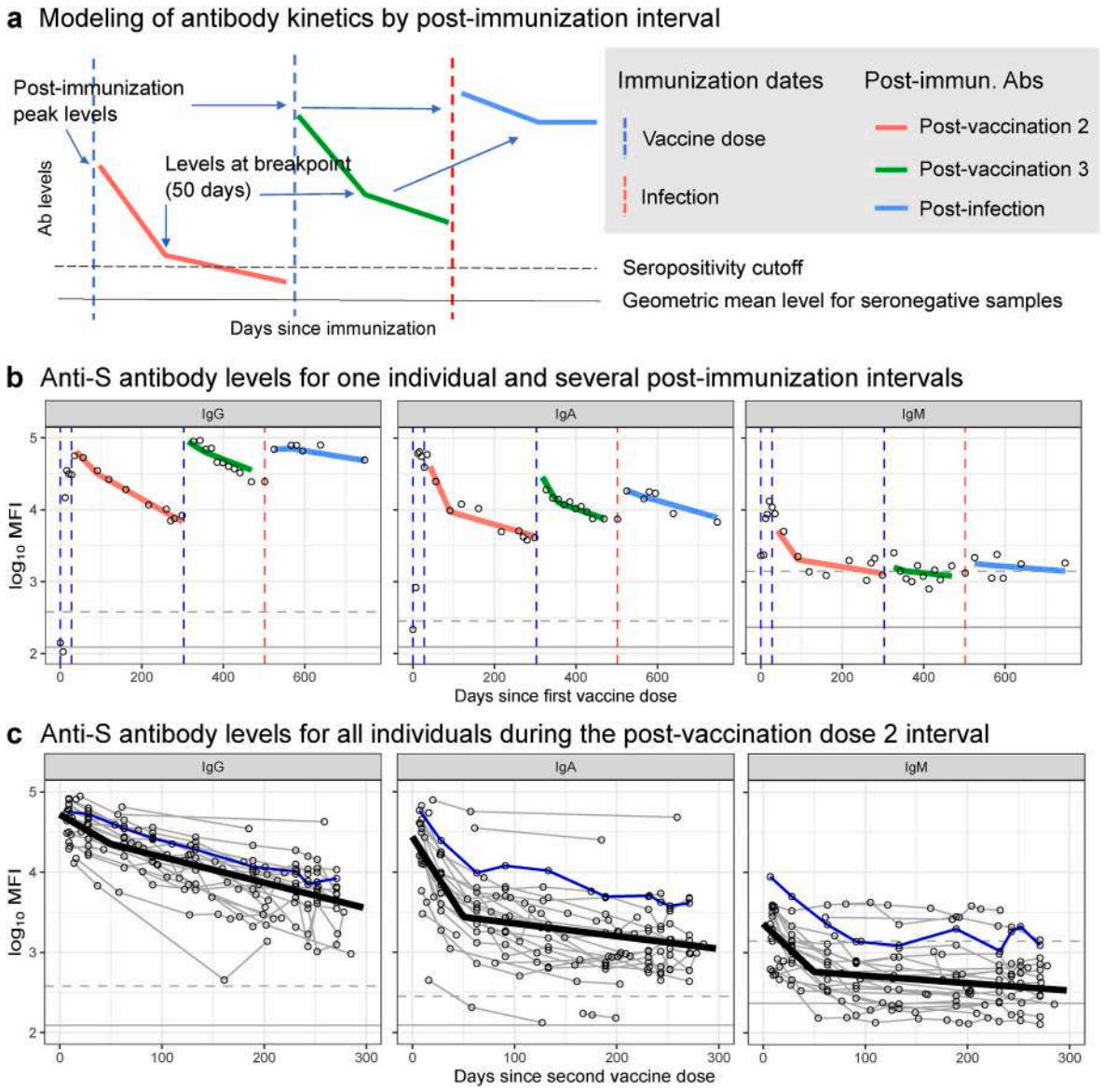

Fig. 1. Segmentation and modeling of the antibody kinetics by antigen, isotype, and immunization interval. (a) Time intervals defined for each participant from the onset of immunization to the subsequent vaccine dose or infection, or until the end of follow-up. **(b)** Example of measurements, segmentation, and model predictions of anti-spike (S) antibody levels for a given study participant. Thick color lines correspond to predictions from our linear mixed model. **(c)** Group measurements of anti-S antibody levels for a given immunization interval (post-2nd dose). Thin blue trajectory corresponds to the subject illustrated in B; thick black line corresponds to the group geometric mean levels predicted by the models.

and N + M respectively, and used at 1 μg/mL. Phytohemagglutinin (PHA) (Merk) was used as positive control in the following assays.

Activation-induced makers (AIM) assay was used for the detection of SARS-CoV-2 antigen-specific $CD4^+$ and $CD8^+$ T-cell responses in 25 participants. They were measured as a percentage of AIM+ ($CD137^+OX40^+$) $CD4^+$ and AIM+ ($CD137^+CD69^+$) $CD8^+$ T cells. Within AIM+ $CD4^+$ and $CD8^+$ T cells, memory subsets were identified through CCR7 and CD45RA expression: T central memory (T_{CM}) ($CD45RA^-CCR7^+$), T effector memory (T_{EM}) ($CD45RA^-CCR7^-$), terminally differentiated effector memory cells re-expressing CD45RA (T_{EMRA}) ($CD45RA^+CCR7^-$) and naïve cells ($CD45RA^+CCR7^+$). After stimulation, PBMCs were stained with the antibody panel shown in Supplementary Table 2 and gating strategy is shown in Supplementary Fig. 1. AIM + $CD4^+$ and $CD8^+$ T cell percentages in unstimulated were subtracted from stimulated samples to account for background responses, and negative values were set to zero. The positivity thresholds were calculated using the median of the 75th percentile of values obtained in the negative controls. Responders were defined when having a positive response to at least one cell type-stimuli combination.

The magnitude of the SARS-CoV-2 Th1 response was measured with the human IFN-γ/IL-2 FluoroSpot kit (Mabtech) in a subset of 20 participants based on availability of PBMCs after AIM assay (Supplementary Fig. 2). Cells that secreted IFN-γ and/or IL-2, were detected and counted as spot-forming units (SFU). Two participants with ≥100 SFU in unstimulated wells for IFN-γ were excluded from the analysis, as they were indeterminate. SFUs in unstimulated were subtracted from stimulated samples to account for background responses, and negative values were set to zero. Results were expressed as SFU per 10^6 PBMCs. Responses were considered positive if the results were ≥ 3-fold the mean of their own unstimulated wells for each cytokine and stimuli. Responders were defined when having a positive response to at least one cytokine-stimuli combination.

2.4. Statistical analyses

2.4.1. Comparative analysis of sociodemographic and clinical data

Sociodemographic and clinical data were compared between study groups using CompareGroups R CRAN package [19]. For continuous normal variables, mean and s.d. were calculated and *t*-test were applied to assess differences. For continuous non-normal variables, median and first and third quartiles were calculated. For categorical variables, differences in proportions were calculated by chi-square test or Fisher's exact test when applicable.

2.4.2. Segmentation and modeling of the antibody kinetics

Antibody kinetics were segmented into 'post-immunization intervals', delimited by consecutive immunization events (Fig. 1). We excluded the initial 7 days following any immunization to ensure that, at the outset of any given interval, antibody levels had nearly reached their peak, thereby consisting of a descending phase within that interval. Intervals following vaccine doses 1 and 4 were excluded from the analysis due to insufficient data points or participants.

We employed linear mixed-effects models to analyze the antibody kinetics. Fixed-effect regressors were coded to accommodate a two-piece linear regression model independently for each post-immunization interval. One slope for an initial phase (from 7 to 50 days) and the other one for the subsequent phase (>50 days). Selection of 50 days as the breakpoint was based on its superior goodness of fit, particularly for IgA, as detailed in Supplementary Fig. 3. This modeling approach enabled us to estimate antibody peak levels (exponentiating intercepts) and the rates of waning (exponentiating slopes and subtracting 1) or half-life ($-\log_{10}(2)$/slope) during the initial and later phases of decay. We estimated differences in antibody levels at day 7 or 50 following different immunizations as interaction terms between post-immunization and a common intercept. We also estimated differences in waning rates for each phase of decay as an interaction between post-immunization and the time since immunization.

Log_{10}-transformed antibody levels (MFI) prior to modelling led to multiplicative effects, representing relative differences. Antibody peak levels were compared as fold-differences in MFI relative to a reference level, obtained by exponentiating the interaction regression coefficient. Waning rates were compared as percentage differences, calculated by exponentiating the interaction regression coefficient, subtracting 1, and multiplying by 100.

To account for the autocorrelation of repeated samples from each participant, we introduced three random-effect intercepts, one for each possible post-immunization interval, with participants as the grouping factor. These three random effects were permitted to covary, as we expected a dependency between antibody levels across intervals within the same individual.

Separate linear mixed-effects models were fitted for each isotype and antigen using the 'lmer' function from R package 'lme4' (version 1.1.27.1). To assess the significance of the fixed-effect regression coefficients, we computed p-values using t-tests applying the Shatterhwaite's approximation for reduced degrees of freedom, as implemented in R package 'lmerTest' (version 3.1.3).

2.4.3. Analysis of antibody levels and cellular responses

To assess antibody responses across SARS-CoV-2 VoCs, the fold change in RBD antibody levels was calculated for each VoC relative to the Wuhan strain in seropositive samples.

Nonparametric tests were used to analyze antibody and T-cell data at 6 months post-booster dose. Comparisons of antibody levels, cytokine-secreting cell numbers, and percentages of T-cell populations between study groups were performed by the Wilcoxon rank-sum test. Nominal p-values (not adjusted for multiple tests due to the small sample size) of <0.05 were considered statistically significant. Correlations between antibody and cellular responses were assessed with Spearman's rank correlation coefficient ρ (rho) and p-values were computed via the asymptotic t approximation.

These analyses were not performed for IgM since all participants had seronegative levels at the timepoints assessed. All data processing and statistical analyses were performed using the R software version 4.2.3.

3. Results

3.1. Participant characteristics

Over half of participants (61.3 %) were female, with an average age of 42.8 years (Table 1). Four participants had COVID-19 before vaccination, 25 had BTIs during the study time, most of them after the emergence of Omicron VoC, and 6 never contracted COVID-19 (Table 1). Thirteen participants had more than one infection during study time. Only 5 infections were asymptomatic, and the rest had mild symptoms. At the end of the study, 58 % of participants received three, 32 % four and 10 % two doses (Table 1). mRNA vaccines were the most common vaccine type (Table 1, Supplementary Table 1). If adverse reactions, they were common side effects, the most frequent being local pain at injection site.

3.2. SARS-CoV-2 seroconversion over time

Seropositivity rates over time, grouped by time intervals after COVID-19 vaccine doses for each antibody isotype-antigen pair, are shown in Fig. 2, and in more detail in Supplementary Table 3. All participants seroconverted for at least one anti-S/isotype within 7–10 days after first dose (Supplementary Fig. 4). While IgG and IgA responses were maintained for up to 25 months, 55 % (17/31) of participants experienced IgM seroreversion, with a median time of 119 [16–587] days after first dose. Seropositivity due to previous SARS-CoV-2 infections before vaccination was detected in some participants. Inclusion of N antigen in the panel allowed the identification of 9 undiagnosed

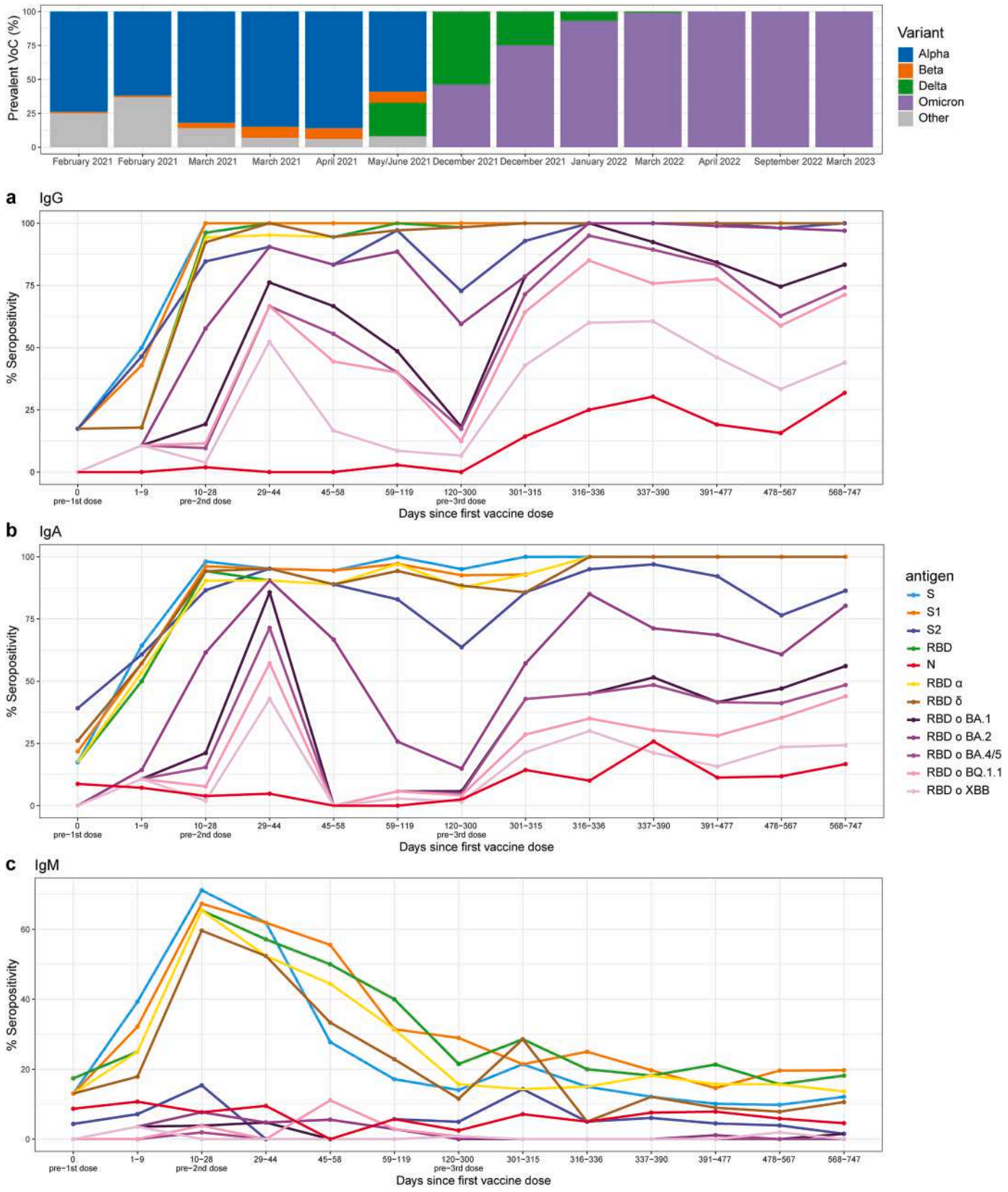

Fig. 2. Antibody seropositivity to SARS-CoV-2 and its VoCs over time since first vaccine dose. Prevalence (%) of circulating SARS-CoV-2 VoCs in Catalonia at the same time that seropositivity rates (source: http://covidtag.paseq.org/). Spaghetti plots depicting IgG **(a)**, IgA **(b)** and IgM **(c)** % of seropositivity over time. Data were grouped by time intervals after first COVID-19 vaccine dose. Nucleocapsid (N), receptor-binding-domain (RBD), spike (S), variants of concern (VoCs).

SARS-CoV-2 exposures (6 symptomatic with no diagnostic test performed or negative result, and 3 asymptomatic). Seropositivity rates for N were much lower than for S antigens, reaching a maximum of 30 % for IgG and IgA when most BTIs occurred, coinciding with the emergence of the Omicron VoC. Consistently, there was also a pronounced increase for IgG and IgA to RBD from Omicron subvariants. However, IgG and IgA seropositivity rates to the Omicron RBDs were lower compared to Wuhan RBD, and exhibited a decreasing trend across Omicron subvariants, with the lowest rates observed in the most recently emerged subvariants. Instead, IgG, IgA, and IgM seropositivity rates for Alpha and Delta RBDs were like those for the ancestral RBD. IgM seropositivities for Omicron subvariants were overall insignificant.

3.3. Antibody kinetics following immunization

3.3.1. Distinct SARS-CoV-2 kinetics by isotype

The kinetics of SARS-CoV-2 IgG and IgA levels exhibited a distinctive sawtooth pattern characterized by abrupt surges, coinciding with successive immunizations. Subsequently, these surges were followed by a gradual decline (Fig. 1). In contrast to IgG and IgA, IgM showed an initial peak after primary vaccination, followed by a rapid decline, resulting in seroreversion without further increases following subsequent immunizations (Figs. 1 and 3).

As anticipated, following an immunization, both IgG and IgA exhibited an initial peak well above the seropositivity threshold,

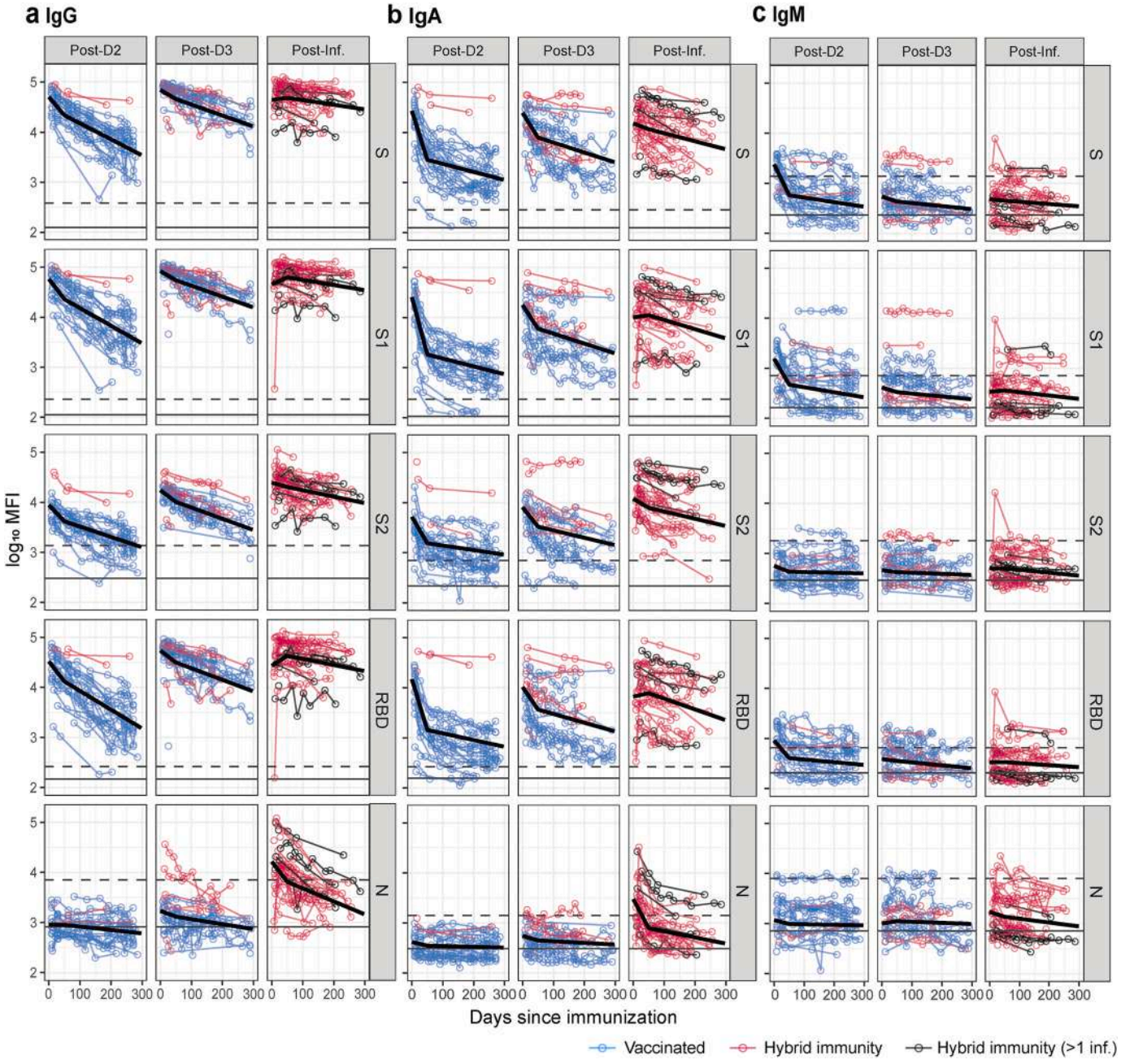

Fig. 3. Wuhan SARS-CoV-2 antibody kinetics following vaccine doses and infections. Thick black lines correspond to the group geometric mean levels predicted by our model fitted for IgG **(a)**, IgA **(b)** and IgM **(c)**, each antigen and immunization interval. Thin lines connect repeated measures from the same participant in the same immunization interval, red ones corresponding to hybrid immunity (at least one infection prior to the vaccine dose). For post-infection data, reinfections are indicated as black trajectories. Dashed black horizontal lines represent seropositivity cutoffs and solid black horizontal lines the geometric mean level for seronegative population. Nucleocapsid (N), receptor-binding-domain (RBD), spike (S), post-2nd dose (Post-D2), post-3rd dose (Post-D3) and post-infection (Post-Inf.).

followed by a subsequent decline (Figs. 1 and 3). Sequential immunizations consistently restored antibody levels, often surpassing previous peaks, especially for IgG (anti-S IgG peak after 2nd and 3rd dose was 424.1 and 603.4 MFI fold-differences over geometric mean seronegativity levels, respectively, Supplementary Table 4). The decline rate after repeated immunizations was generally slower compared to that observed following previous immunizations (e.g. anti-S IgG half-life after 2nd and 3rd dose was 3.1 and 4.6 months, respectively, Supplementary Table 5). Notably, IgG reached the highest peak levels and showed a lower monthly waning compared to IgA (e.g. In the first phase of decline anti-S IgG and IgA after 3rd dose: 603.4 and 196.2 antibody peak, and 2.7 and 1 months of half-life, respectively, Supplementary Table 4 and Supplementary Table 5). The antibody waning rate within post-immunization intervals was not constant over time (Fig. 3, Supplementary Fig. 5). IgG waning exhibited minimal deceleration and it was relatively constant. By contrast, IgA levels showed a highly non-constant antibody waning rate made of a rapid initial decay over the first few months, after which decelerated, and antibody levels remained, albeit still slowly waning, neatly above the seropositivity cutoff for many months. For initial and subsequent phases of declines, the antibodies half-life was: anti-S IgG after 2nd dose 1.4 and 3.1 months and after 3rd dose 2.7 and 4.6 months, anti-S IgA after 2nd dose 0.5 and 6.3 months and after 3rd dose 1 and 5 months; anti-N after infection IgG 1.4 and 3.7 days and IgA 7 and 7.6 months (Supplementary Table 5). Finally, IgM waning following the initial immunizations was rapid within the first phase of decline with anti-S half-life after 2nd and 3rd doses of 0.8 and 5.6 months, respectively, and anti-N 6.3 months after infection (Supplementary Table 5), falling below the seropositivity threshold.

The same pattern of an initial antibody peak followed by waning after immunization was observed for antibodies to SARS-CoV-2 VoCs, although with some differences when compared to the ancestral virus. Antibodies to RBD from Alpha and Delta exhibited similar kinetics to those of Wuhan (Supplementary Fig. 5) but displayed lower levels of IgG and IgA to Alpha and IgG to Delta variants (Supplementary Fig. 6). In sharp contrast, the IgG and IgA post-immunization peaks to RBD from Omicron subvariants were much lower (e.g. after infection, IgG anti-RBD from Omicron range was 7.4–14.1 vs. Wuhan 164.3 MFI fold-differences over geometric mean seronegativity levels, Supplementary Table 4), and levels rapidly reached seroreversion (Supplementary Fig. 5) compared to Wuhan, Alpha and Delta. In addition, IgM levels to all Omicron subvariants remained negligible even after an infection (IgG anti-RBD peak range 1–2.3 MFI fold-differences over geometric mean seronegativity levels Supplementary Table 4, Supplementary Fig. 5). This resulted in shorter persistence of antibodies against Omicron subvariants compared to the ones against the ancestral RBD. The lower recognition of VoCs relative to the Wuhan strain did not change substantially over time (Supplementary Fig. 6).

Despite the clear distinctive patterns by isotype and SARS-CoV-2 variants, antibody responses were highly heterogeneous at the individual level (Fig. 1c and 3, Supplementary Fig. 5), which was already detected early after the 1st dose (Supplementary Fig. 4). This suggests that host factors play an important role in defining antibody responses. Furthermore, this heterogeneity between individuals was partly maintained across different immunization intervals. Thus, individuals exhibiting strong antibody responses following 2nd dose were more likely to exhibit strong responses after 3rd dose. Also, strong antibody responses to the vaccine, especially to 3rd dose, made individuals more likely to respond strongly to an infection (Supplementary Fig. 7, Supplementary Table 6). This correlation was particularly strong for IgA levels. In addition, individuals who reached higher antibody levels after immunization maintained higher levels for a longer period, and those with hybrid immunity tended to have higher levels at post-vaccination intervals (Fig. 3, Supplementary Fig. 5).

3.3.2. *Differences in antibody peak and waning by immunization type*

To evaluate the contribution of each immunization to the antibody response, we compared antibody peak levels and their monthly waning rate between vaccine doses and infections. Differences in antibody peak levels between post-immunization intervals against SARS-CoV-2 and VoCs antigens are shown in Supplementary Tables 7 and 8, respectively. Both 3rd dose and infection conferred approximately double IgG and IgA MFI levels against S2 after 7 days compared to a 2nd dose. However, differences in IgG and IgA levels against all SARS-CoV-2 antigens were more notable 50 days after immunization: antibody levels after 3rd dose were significantly higher than 2nd dose, and they were also higher after an infection than vaccination, particularly for 2nd dose. E.g., anti-S IgG levels at 50 days were 2.4-fold higher after 3rd dose than 2nd dose; 2.9-fold higher after infection vs. 2nd dose; and 1.2-fold higher after infection vs. 3rd dose. In contrast, IgM levels against all SARS-CoV-2 S antigens were markedly lower 7 days after 3rd dose or infection compared to 2nd dose (anti-S 0.2-fold), and they were also lower 50 days after 3rd than 2nd dose (anti-S 0.8-fold). Similar patterns were found for antibodies against RBD from VoCs. As expected, anti-N antibody levels were higher following infection than vaccination, especially at 7 days, e.g., IgG levels were found to be 18.1 and 9.7 times higher after infection than after 2nd and 3rd doses, respectively. All these findings remained unchanged when the analysis was performed excluding participants with hybrid immunity after the vaccine immunization intervals (Supplementary Table 9). Anti-N IgG and IgA levels were also slightly higher following the 3rd vs. 2nd dose, which we attribute to cross-reactivity of antibodies against N after administering S-based vaccines, as seen in our previous work [20].

Differences in antibody waning between intervals against SARS-CoV-2 and VoCs antigens are shown in Supplementary Tables 10 and 11, respectively. IgG against all SARS-CoV-2 S antigens waned more slowly after infection compared to vaccination, and after 3rd vs. 2nd doses. These differences were observed in both antibody decay phases but were greater in the initial phase (7–50 days). E.g., in the initial phase, anti-S IgG had a 75.8 % and 36.9 % less decay rate after infection than 2nd and 3rd dose, respectively. When comparing vaccination, the waning rate after 3rd dose was 28.4 % less than 2nd dose. Even greater differences in decay rates were observed for IgA against S antigens in the same order: infections <3rd dose <2nd dose. Similar differences in waning between intervals were found for VoCs antibodies, detectable in both decay phases for IgG and only significantly during the initial phase for IgA and IgM. However, around 9 % more waning was observed for anti-RBD IgA from Omicron subvariants after infection compared to vaccination in the second decay phase.

As expected, anti-N antibodies exhibited a higher decay rate after infection compared to vaccination in both decline phases. Of note, vaccines included in this study contained only Wuhan S antigen and did not immunize against N antigen. Thus, post-vaccination decline in anti-N antibodies simply measures the “no slope” of a seronegative individual, the small slope of residual anti-N antibodies from a previous infection, or the antibody decay in the case of anti-N cross-reactive responses after vaccination [20]. All these findings remained unchanged when the analysis was performed excluding participants with hybrid immunity after vaccine immunization intervals (Supplementary Table 12).

3.4. *Humoral and cellular immune responses 6 months after booster dose*

All individuals tested had detectable T-cell responses against S or N + M (Supplementary Table 13) six months after 3rd dose. S-specific T-cell responses were higher than those to N + M (Supplementary Fig. 8), with higher frequencies of S-specific AIM + $CD4^+$ and AIM+ $CD8^+$ T cells, and a larger number of S-specific IFN-γ, IL-2 and IFN-γ+IL-2 (polyfunctional) secreting T cells. Males showed increased IL-2 secreting T cells to S and N + M, S-specific polyfunctional T cells, and N + M-specific AIM + $CD8^+$ compared to females (Supplementary Fig. 9). No

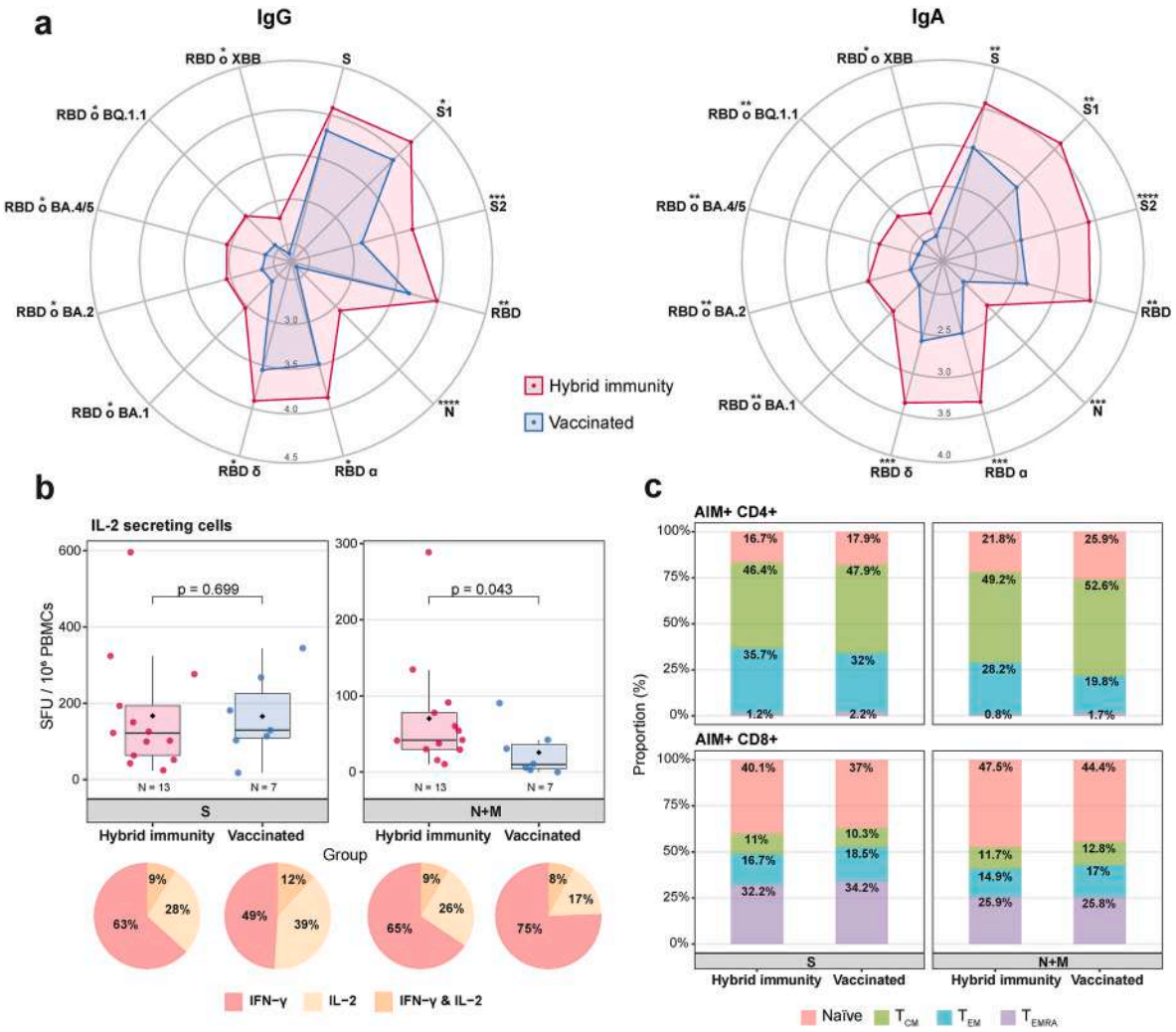

Fig. 4. SARS-CoV-2 adaptive immune responses 6 months after 3rd dose by immunity groups. (a) Radar plot summarizing IgG and IgA responses to different antigens. Colored lines represent the median of antibody levels (log_{10}MFI) of hybrid immunity in red and vaccinated in blue. Groups were compared by Wilcoxon rank-sum test. **(b)** Magnitude of the SARS-CoV-2 Th1 response. Total number of cells, expressed as SFU/10^6 PBMCs, that responded to SARS-CoV-2 S or N + M by secreting IL-2. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Groups were compared by Wilcoxon rank-sum test. Pie charts show the average proportion (%) of secreting T cells by cytokine: IFN-γ (red), IL-2 (beige), or both (orange) by immunity groups. Proportions between groups were compared by Chi-square test, but there were not statistically significant differences. **(c)** SARS-CoV-2 AIM + $CD4^+$ and AIM+ $CD8^+$ T cell responses. Stacked bar chart representing the proportion (%) of AIM + $CD4^+$ and AIM+ $CD8^+$ T cell memory subsets upon S or N+M stimuli. Naïve (red), T_{CM} (green), T_{EM} (blue), and T_{EMRA} (purple). P-values: * ≤0.05, ** ≤0.01 and *** ≤0.001. Activation-induced markers (AIM), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMC), receptor-binding-domain (RBD), spike (S), spots-forming units (SFU), T central memory (T_{CM}), T effector memory (T_{EM}), terminally differentiated effector memory cells re-expressing CD45RA (T_{EMRA}). Not statistically significant results are shown in Supplementary Fig. 9.

differences were found by age (Supplementary Fig. 10).

Comparing between immunity groups (Fig. 4), hybrid immunity conferred higher levels of IgG and IgA antibodies to SARS-CoV-2 antigens and higher numbers of N + M-specific IL-2 secreting T cells than vaccination alone. However, no significant differences were observed in terms of the numbers of IFN-γ and polyfunctional secreting T cells, or AIM + $CD4^+$ and $CD8^+$ frequencies (Supplementary Fig. 11). In both groups most SARS-CoV-2-specific T cells secreted IFN-γ. The vaccinated group had a higher proportion of S-specific polyfunctional (12 %) and IL-2 (39 %) secreting T cells than the hybrid immunity (9 % and 28 %, respectively), while the hybrid immunity had higher N + M-specific IL-2 secreting T cells (26 %) than the vaccinated (17 %), although differences were not statistically significant. Finally, the distribution of the AIM + $CD4^+$ and $CD8^+$ memory T-cell subsets was similar in both groups, with approximately half of AIM + $CD4^+$ cells presenting T_{CM} and around 30 % a T_{EM}; for AIM + $CD8^+$, naïve T cells were the most common (43 %), followed by 30 % of T_{EMRA}.

3.5. Correlations of cellular responses with later antibody responses

We assessed the relationship between the SARS-CoV-2-specific T-cell responses 6 months after 3rd dose and antibody levels to SARS-CoV-2 and VoCs measured one month later (Fig. 5a), and one month after a subsequent immunization (Fig. 5b). Cellular responses exhibited a moderate positive correlation with anti-SARS-CoV-2 IgG and IgA levels measured one month later. Notably, the frequencies of S-specific AIM + $CD4^+$ were positively correlated with anti-S IgG ($\rho = 0.56$), and N-specific AIM + $CD4^+$ with anti-N IgA ($\rho = 0.48$). Correlations were stronger when assessing SARS-CoV-2 antibody levels one month after subsequent immunization. In this case, the frequencies of S-specific AIM + $CD8^+$ were positively correlated with anti-S IgG ($\rho = 0.52$), S-specific polyfunctional T cells with IgA to all SARS-CoV-2 antigens ($\rho \sim 0.59$), and N + M-specific IL-2 secreting T cells with anti-N IgG and IgA ($\rho \sim 0.57$). Nevertheless, cellular responses showed a weaker positive correlation with anti-VoCs antibodies measured one month later

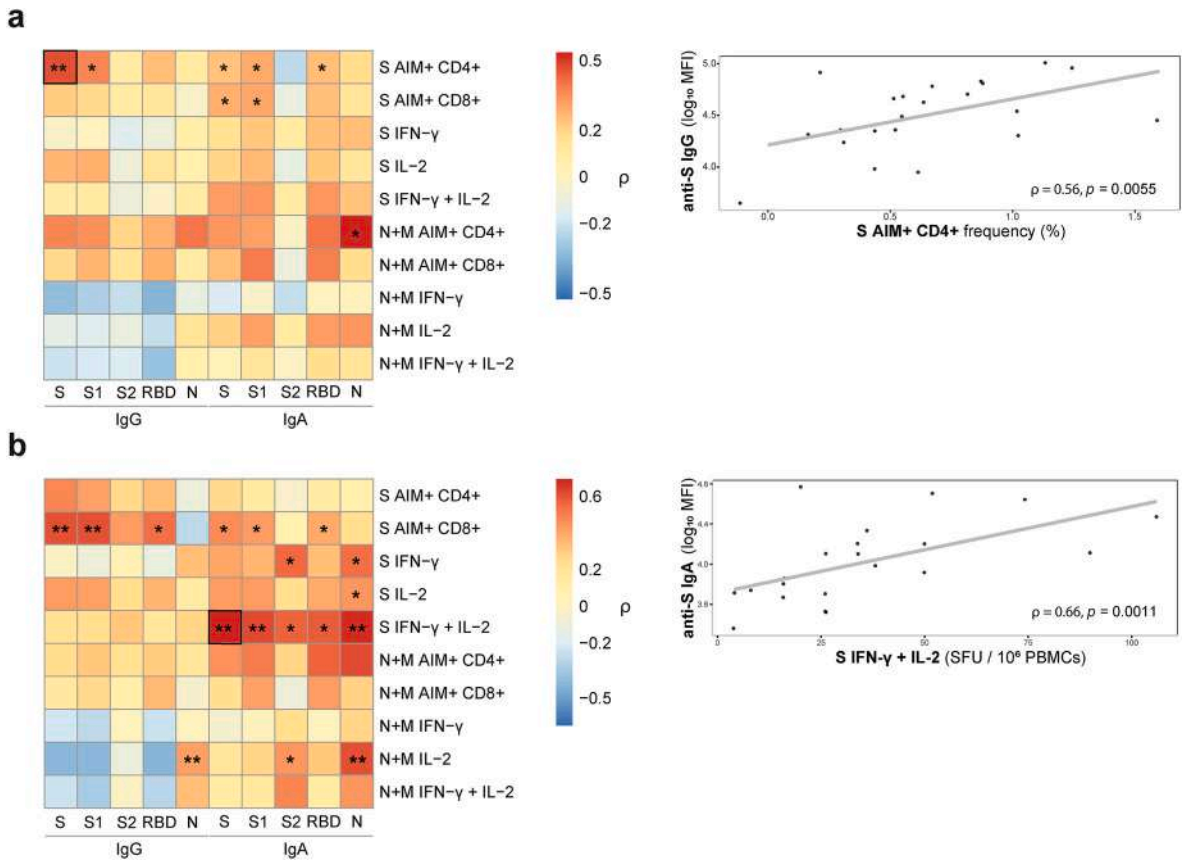

Fig. 5. Correlations between cellular responses and subsequent antibody responses to SARS-CoV-2 after one month **(a)** and one month after subsequent immunization **(b)**. Heatmaps illustrate the Spearman's correlation coefficient (ρ) between cellular responses, including frequencies of SARS-CoV-2-specific AIM + $CD4^+$ and AIM+ $CD8^+$ T cells, and the magnitude of Th1 response to SARS-CoV-2 (SFU/10^6 PBMCs) that secrete IFN-γ, IL-2 and IFN-γ+IL-2, with anti-SARS-CoV-2 IgG and IgA (log_{10}MFI) responses. Scatter plots with linear model display a correlation example (highlighted blacked squares) and show the ρ and p-values: * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001. Activation-induced markers (AIM), interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMC), receptor-binding-domain (RBD), spike (S).

(Supplementary Fig. 12a). The only statistically significant correlations were between SARS-CoV-2 S-specific AIM + $CD4^+$ and $CD8^+$ responses and IgA anti-RBD from Alpha (ρ ~ 0.30). Conversely, the positive correlations between SARS-CoV-2-cellular responses and anti-VoCs antibodies one month after the following immunization were much stronger (Supplementary Fig. 12b); especially S-specific polyfunctional T cells were positively correlated with IgA against all VoCs (ρ ~ 0.55). These results suggest an association between SARS-CoV-2-specific polyfunctional T cells and future IgA responses to COVID-19 vaccination or infection by different variants.

4. Discussion

We followed 31 individuals for an extended period of 747 days after COVID-19 vaccination, with multiple closely repeated measures of antibodies, shedding light on the kinetics of different antibody isotypes and exposures. Our study revealed distinct SARS-CoV-2 kinetic patterns for IgG, IgA and IgM. For IgG, the decay rate was slow and decelerated to even slower rates following the first months. Instead, IgA exhibited a faster decay that abruptly decelerated, exhibiting a first phase of rapid decay followed by a slower decay, maintaining seropositivity for many months. Conversely, IgM levels rapidly declined below the seropositivity threshold. Another key finding was that the highest antibody peak and persistence were induced by infection, followed by three and finally by two vaccine doses.

Seroconversion responses vary depending on the assay, disease severity, and antigen target [21]. However, several studies, including ours, have shown that IgM, IgG and IgA isotypes seroconvert simultaneously within 7–14 days after COVID-19 vaccination or infection [21–23]. All participants had detectable antibody responses 25 and 15 months after 1st and 3rd doses, respectively, which is in line with our previous study [16]. Our findings confirm the kinetic pattern of IgG and IgA surges following subsequent immunizations, which restore and increase humoral responses, previously observed [7,24]. It also aligns with a few studies that have reported a non-constant decelerating waning of antibodies after vaccination and infection [6,9,25], which results in a biphasic decline with an initial rapid decay during the first few months, followed by a phase of slower decline and stabilization. This phenomenon has also been observed in immune responses to other vaccines such as the malaria RTS,S [26] and the trivalent inactivated influenza [27]. Regarding IgM, some studies have noted that levels drop sharply to seronegativity after immunization and some individuals never achieve IgM seroconversion [24,28]. The kinetics of IgG and IgA followed the expected pattern of primary and secondary immune responses [9], and the non-constant decelerating decline after immunization may be a consequence of B-cell development [29]. After immunization, plasmablasts become an early source of antibodies [29]. These cells proliferate quickly, circulate in the periphery, and produce large amounts of antibodies but are short-lived [9], which may be reflected in the first rapid antibody decay phase. Over time, long-lived plasma cells in the bone marrow producing high affinity antibodies are responsible for long-lasting antibody responses that reach a steady state [30], which may be reflected in a second slower antibody decay phase. Higher and longer IgG responses than IgA are attributed to the fact that IgG is the

most abundant class in the bloodstream and has a longer half-life than IgA [31]. IgM antibodies are produced early during the humoral response [28]. Following maturation and isotype class switching, memory IgG and IgA antibodies with increased affinities are produced [28,29]. Thus, the IgM response after subsequent immunizations might be suppressed by pre-existing immunity.

Consistent with previous reports indicating a reduction in vaccine efficacy, neutralization and durability of humoral responses against VoCs, particularly to Omicron [10], our study detected lower antibody levels compared to the Wuhan strain and, in the case of Omicron subvariants, a faster antibody decay after vaccination. BTIs, most likely caused by Omicron, induced lower antibody levels to this VoC than to Wuhan. This observation might be the result of a combination of: i) lower immunogenicity of Omicron S compared to earlier variants, leading to reduced stimulation of immune responses [29], ii) accelerated antigen clearance due to pre-existing immunity [32], and iii) the impact of immune imprinting. It refers to a preferential immune response to the first variant encountered which limits new responses to new variants [28].

Our research reinforces the benefits of administering a third dose, as it significantly increased the antibody levels and their persistence against both Wuhan and VoCs compared to two doses. Furthermore, the highest antibody responses and maintenance observed in response to infection underscore the importance of hybrid immunity, which needs to be taken into consideration by future vaccination strategies. These findings corroborate those of other studies showing increased humoral responses after booster dose [9,10,33] and when having hybrid immunity [12,14,33]. This increase in the magnitude and persistence of antibody responses may be attributed to immunization-induced cumulative affinity-enhancement of somatic hypermutations that differentiate into specific-memory B cells and long-lived bone marrow plasma cells [34]. This process occurs over time, as was shown by Bellusci et al. particularly after the third COVID-19 mRNA vaccination [35].

The induction of SARS-CoV-2-specific memory T cells is vital for long-term protection [36]. All participants had detectable T-cell responses for up to 6 months after booster dose, consistent with previous research [10]. In parallel with other studies, T cell responses after COVID-19 vaccination were mainly directed to the S antigen [37], with the secretion of IFN-γ, followed by IL-2 [38]. SARS-CoV-2-specific $CD4^+$ T cells had a T_{CM} and T_{EM} phenotype [39–41], while $CD8^+$ T cells were predominantly naïve-like and T_{EMRA} [41,42]. The memory phenotypes of $CD4^+$ T cells indicate long-term protection [39]. Regarding phenotypes of $CD8^+$ T cells, Kared et al. described a unique phenotype for SARS-CoV-2-specific T cells, characterized by an enrichment of $CD8^+$ T cells with a naïve-like or stem cell phenotype [42]. These cells have the potential to differentiate into various T-cell memory subsets, contributing to durable protection [42]. However, their role in SARS-CoV-2 immune protection remains to be elucidated.

Although the hybrid immunity group had higher IgG and IgA levels against Wuhan and VoCs compared to vaccinated, there were no differences in T-cell responses 6 months post-booster, as seen previously [43]. N-specific cellular responses in the vaccinated group are indicative of cross-reactivity with N from common cold coronaviruses observed in prepandemic samples [44]. In agreement with other studies [25,45], stronger SARS-CoV-2-specific T-cell responses were positively associated with subsequent IgG and IgA titers which suggest that antibodies are influenced by cellular responses [10]. Although polyfunctional T cells were present in lower proportions, we observed that they may play an important role in the IgA response to SARS-CoV-2. Additional research is required to investigate underlying mechanisms and implications.

Finally, even when individuals received the same vaccine type and regimen, we still observed high heterogeneity, in agreement with other studies [5,46]. This variability suggests that while the antigen itself and vaccine formulation play a crucial role in immune response, host-specific factors also influence it [47]. The immune status at baseline, and immediately prior to vaccination may determine the immune response to vaccination [48]. This may also help explain why potent vaccine responses following a given dose predict strong responses to the subsequent doses or to an infection in the same individual. Further investigation is required to better understand these host factors.

The limitations of our study are, first, the small sample size that prevented the assessment of differences in immune responses by vaccine type or by VoC infections, and second, that conclusions cannot be extrapolated to certain population groups as the elderly or immunocompromised individuals. Additionally, the lack of a direct conversion between MFI units and standard units makes it more difficult to compare the results to other studies. It remains to be seen if antibody kinetics reflects antibody functionality, and how memory B cells and T cells evolve over time and successive exposures.

In summary, we characterized the antibody kinetics after immunization in a small group of individuals with high temporal resolution, deciphering distinct isotype kinetics and non-constant decelerating decay, which are important to consider when evaluating the durability of humoral responses.

CRediT authorship contribution statement

Rocío Rubio: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Dídac Macià:** Writing – review & editing, Writing – original draft, Formal analysis. **Diana Barrios:** Methodology. **Marta Vidal:** Methodology. **Alfons Jiménez:** Methodology. **Luis M. Molinos-Albert:** Writing – review & editing, Methodology. **Natalia Díaz:** Methodology. **Mar Canyelles:** Writing – review & editing, Methodology. **Maria Lara-Escandell:** Methodology. **Cyril Planchais:** Resources. **Pere Santamaria:** Resources. **Carlo Carolis:** Resources. **Luis Izquierdo:** Resources. **Ruth Aguilar:** Writing – review & editing, Supervision. **Gemma Moncunill:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Carlota Dobaño:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

PS is founder, scientific officer and stockholder of Parvus Therapeutics and receives funding from the company. He also has a consulting agreement with Sanofi. All other authors declare non-financial competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2024.105423.

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Supplementary material for

High-resolution kinetics and cellular determinants of SARS-CoV-2 antibody response over two years after COVID-19 vaccination

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Supplementary Materials and methods

Supplementary Table 1 to Table 13

Supplementary Fig. 1 to Fig. 12

Supplementary Materials and methods

T cellular assays

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood samples by density-gradient centrifugation using Ficoll-Paque (Merck), cryopreserved in heat-inactivated fetal bovine serum (HI-FBS) (Thermo Fisher Scientific) with 10 % dimethyl sulfoxide (DMSO) (Merck), and stored in liquid nitrogen until use. Frozen PBMCs were rapidly thawed and diluted into 5 ml TexMACS medium (Miltenyi Biotech) with 1% penicillin/streptomycin (Thermo Fisher Scientific) and 10 U/mL DNase (Benzonase® Nuclease, Merk). After washing twice with culture medium (TexMACS medium-1% penicillin/streptomycin), live PBMCs were counted on the Guava® easyCyte™ Flow Cytometer (Luminex) at 1:40 dilution with Guava® ViaCount™ Reagent (Luminex). The PBMCs were then rested at $2x10^6$ cells/mL with culture medium at 37 °C and 5 % CO_2 overnight (ON) in 50 mL tubes. After ON resting, live PBMCs were counted on the Guava as explained above and were ready to be used in the following assays. Cell viability was always maintained ≥ 70% after ON resting.

Peptides were 15-mer sequences with 11 amino acids overlap and were dissolved in sterile water according to the manufacturer's instructions.

For AIM assay, one million PBMCs from each participant were incubated with the stimuli, culture medium only (unstimulated), and positive control (PHA, 10 µg/ml) in the respective wells for 24h at 37°C 5% CO_2. PBMCs were stained with 1:1000 Fixable Viability Dye eFluor 506 (Thermofisher) 1:1000 for 30min at 4°C to exclude dead cells, and with the antibody panel shown in **Supplementary Table 2** for 30min at 4°C. Cells were fixed with 1% paraformaldehyde (Fisher Scientific) for 20min at room temperature. Finally, cells were resuspended in phosphate-buffered saline and acquired in the LSRFortessa™ Cell Analyzer 5L (BD Biosciences). Sphero™ Rainbow Calibration Particles (8 peaks) (BD Biosciences) were used for performance verification of flow cytometry settings and fluorescence. Data were analyzed using the BD FACSDiva™ Software (BD Biosciences) and the FlowJo software version 10.8.1 (BD Biosciences).

Human IFN-γ/IL-2 FluoroSpot assay was performed according to the manufacturer's instructions using $2.5x10^5$ PBMCs for the stimuli or unstimulated wells, and $5x10^4$ PBMCs to the positive control (PHA, 5 µg/ml) wells, in duplicate, and were incubated for 20 h at 37°C 5% CO_2. Fluorescent spots, indicating cells that secreted IFN-γ and/or IL-2, were detected and counted with the AID vSpot Spectrum reader and AID EliSpot v7 software (Autoimmun Diagnostika) (**Supplementary Fig. 2**).

Supplementary Table 1. *Detailed vaccine regimens of study population*

	Entire cohort ***N=31***
Vaccine regimens: n (%)	
Spikevax-Spikevax	1 (3.23%)
Comirnaty-Comirnaty	1 (3.23%)
Jcovden-Spikevax	1 (3.23%)
Spikevax-Spikevax-Spikevax	11 (35.50%)
Comirnaty-Comirnaty-Spikevax	2 (6.45%)
Comirnaty-Comirnaty-Comirnaty	1 (3.23%)
Vaxzevria-Comirnaty-Spikevax	2 (6.45%)
Vaxzevria-Spikevax-Spikevax	1 (3.23%)
CoronaVac-CoronaVac-Spikevax	1 (3.23%)
Vaxzevria-Vaxzevria-Spikevax-Comirnaty bivalent*	3 (9.68%)
Spikevax-Spikevax-Spikevax-Comirnaty bivalent*	3 (9.68%)
Vaxzevria-Vaxzevria-Comirnaty-Comirnaty bivalent*	1 (3.23%)
Comirnaty-Comirnaty-Spikevax-Comirnaty bivalent*	1 (3.23%)
Comirnaty-Comirnaty-Spikevax-Spikevax	1 (3.23%)
Spikevax-Spikevax-Spikevax-Spikevax	1 (3.23%)

*Original+Omicron BA.4-5

Supplementary Table 2. *AIM antibody panel*

Reagents	**Source**	**Cat#**	**Lot #**	**Dilution**
Mouse anti-Human CD14 BV510 (clone M5E2)	BD Biosciences	740163	2067107	1:50
Mouse anti-human CD19 BV510 (clone HIB19)	BD Biosciences	740164	2133194	1:50
Mouse anti-human CD4 BV605 (clone RPA-T4)	BD Biosciences	562658	1173914	1:25
Mouse anti-human CD69 PE (clone FN50)	BD Biosciences	555531	1131947	1:10
Mouse anti-human CD45RA BV421 (clone HI100)	Biolegend	304130	B337099	1:50
Mouse anti-human CD8a BV650 (clone RPA-T8)	Biolegend	301042	B328291	1:50
Mouse anti-human CCR7 FITC (clone G043H7)	Biolegend	353216	B345204	1:50
Mouse anti-human OX40 PECy7 (clone Ber-ACT35)	Biolegend	350012	B339312	1:50
Mouse anti-human CD137 APC (clone 4B4-1)	Biolegend	309810	B336657	1:25
Mouse anti-human CD3 AF700 (clone UCHT1)	Thermofisher	56-0038-42	2286408	1:25

Supplementary Table 3. *Percentage (%) of seropositivity over time by time intervals before and after COVID-19 vaccination*

	pre-1st 0d (n=23)	1st + 9d 1-9d (n=28)	pre-2nd 10-28d (n=52)	2nd + 15d 29-44d (n=21)	2nd + 1m 45-58d (n=18)	2nd + 3m 59-119d (n=35)	pre-3rd 120-300d (n=121)	3rd + 15d 301-315d (n=14)	3rd + 1m 316-336d (n=20)	3rd + 3m 337-390d (n=66)	3rd + 6m 391-477d (n=89)	3rd + 9m 478-567d (n=51)	3rd + 15m 568-747d (n=66)
final result	47.83	75	100	100	100	100	100	100	100	100	100	100	100
IgA	43.48	67.86	98.08	100	100	100	99.17	100	100	100	100	100	100
S	17.39	64.29	98.08	95.24	94.44	100	95.04	100	100	100	100	100	100
S1	21.74	57.14	96.15	95.24	94.44	97.14	92.56	92.86	100	100	100	100	100
S2	39.13	60.71	86.54	95.24	88.89	82.86	63.64	85.71	95	96.97	92.13	76.47	86.36
RBD	17.39	50	94.23	90.48	88.89	97.14	87.6	92.86	100	100	100	100	100
N	8.7	7.14	3.85	4.76	0	0	2.48	14.29	10	25.76	11.24	11.76	16.67
IgG	17.39	57.14	100	100	100	100	100	100	100	100	100	100	100
S	17.39	50	100	100	94.44	100	100	100	100	100	100	100	100
S1	17.39	42.86	100	100	100	100	100	100	100	100	100	100	100
S2	17.39	46.43	84.62	90.48	83.33	97.14	72.73	92.86	100	100	100	98.04	100
RBD	17.39	17.86	96.15	100	94.44	100	98.35	100	100	100	100	100	100
N	0	0	1.92	0	0	2.86	0	14.29	25	30.3	19.1	15.69	31.82
IgM	26.09	46.43	76.92	71.43	55.56	45.71	34.71	35.71	30	33.33	32.58	29.41	28.79
S	13.04	39.29	71.15	61.9	27.78	17.14	14.05	21.43	15	12.12	10.11	9.8	12.12
S1	13.04	32.14	67.31	61.9	55.56	31.43	28.93	21.43	25	19.7	14.61	19.61	19.7
S2	4.35	7.14	15.38	0	0	5.71	4.96	14.29	5	6.06	4.49	3.92	1.52
RBD	17.39	25	65.38	57.14	50	40	21.49	28.57	20	18.18	21.35	15.69	18.18
N	8.7	10.71	7.69	9.52	0	5.71	2.48	7.14	5	7.58	7.87	5.88	4.55
VoCs													
IgA RBD													
α	17.39	53.57	90.38	90.48	88.89	97.14	87.6	92.86	100	100	100	100	100
δ	26.09	57.14	94.23	95.24	88.89	94.29	88.43	85.71	100	100	100	100	100
o BA.1	0	10.71	21.15	85.71	0	5.71	5.79	42.86	45	51.52	41.57	47.06	56.06
o BA.2	0	14.29	61.54	90.48	66.67	25.71	14.88	57.14	85	71.21	68.54	60.78	80.3
o BA.4/5	0	10.71	15.38	71.43	0	5.71	4.96	42.86	45	48.48	41.57	41.18	48.48
o BQ.1.1	0	10.71	7.69	57.14	0	5.71	4.13	28.57	35	30.3	28.09	35.29	43.94
o XBB	0	10.71	1.92	42.86	0	2.86	1.65	21.43	30	21.21	15.73	23.53	24.24

IgG RBD													
α	17.39	17.86	94.23	95.24	94.44	97.14	98.35	100	100	100	100	100	100
δ	17.39	17.86	92.31	100	94.44	97.14	98.35	100	100	100	100	100	100
o BA.1	0	10.71	19.23	76.19	66.67	48.57	18.18	78.57	100	92.42	84.27	74.51	83.33
o BA.2	0	10.71	57.69	90.48	83.33	88.57	59.5	78.57	100	100	98.88	98.04	96.97
o BA.4/5	0	10.71	9.62	66.67	55.56	40	17.36	71.43	95	89.39	83.15	62.75	74.24
o BQ.1.1	0	10.71	11.54	66.67	44.44	40	12.4	64.29	85	75.76	77.53	58.82	71.21
o XBB	0	10.71	3.85	52.38	16.67	8.57	6.61	42.86	60	60.61	46.07	33.33	43.94
IgM RBD													
α	13.04	25	65.38	52.38	44.44	31.43	15.7	14.29	15	18.18	15.73	15.69	13.64
δ	13.04	17.86	59.62	52.38	33.33	22.86	11.57	28.57	5	12.12	8.99	7.84	10.61
o BA.1	0	3.57	3.85	4.76	0	0	0.83	0	0	0	0	0	1.52
o BA.2	0	3.57	7.69	4.76	5.56	2.86	0	0	0	0	1.12	0	0
o BA.4/5	0	0	1.92	0	0	0	0.83	0	0	0	0	0	0
o BQ.1.1	0	0	3.85	0	11.11	2.86	0.83	0	0	0	0	1.96	0
o XBB	0	3.57	0	0	0	0	0.83	0	0	0	0	1.96	0

Abbreviations: days (d), months (m), 1st 2nd and 3rd vaccine dose, nucleocapsid (N), receptor-binding-domain (RBD), spike (S), variants of concern (VoCs)

Supplementary Table 4. *Isotype-antigen pair antibody peak (MFI fold-differences over geometric mean seronegativity levels) after each immunization*

		after 2nd dose		after 3rd dose		after infection	
		Day 7	Day 50	Day 7	Day 50	Day 7	Day 50
IgG	S	**424.1 [291.2, 617.5]**	**80.4 [48.5, 133]**	**603.4 [453, 803.6]**	**114.3 [74, 176.6]**	**281.8 [203.8, 389.6]**	**53.4 [33.9, 84.1]**
	S1	**496.8 [328.4, 751.4]**	**94.8 [55.4, 162.2]**	**769.6 [558.1, 1061.4]**	**146.9 [92.4, 233.4]**	**345.1 [231.4, 514.7]**	**65.9 [39.5, 109.9]**
	S2	**30.4 [20.3, 45.4]**	**4.5 [2.6, 7.5]**	**57.5 [41.6, 79.5]**	**8.4 [5.3, 13.4]**	**66.7 [50, 89]**	**9.8 [6.4, 15]**
	RBD	**231.9 [141.8, 379.3]**	**39.2 [21.2, 72.5]**	**377.5 [246.9, 577.2]**	**63.8 [36.6, 111.2]**	**164.3 [105.3, 256.2]**	**27.8 [15.8, 48.8]**
	N	**1.1 [0.8, 1.6]**	**0.2 [0.1, 0.4]**	**2 [1.3, 3]**	**0.4 [0.2, 0.7]**	**17.9 [11.5, 27.9]**	**3.6 [2, 6.6]**
	RBD α	**157.8 [97, 256.8]**	**25.8 [14.1, 47.2]**	**259.5 [169, 398.4]**	**42.4 [24.4, 73.6]**	**121.6 [78, 189.6]**	**19.9 [11.4, 34.7]**
	RBD δ	**130 [81.3, 207.8]**	**20.9 [11.5, 38.1]**	**210.3 [138.8, 318.5]**	**33.8 [19.5, 58.8]**	**99.4 [66.4, 148.8]**	**16 [9.4, 27.3]**
	RBD BA.1	**10.9 [7.4, 16.1]**	1 [0.6, 1.6]	**29.9 [19.8, 45.2]**	**2.7 [1.6, 4.5]**	**14.1 [8.7, 22.7]**	1.3 [0.7, 2.2]
	RBD BA.2	**16.4 [11.3, 23.9]**	1.4 [0.8, 2.3]	**34.2 [23.4, 50]**	**2.9 [1.8, 4.7]**	**17.1 [11, 26.6]**	1.4 [0.8, 2.5]
	RBD BA.4/5	**12.4 [8.1, 18.9]**	1.3 [0.8, 2.4]	**32.3 [20.9, 49.8]**	**3.5 [2, 6.2]**	**17 [10.4, 27.7]**	1.8 [1, 3.4]
	RBD BQ.1.1	**13.2 [8.7, 20.2]**	1.2 [0.7, 2.1]	**28.8 [18.6, 44.4]**	**2.5 [1.4, 4.5]**	**15.9 [9.7, 26.2]**	1.4 [0.8, 2.6]
	RBD XBB	**6.1 [4.3, 8.6]**	0.7 [0.4, 1.2]	**16.1 [10.8, 23.9]**	**1.9 [1.1, 3.2]**	**7.4 [4.6, 11.7]**	0.9 [0.5, 1.6]
IgA	S	**221.8 [131, 375.7]**	**3.9 [2, 7.3]**	**196.2 [122.7, 313.8]**	**3.4 [1.9, 6.1]**	**98.5 [65.4, 148.3]**	1.7 [1, 2.9]
	S1	**243.8 [135.5, 438.5]**	**3.7 [1.9, 7.2]**	**171.8 [98.1, 300.8]**	**2.6 [1.3, 4.9]**	**84.9 [50.9, 141.7]**	1.3 [0.7, 2.3]
	S2	**28.5 [18.3, 44.6]**	1.5 [0.8, 2.6]	**37.8 [23.4, 61.1]**	**1.9 [1.1, 3.5]**	**45.8 [30.5, 68.9]**	**2.4 [1.4, 4]**
	RBD	**98.8 [49.4, 197.6]**	2.2 [1, 4.8]	**66 [36.7, 118.9]**	1.5 [0.8, 2.9]	**39.6 [23.4, 67.3]**	0.9 [0.5, 1.6]
	N	**1.4 [1.1, 1.8]**	**0.2 [0.2, 0.3]**	**1.7 [1.3, 2.3]**	**0.3 [0.2, 0.4]**	**8.4 [5.8, 12.2]**	1.4 [0.9, 2.2]
	RBD α	**82.6 [43.1, 158]**	1.6 [0.8, 3.3]	**57.2 [31.6, 103.3]**	1.1 [0.6, 2.2]	**37.6 [22.3, 63.5]**	0.7 [0.4, 1.3]
	RBD δ	**65.9 [35.3, 123.1]**	1.6 [0.8, 3.4]	**43.1 [23.8, 78.3]**	1.1 [0.5, 2.1]	**27 [16.5, 44.1]**	0.7 [0.4, 1.2]
	RBD BA.1	**3.9 [2.9, 5.2]**	**0.4 [0.3, 0.6]**	**3.8 [2.7, 5.5]**	**0.4 [0.3, 0.6]**	**3.6 [2.5, 5.1]**	**0.4 [0.2, 0.5]**
	RBD BA.2	**6.1 [4.3, 8.8]**	**0.5 [0.3, 0.7]**	**5.7 [3.9, 8.4]**	**0.5 [0.3, 0.7]**	**5.1 [3.4, 7.5]**	**0.4 [0.3, 0.6]**
	RBD BA.4/5	**4.3 [3.1, 5.8]**	**0.3 [0.2, 0.5]**	**3.7 [2.6, 5.3]**	**0.3 [0.2, 0.5]**	**3.8 [2.6, 5.4]**	**0.3 [0.2, 0.5]**
	RBD BQ.1.1	**3.9 [2.9, 5.2]**	**0.3 [0.2, 0.5]**	**3.2 [2.3, 4.4]**	**0.3 [0.2, 0.4]**	**3.5 [2.5, 5]**	**0.3 [0.2, 0.4]**
	RBD XBB	**2.4 [2, 2.9]**	**0.4 [0.3, 0.6]**	**2.2 [1.6, 2.8]**	**0.4 [0.3, 0.5]**	**2 [1.5, 2.7]**	**0.4 [0.3, 0.5]**
IgM	S	**9.8 [6.9, 13.8]**	**1.6 [1.1, 2.5]**	**2.3 [1.7, 3.1]**	**0.4 [0.3, 0.6]**	**1.9 [1.3, 2.7]**	**0.3 [0.2, 0.5]**
	S1	**8.4 [5.5, 13]**	**2.2 [1.3, 3.7]**	**2.5 [1.7, 3.8]**	0.7 [0.4, 1.1]	**1.9 [1.2, 2.9]**	**0.5 [0.3, 0.8]**
	S2	**2 [1.5, 2.6]**	1.3 [1, 1.8]	**1.6 [1.2, 2]**	1.1 [0.8, 1.5]	**1.6 [1.2, 2.1]**	1.1 [0.8, 1.5]
	RBD	**4.3 [3.2, 5.7]**	**1.7 [1.2, 2.5]**	**1.8 [1.4, 2.4]**	0.7 [0.5, 1]	**1.5 [1.1, 2]**	**0.6 [0.4, 0.8]**
	N	**1.7 [1.2, 2.4]**	1.1 [0.7, 1.7]	1.4 [1, 2]	0.9 [0.6, 1.4]	**2.3 [1.6, 3.4]**	1.5 [1, 2.4]
	RBD α	**3.9 [2.9, 5.2]**	**1.7 [1.2, 2.5]**	**1.8 [1.3, 2.3]**	0.8 [0.6, 1.1]	**1.6 [1.2, 2.1]**	**0.7 [0.5, 1]**
	RBD δ	**3.7 [2.8, 4.9]**	**1.8 [1.2, 2.6]**	**1.7 [1.3, 2.2]**	0.9 [0.6, 1.2]	**1.4 [1.1, 1.9]**	0.7 [0.5, 1]
	RBD BA.1	**1.4 [1.1, 1.7]**	1.2 [0.9, 1.7]	1.2 [0.9, 1.5]	1.1 [0.8, 1.4]	1 [0.8, 1.3]	0.9 [0.7, 1.2]
	RBD BA.2	**1.4 [1.2, 1.8]**	1.2 [0.9, 1.6]	1.1 [1, 1.4]	1 [0.8, 1.2]	1.2 [1, 1.4]	1 [0.8, 1.2]
	RBD BA.4/5	**1.3 [1, 1.7]**	1.3 [0.9, 1.8]	1.2 [0.9, 1.5]	1.1 [0.8, 1.6]	1 [0.8, 1.3]	1 [0.7, 1.4]
	RBD BQ.1.1	**1.4 [1.1, 1.7]**	1.2 [0.9, 1.7]	1.1 [0.9, 1.5]	1 [0.7, 1.4]	1 [0.8, 1.3]	0.9 [0.7, 1.2]
	RBD XBB	1.3 [1, 1.6]	1.3 [0.9, 1.7]	1.2 [0.9, 1.5]	1.2 [0.9, 1.6]	1 [0.8, 1.3]	1 [0.7, 1.3]

Antibody levels that are significantly different from the seronegativity mean are indicated in bold.
Abbreviations: Nucleocapsid (N), receptor-binding-domain (RBD), spike (S)

Supplementary Table 5. *Isotype-antigen pair half-life (in months) after each immunization*

		after 2nd dose		after 3rd dose		after infection	
		Day 7	Day 50	Day 7	Day 50	Day 7	Day 50
IgG	S	**1.4 [1, 2.1]**	**3.1 [2.8, 3.6]**	**2.7 [1.6, 8.1]**	**4.6 [3.7, 5.8]**	**>1000 [>1000, >1000]**	**8.6 [5.7, 18]**
	S1	**1.2 [1, 1.8]**	**2.8 [2.5, 3.2]**	**2.7 [1.6, 8.4]**	**4.6 [3.8, 5.9]**	**>1000 [>1000, >1000]**	**8.4 [5.6, 17]**
	S2	**1.5 [1.1, 2.3]**	**4.9 [4.1, 6.1]**	**2.2 [1.4, 5.1]**	**4.5 [3.7, 5.7]**	>1000 [4.6, >1000]	**6.8 [4.8, 11.4]**
	RBD	**1.2 [0.9, 1.8]**	**2.7 [2.4, 3]**	**2.1 [1.3, 4.8]**	**4.4 [3.5, 5.7]**	**>1000 [>1000, >1000]**	**7.5 [5.1, 14.5]**
	N	19.8 [2.7, >1000]	**14.9 [8.4, 67.9]**	4.8 [1.8, >1000]	**10.4 [6.3, 30]**	**1.4 [1, 2.7]**	**3.7 [2.9, 5.2]**
	RBD α	**1.2 [0.9, 1.6]**	**2.8 [2.5, 3.1]**	**2.1 [1.3, 5]**	**4.2 [3.5, 5.4]**	**>1000 [>1000, >1000]**	**7 [4.9, 12.6]**
	RBD δ	**1.2 [0.9, 1.8]**	**2.7 [2.4, 3]**	**2 [1.3, 4.9]**	**4.2 [3.4, 5.4]**	**>1000 [>1000, >1000]**	**8 [5.2, 16.7]**
	RBD BA.1	**1.1 [0.9, 1.6]**	**4.6 [3.9, 5.7]**	**1.3 [1, 1.9]**	**3.7 [3.1, 4.5]**	>1000 [35.2, >1000]	**5.6 [4.2, 8.5]**
	RBD BA.2	**1.1 [0.9, 1.4]**	**4 [3.5, 4.8]**	**1.3 [1, 1.9]**	**3.8 [3.2, 4.5]**	>1000 [279.5, >1000]	**5.8 [4.4, 8.7]**
	RBD BA.4/5	**1.2 [0.9, 1.9]**	**4 [3.4, 4.9]**	**1.6 [1.1, 2.9]**	**3.4 [2.8, 4.1]**	>1000 [23.8, >1000]	**5.6 [4, 9]**
	RBD BQ.1.1	**1.1 [0.9, 1.7]**	**4 [3.4, 5]**	**1.5 [1, 2.7]**	**3.4 [2.9, 4.2]**	>1000 [5.9, >1000]	**6.6 [4.5, 11.9]**
	RBD XBB	**1.4 [1, 2.2]**	**5.4 [4.4, 7]**	**1.4 [1, 2.2]**	**3.8 [3.2, 4.6]**	>1000 [16.9, >1000]	**6 [4.4, 9.6]**
IgA	S	**0.5 [0.4, 0.6]**	**6.3 [5, 8.7]**	**1 [0.8, 1.5]**	**5 [4, 6.7]**	72.8 [3.5, >1000]	**5.5 [4.1, 8.5]**
	S1	**0.4 [0.4, 0.5]**	**6.4 [5.1, 8.7]**	**1.1 [0.8, 1.4]**	**4.9 [4, 6.5]**	>1000 [15.5, >1000]	**5.1 [3.9, 7.3]**
	S2	**0.8 [0.7, 1]**	**12.1 [8.1, 23.7]**	**1.3 [1, 2]**	**6.8 [5.1, 10.1]**	6.1 [2.4, >1000]	**6.2 [4.5, 9.8]**
	RBD	**0.5 [0.4, 0.5]**	**7.6 [5.8, 11]**	**1.1 [0.9, 1.6]**	**5.6 [4.4, 7.8]**	>1000 [21.5, >1000]	**4.5 [3.5, 6.2]**
	N	5 [2.3, >1000]	75.9 [19.8, >1000]	6.5 [2.6, >1000]	32.9 [13.4, >1000]	**1 [0.8, 1.2]**	**7.6 [5.3, 13]**
	RBD α	**0.5 [0.4, 0.5]**	**8.8 [6.5, 13.7]**	**1.1 [0.9, 1.5]**	**5.7 [4.5, 8]**	>1000 [7.9, >1000]	**5.3 [4, 7.7]**
	RBD δ	**0.5 [0.4, 0.6]**	**9.3 [6.7, 15]**	**1.1 [0.8, 1.5]**	**6.5 [4.9, 9.6]**	>1000 [23.3, >1000]	**5 [3.9, 7.3]**
	RBD BA.1	**1 [0.9, 1.2]**	69.1 [26.4, >1000]	**1.6 [1.3, 2.1]**	**27.8 [15.6, 131.3]**	7.7 [3.6, >1000]	**6.1 [5.1, 7.8]**
	RBD BA.2	**0.8 [0.7, 1]**	40.3 [19.9, >1000]	**1.4 [1.1, 1.8]**	**20 [12.4, 52.4]**	20.3 [4.7, >1000]	**5.9 [4.8, 7.5]**
	RBD BA.4/5	**0.9 [0.8, 1]**	66.8 [24.1, >1000]	**1.5 [1.2, 2]**	**23.5 [13.4, 94.3]**	6.7 [3.1, >1000]	**5.7 [4.6, 7.3]**
	RBD BQ.1.1	**0.9 [0.8, 1.1]**	199.6 [33.6, >1000]	**1.5 [1.2, 2]**	**27.2 [15, 146.5]**	**4.8 [2.7, 20.4]**	**6.2 [5, 7.9]**
	RBD XBB	**1.3 [1.1, 1.5]**	205.9 [39.7, >1000]	**2.3 [1.8, 3.3]**	**39.4 [20, >1000]**	**7.4 [3.8, 248.7]**	**8.7 [6.9, 11.8]**
IgM	S	**0.8 [0.7, 1]**	**10.8 [8.2, 16.1]**	5.6 [2.8, >1000]	**16.4 [10.3, 39.5]**	>1000 [6.4, >1000]	**18.4 [10.1, 102.4]**
	S1	**1.1 [0.9, 1.4]**	**9.8 [7.2, 15]**	6.1 [2.6, >1000]	**17.4 [10, 65.8]**	>1000 [13.6, >1000]	**13.3 [7.8, 43.8]**
	S2	**3.9 [2.5, 8.9]**	78.1 [27.3, >1000]	16.5 [4.8, >1000]	41.5 [19.2, >1000]	>1000 [14.2, >1000]	**15.1 [9.9, 32.6]**
	RBD	**1.5 [1.2, 2]**	**18.7 [12.2, 40.1]**	20.6 [4.5, >1000]	**17.5 [11, 42.5]**	>1000 [11.6, >1000]	**20.8 [11.1, 168.7]**
	N	**5.5 [2.9, 62.5]**	109.6 [27.2, >1000]	>1000 [14.4, >1000]	48.1 [18.7, >1000]	6.3 [3, >1000]	**12.4 [8.2, 25.2]**
	RBD α	**1.6 [1.3, 2.1]**	**30.4 [16.7, 164.8]**	>1000 [6.5, >1000]	**21.4 [12.7, 66.9]**	>1000 [10.9, >1000]	**15.9 [9.8, 42.5]**
	RBD δ	**1.6 [1.2, 2.2]**	35.3 [16.8, >1000]	>1000 [5.4, >1000]	**20.2 [11.6, 78.2]**	>1000 [25, >1000]	**18.8 [10.1, 137.5]**
	RBD BA.1	8 [4, >1000]	117.3 [33.8, >1000]	>1000 [11.4, >1000]	40.3 [20.1, >1000]	>1000 [12.5, >1000]	**31.2 [15.8, >1000]**
	RBD BA.2	**5.2 [3.3, 12.1**]	76.7 [32.3, >1000]	>1000 [21.8, >1000]	55.3 [25.2, >1000]	>1000 [10.7, >1000]	68.4 [24.5, >1000]
	RBD BA.4/5	10.4 [4.2, >1000]	129.9 [31.6, >1000]	>1000 [19, >1000]	**30.7 [16.5, 228.9]**	>1000 [11.5, >1000]	29.7 [14.5, >1000]
	RBD BQ.1.1	**6.5 [3.3, 154.1]**	265.7 [35.1, >1000]	>1000 [12.1, >1000]	**31.8 [16.5, 403.7]**	>1000 [10.5, >1000]	29.4 [14.2, >1000]
	RBD XBB	12.4 [4.6, >1000]	246.9 [37.2, >1000]	>1000 [14.6, >1000]	39.3 [19.2, >1000]	>1000 [12.8, >1000]	34.2 [15.9, >1000]

Decay rates that are significantly different from 0 (corresponding to an infinite half-life) are indicated in bold. Abbreviations: Nucleocapsid (N), receptor-binding-domain (RBD), spike (S)

Supplementary Table 6. *Correlation of estimated peak IgG and IgA levels (day 7) between different immunization intervals for all other COVID-19 antigens*

		2nd vs. 3rd dose	2nd dose vs. inf	3rd dose vs. Inf	Chi^2	Df	p val
IgG	**N**	0.72	0.59	0.57	16.28	3	0.001
	S	0.77	0.03	0.55	13.55	3	0.004
	S1	0.69	0.27	0.15	9.6	3	0.022
	S2	0.78	0	0.35	15.08	3	0.002
	RBD	0.71	-0.16	0.26	12.23	3	0.007
	RBD α	0.73	-0.17	0.25	13.67	3	0.003
	RBD δ	0.71	0	0.25	11.38	3	0.01
	RBD o BA.1	0.73	-0.02	0.48	17.14	3	0.001
	RBD o BA.2	0.68	-0.04	0.47	13.15	3	0.004
	RBD o BA.4/5	0.72	0.05	0.41	12.22	3	0.007
	RBD o BQ.1.1	0.71	0.03	0.46	13.7	3	0.003
	RBD o XBB	0.78	0.13	0.46	16.2	3	0.001
IgA	**N**	0.82	0.59	0.85	32.91	3	3.37E-07
	S	0.7	0.65	0.69	22.82	3	4.41E-05
	S1	0.71	0.53	0.44	17.83	3	4.76E-04
	S2	0.84	0.72	0.81	37.47	3	3.65E-08
	RBD	0.8	0.02	0.55	22.09	3	6.26E-05
	RBD α	0.79	0.07	0.56	19.4	3	2.26E-04
	RBD δ	0.83	0.25	0.61	22.25	3	5.78E-05
	RBD o BA.1	0.73	0.06	0.6	17.4	3	5.85E-04
	RBD o BA.2	0.75	0.07	0.6	21.28	3	9.22E-05
	RBD o BA.4/5	0.77	0	0.45	12.09	3	7.09E-03
	RBD o BQ.1.1	0.77	0.07	0.46	13.27	3	4.09E-03
	RBD o XBB	0.77	0.05	0.52	13.61	3	3.49E-03

A log-likelihood ratio test was used to assess the significance of non-null correlations between random intercepts in the linear mixed models by comparing a full model with correlated random intercepts and a competing reduced model (3 degrees of freedom less) without it.

Supplementary Table 7. *Fold differences in Wuhan SARS-CoV-2 antibody levels between post-immunization segments*

		3rd vs. 2nd dose		infection vs. 2nd dose		infection vs. 3rd dose	
		Day 7	Day 50	Day 7	Day 50	Day 7	Day 50
IgG	S	1.4 [1, 2]	**2.4 [1.8, 3.1]**	0.9 [0.6, 1.3]	**2.9 [2.1, 4.1]**	**0.6 [0.5, 0.8]**	**1.2 [1, 1.5]**
	S1	1.4 [1, 2.2]	**2.8 [2, 3.9]**	0.8 [0.5, 1.3]	**3.7 [2.3, 5.7]**	**0.5 [0.4, 0.9]**	1.3 [0.9, 1.9]
	S2	**2 [1.4, 2.9]**	**2.4 [1.8, 3.2]**	**2.8 [1.8, 4.4]**	**5.3 [3.6, 7.8]**	**1.4 [1, 2]**	**2.2 [1.7, 2.8]**
	RBD	**1.7 [1, 2.7]**	**2.9 [2, 4.2]**	0.8 [0.4, 1.6]	**4.4 [2.4, 7.9]**	**0.5 [0.3, 0.9]**	1.5 [1, 2.4]
	N	**1.9 [1.2, 3]**	**1.4 [1, 1.9]**	**18.1 [10.9, 29.9]**	**5.8 [4.1, 8.2]**	**9.7 [5.7, 16.3]**	**4.1 [2.8, 6.1]**
IgA	S	0.9 [0.6, 1.5]	**2.7 [1.8, 4]**	**0.6 [0.3, 0.9]**	**4.1 [2.7, 6.3]**	**0.6 [0.4, 0.9]**	**1.5 [1.1, 2.1]**
	S1	0.7 [0.4, 1.2]	3.1 [1.9, 5.1]	**0.4 [0.2, 0.7]**	**5.9 [3.3, 10.5]**	0.6 [0.3, 1.1]	**1.9 [1.1, 3.3]**
	S2	**1.5 [1, 2.3]**	**2 [1.5, 2.8]**	**2.3 [1.5, 3.5]**	**4.8 [3.4, 6.8]**	1.5 [1, 2.2]	**2.4 [1.7, 3.3]**
	RBD	0.7 [0.4, 1.2]	**2.5 [1.6, 3.8]**	0.4 [0.2, 1.1]	**5 [2.2, 11.1]**	0.6 [0.4, 1.2]	**2 [1.2, 3.4]**
	N	1.3 [1, 1.8]	**1.2 [1, 1.5]**	**7.2 [4.9, 10.7]**	**2 [1.5, 2.7]**	**5.5 [3.8, 7.7]**	**1.6 [1.2, 2]**
IgM	S	**0.2 [0.2, 0.3]**	**0.8 [0.7, 0.9]**	**0.2 [0.1, 0.3]**	0.8 [0.6, 1.1]	0.9 [0.6, 1.2]	1.1 [0.8, 1.4]
	S1	**0.3 [0.2, 0.4]**	**0.7 [0.6, 0.9]**	**0.2 [0.2, 0.3]**	0.8 [0.6, 1.1]	0.8 [0.6, 1.1]	1.1 [0.8, 1.4]
	S2	**0.8 [0.7, 1]**	1 [0.9, 1.1]	0.9 [0.7, 1.1]	1.1 [0.9, 1.3]	1.1 [0.9, 1.4]	1.1 [0.9, 1.4]
	RBD	**0.4 [0.3, 0.5]**	**0.9 [0.8, 1]**	**0.4 [0.3, 0.5]**	0.8 [0.7, 1]	0.9 [0.7, 1.1]	1 [0.8, 1.2]
	N	0.9 [0.7, 1.1]	1.1 [1, 1.3]	**1.5 [1.1, 1.9]**	**1.3 [1, 1.6]**	**1.7 [1.3, 2.2]**	1.1 [0.9, 1.4]

Differences expressed as fold-changes [CI] at day 7 and 50 after immunizations. Statistically significant differences are in bold. Abbreviations: Nucleocapsid (N), receptor-binding-domain (RBD), spike (S)

Supplementary Table 8. *Fold differences in SARS-CoV-2 VoCs antibody levels between post-immunization intervals*

		3rd vs. 2nd dose		infection vs. 2nd dose		infection vs. 3rd dose	
		Day 7	Day 50	Day 7	Day 50	Day 7	Day 50
IgG	RBD α	**1.7 [1, 2.6]**	**3 [2.1, 4.3]**	0.9 [0.5, 1.7]	**4.9 [2.7, 8.9]**	**0.5 [0.3, 0.9]**	1.6 [1, 2.6]
	RBD δ	1.6 [1, 2.5]	**2.7 [1.9, 4]**	0.9 [0.5, 1.6]	**4.6 [2.7, 7.7]**	**0.6 [0.3, 0.9]**	**1.7 [1.1, 2.5]**
	RBD o BA.1	**3 [2, 4.6]**	**2.9 [2.1, 3.9]**	1.4 [0.8, 2.7]	**4.9 [2.8, 8.6]**	**0.5 [0.3, 0.8]**	**1.7 [1.1, 2.7]**
	RBD o BA.2	**2.1 [1.4, 3.2]**	**2.5 [1.8, 3.4]**	1.1 [0.6, 2]	**4.4 [2.6, 7.5]**	**0.5 [0.3, 0.9]**	**1.8 [1.2, 2.7**
	RBD o BA.4/5	**2.9 [1.8, 4.5]**	**3 [2.2, 4.2]**	1.5 [0.8, 2.9]	**5.1 [2.9, 8.9]**	**0.5 [0.3, 1]**	**1.7 [1, 2.7]**
	RBD o BQ.1.1	**2.4 [1.5, 3.8]**	**2.7 [1.9, 3.7]**	1.3 [0.7, 2.6]	**4.3 [2.5, 7.6]**	0.6 [0.3, 1]	1.6 [1, 2.6]
	RBD o XBB	**2.9 [1.9, 4.3]**	**2.4 [1.8, 3.2]**	1.3 [0.7, 2.3]	**3.6 [2.2, 5.9]**	**0.4 [0.3, 0.8]**	1.5 [0.9, 2.4]
IgA	RBD α	0.7 [0.4, 1.3]	**2.6 [1.7, 4.1]**	0.5 [0.2, 1.2]	**5.6 [2.7, 12]**	0.7 [0.4, 1.3]	**2.1 [1.2, 3.7]**
	RBD δ	0.7 [0.4, 1.1]	**2.2 [1.5, 3.3]**	0.5 [0.2, 1]	**4.9 [2.6, 9.3]**	0.7 [0.4, 1.2]	**2.2 [1.3, 3.6]**
	RBD o BA.1	1 [0.8, 1.4]	**1.4 [1.1, 1.9]**	1 [0.6, 1.6]	**2.1 [1.3, 3.2]**	0.9 [0.6, 1.4]	1.4 [1, 2]
	RBD o BA.2	1 [0.7, 1.4]	**1.5 [1.1, 2]**	0.9 [0.5, 1.5]	**2.5 [1.5, 4.2]**	0.9 [0.6, 1.4]	**1.7 [1.2, 2.5]**
	RBD o BA.4/5	1 [0.7, 1.3]	**1.4 [1, 1.8]**	1 [0.6, 1.6]	**2.2 [1.4, 3.4]**	1 [0.7, 1.6]	**1.6 [1.1, 2.4]**
	RBD o BQ.1.1	0.9 [0.6, 1.2]	1.3 [1, 1.6]	1 [0.6, 1.5]	**2 [1.3, 3.1]**	1.1 [0.8, 1.7]	**1.6 [1.1, 2.3]**
	RBD o XBB	0.9 [0.7, 1.2]	**1.3 [1, 1.5]**	0.9 [0.6, 1.3]	**1.5 [1.1, 2.1]**	1 [0.7, 1.3]	1.2 [0.9, 1.6]
IgM	RBD α	**0.4 [0.4, 0.6]**	0.9 [0.8, 1.1]	**0.4 [0.3, 0.5]**	0.9 [0.8, 1.1]	0.9 [0.7, 1.2]	1 [0.8, 1.1]
	RBD δ	**0.5 [0.4, 0.6]**	1 [0.8, 1.1]	**0.4 [0.3, 0.5]**	0.9 [0.8, 1.1]	0.9 [0.7, 1.2]	1 [0.8, 1.2]
	RBD o BA.1	0.9 [0.7, 1]	1 [0.9, 1.1]	**0.8 [0.6, 0.9]**	0.9 [0.8, 1]	0.9 [0.7, 1.1]	**0.9 [0.8, 1]**
	RBD o BA.2	**0.8 [0.7, 0.9]**	1.1 [0.9, 1.2]	**0.8 [0.7, 0.9]**	1 [0.9, 1.1]	1 [0.9, 1.2]	1 [0.9, 1.1]
	RBD o BA.4/5	0.9 [0.7, 1.1]	1 [1, 1.1]	**0.8 [0.6, 1]**	0.9 [0.8, 1]	0.9 [0.7, 1.1]	**0.8 [0.7, 1]**
	RBD o BQ.1.1	0.9 [0.7, 1]	1.1 [1, 1.2]	**0.8 [0.6, 1]**	0.9 [0.8, 1]	0.9 [0.7, 1.1]	**0.9 [0.8, 1]**
	RBD o XBB	0.9 [0.8, 1.1]	1.1 [1, 1.2]	**0.8 [0.6, 1]**	0.9 [0.8, 1]	0.9 [0.7, 1]	**0.9 [0.8, 1]**

Differences expressed as fold-change [CI] at day 7 and 50 after immunization. Statistically significant differences are in bold

Supplementary Table 9. *Fold differences in antibody levels between post-immunization intervals excluding participants with hybrid immunity after vaccine immunization intervals*

		3rd vs. 2nd dose		infection vs. 2nd dose		infection vs. 3rd dose	
		Day 7	Day 50	Day 7	Day 50	Day 7	Day 50
IgG	S	**1.6 [1.1, 2.2]**	**2.6 [2, 3.3]**	1.2 [0.8, 1.8]	**3.4 [2.5, 4.5]**	0.8 [0.6, 1]	**1.3 [1.1, 1.6]**
	S1	1.4 [0.9, 2.1]	**2.6 [1.9, 3.6]**	1 [0.7, 1.6]	**4.2 [3, 5.9]**	0.7 [0.5, 1]	**1.6 [1.4, 1.9]**
	S2	**2.3 [1.5, 3.3]**	**2.7 [2, 3.5]**	**4.3 [3, 6.4]**	**6.4 [4.8, 8.4]**	**1.9 [1.4, 2.6]**	**2.4 [2, 2.8]**
	RBD	**1.7 [1.1, 2.7]**	**2.8 [2, 4.1]**	1.2 [0.7, 2]	**5.7 [3.9, 8.4]**	0.7 [0.5, 1.1]	**2 [1.6, 2.5]**
	N	1.4 [0.9, 2.1]	**1.3 [1.1, 1.5]**	**15.2 [9, 25.7]**	**5 [3.4, 7.2]**	**11.2 [6.5, 19.4]**	**3.8 [2.6, 5.6]**
	RBD α	**1.8 [1.1, 2.8]**	**3 [2.2, 4.3]**	1.2 [0.8, 2.1]	**6.3 [4.2, 9.5]**	0.7 [0.5, 1.1]	**2.1 [1.6, 2.7]**
	RBD δ	1.6 [1, 2.6]	**2.7 [1.9, 3.9]**	1.3 [0.8, 2.1]	**5.9 [4.1, 8.5]**	0.8 [0.5, 1.2]	**2.2 [1.7, 2.7]**
	RBD o BA.1	**3.2 [2.1, 4.9]**	**3.2 [2.4, 4.2]**	**2.1 [1.2, 3.6]**	**6.5 [4.1, 10.3]**	0.7 [0.4, 1]	**2.1 [1.5, 2.9]**
	RBD o BA.2	**2.2 [1.5, 3.3]**	**2.6 [2, 3.5]**	1.6 [0.9, 2.7]	**5.5 [3.5, 8.5]**	0.7 [0.5, 1.1]	**2.1 [1.5, 2.8]**
	RBD o BA.4/5	**3 [1.9, 4.7]**	**3.1 [2.3, 4.2]**	**2.3 [1.3, 4.1]**	**6.6 [4.1, 10.4]**	0.8 [0.5, 1.3]	**2.1 [1.4, 3.1]**
	RBD o BQ.1.1	**2.5 [1.6, 3.9]**	**2.8 [2.1, 3.8]**	**2 [1.1, 3.5]**	**5.7 [3.6, 9]**	0.8 [0.5, 1.3]	**2 [1.4, 2.9]**
	RBD o XBB	**3 [2, 4.5]**	**2.5 [1.9, 3.3]**	**1.9 [1.1, 3.2]**	**4.7 [3.1, 7]**	0.6 [0.4, 1]	**1.8 [1.3, 2.7]**
IgA	S	1.1 [0.7, 1.7]	**2.6 [1.7, 3.9]**	0.8 [0.5, 1.2]	**4.8 [3.3, 7]**	0.7 [0.5, 1.1]	**1.8 [1.3, 2.6]**
	S1	0.7 [0.4, 1.2]	**2.8 [1.7, 4.4]**	0.6 [0.3, 1]	**7.9 [4.9, 12.8]**	0.8 [0.5, 1.2]	**2.9 [2.1, 3.9]**
	S2	**2 [1.3, 2.9]**	**2 [1.5, 2.8]**	**3.3 [2.1, 5.1]**	**5.8 [4.1, 8.3]**	**1.7 [1, 2.7]**	**2.9 [2, 4.3]**
	RBD	0.8 [0.5, 1.3]	**2.4 [1.6, 3.7]**	0.7 [0.4, 1.3]	**7.7 [4.6, 13.1]**	0.9 [0.6, 1.4]	**3.2 [2.5, 4.1]**
	N	1.1 [0.8, 1.5]	1.1 [1, 1.2]	**7.4 [4.8, 11.3]**	**1.7 [1.2, 2.4]**	**6.7 [4.4, 10.3]**	**1.6 [1.1, 2.2]**
	RBD α	0.8 [0.5, 1.3]	**2.6 [1.7, 3.8]**	0.8 [0.4, 1.4]	**8.6 [5.2, 14.3]**	1 [0.7, 1.5]	**3.4 [2.6, 4.3]**
	RBD δ	0.7 [0.4, 1.2]	**2.1 [1.4, 3.1]**	0.7 [0.4, 1.2]	**7.2 [4.6, 11.3]**	1 [0.6, 1.5]	**3.4 [2.6, 4.6]**
	RBD o BA.1	1 [0.7, 1.4]	**1.4 [1.1, 1.9]**	1.1 [0.8, 1.7]	**2.7 [1.9, 3.9]**	1.1 [0.8, 1.6]	**1.9 [1.4, 2.5]**
	RBD o BA.2	0.9 [0.7, 1.3]	**1.5 [1.2, 2]**	1.1 [0.7, 1.7]	**3.4 [2.3, 5.2]**	1.1 [0.8, 1.6]	**2.2 [1.7, 3]**
	RBD o BA.4/5	0.9 [0.7, 1.3]	**1.4 [1.1, 1.8]**	1.2 [0.8, 1.8]	**2.8 [2, 4]**	1.3 [0.9, 1.8]	**2 [1.5, 2.7]**
	RBD o BQ.1.1	0.9 [0.7, 1.2]	**1.3 [1.1, 1.7]**	1.1 [0.8, 1.6]	**2.6 [1.9, 3.6]**	1.2 [0.9, 1.8]	**2 [1.5, 2.6]**
	RBD o XBB	0.9 [0.7, 1.2]	**1.3 [1.1, 1.6]**	1 [0.7, 1.4]	**1.8 [1.3, 2.5]**	1 [0.7, 1.4]	**1.4 [1.1, 1.8]**
IgM	S	**0.2 [0.2, 0.3]**	**0.8 [0.7, 0.9]**	**0.2 [0.1, 0.2]**	**0.7 [0.6, 1]**	0.8 [0.6, 1]	0.9 [0.8, 1.1]
	S1	**0.2 [0.2, 0.3]**	**0.7 [0.6, 0.8]**	**0.2 [0.1, 0.3]**	**0.7 [0.5, 1]**	0.8 [0.6, 1.1]	1 [0.9, 1.3]
	S2	0.8 [0.7, 1]	1 [0.9, 1.1]	0.8 [0.7, 1]	1.1 [1, 1.2]	1 [0.8, 1.3]	1.1 [1, 1.2]
	RBD	**0.4 [0.3, 0.5]**	0.9 [0.8, 1]	**0.3 [0.2, 0.4]**	**0.8 [0.6, 0.9]**	**0.8 [0.6, 1]**	0.9 [0.8, 1]
	N	0.8 [0.7, 1.1]	1.1 [1, 1.2]	**1.6 [1.1, 2.2]**	**1.4 [1.1, 1.8]**	**1.9 [1.4, 2.6]**	**1.3 [1, 1.6]**
	RBD α	**0.4 [0.3, 0.5]**	0.9 [0.8, 1.1]	**0.4 [0.3, 0.5]**	0.9 [0.7, 1]	0.9 [0.7, 1.1]	0.9 [0.8, 1]
	RBD δ	**0.5 [0.4, 0.6]**	0.9 [0.8, 1.1]	**0.4 [0.3, 0.5]**	0.9 [0.8, 1.1]	0.8 [0.6, 1.1]	1 [0.8, 1.1]
	RBD o BA.1	0.9 [0.7, 1.1]	1 [0.9, 1.1]	**0.7 [0.6, 0.9]**	**0.9 [0.8, 1]**	0.8 [0.7, 1]	**0.9 [0.8, 1]**
	RBD o BA.2	**0.8 [0.7, 0.9]**	1.1 [1, 1.1]	**0.8 [0.7, 0.9]**	1 [0.9, 1.1]	1 [0.9, 1.2]	1 [0.9, 1]
	RBD o BA.4/5	0.9 [0.7, 1.1]	1 [0.9, 1.2]	**0.7 [0.6, 0.9]**	**0.9 [0.8, 1]**	0.8 [0.6, 1]	**0.8 [0.7, 0.9]**
	RBD o BQ.1.1	0.9 [0.7, 1.1]	1.1 [1, 1.2]	**0.7 [0.6, 0.9]**	0.9 [0.8, 1]	0.8 [0.7, 1.1]	**0.9 [0.8, 1]**
	RBD o XBB	0.9 [0.7, 1.1]	1.1 [1, 1.2]	**0.7 [0.6, 0.9]**	0.9 [0.8, 1]	0.8 [0.6, 1]	**0.8 [0.7, 0.9]**

Differences expressed as fold-change [CI] at day 7 and 50 after immunization. Statistically significant differences are in bold

Supplementary Table 10. *Relative differences in SARS-CoV-2 antibody waning as % monthly decay rates between post-immunization intervals*

		3rd vs. 2nd dose		infection vs. 2nd dose		infection vs. 3rd dose	
		Day 7 to 50	Day >50	Day 7 to 50	Day >50	Day 7 to 50	Day >50
IgG	S	**-28.4 [-4.1, -58.5]**	**-7.3 [-3.5, -11.2]**	**-75.8 [-42.5, -117]**	**-17.1 [-12.3, -22]**	**-36.9 [-11.1, -68.6]**	**-9.1 [-4.4, -14]**
	S1	**-35.9 [-6.9, -72.7]**	**-9.8 [-5.4, -14.3]**	**-111.3 [-66.4, -168.3]**	**-18.9 [-13.5, -24.5]**	**-55.5 [-22.5, -97.4]**	**-8.3 [-3.1, -13.8]**
	S2	-13.8 [8.6, -41.8]	1.2 [4.9, -2.5]	**-38.9 [-11.6, -72.9]**	**-5.6 [-1.2, -10.2]**	-22.1 [1.9, -51.8]	**-6.9 [-2.1, -11.9]**
	RBD	-25.1 [4.8, -64.3]	**-10.5 [-5.5, -15.7]**	**-128.7 [-74.3, -199.9]**	**-19.1 [-13, -25.6]**	**-82.8 [-39.3, -139.9]**	**-7.8 [-1.9, -14.1]**
	N	13.9 [38, -19.4]	2.2 [7.5, -3.5]	**42.2 [58.3, 19.9]**	**12.4 [17.9, 6.6]**	**32.9 [51.8, 6.6]**	**10.5 [16.4, 4.1]**
IgA	S	**-96.1 [-54.4, -149]**	2.5 [6.4, -1.5]	**-229.9 [-160.3, -318]**	-0.1 [4.4, -4.8]	**-68.2 [-32.7, -113.3]**	-2.7 [2.3, -7.8]
	S1	**-154.2 [-98, -226.4]**	2.8 [6.9, -1.4]	**-417.8 [-304.3, -563.2]**	2 [6.6, -2.9]	**-103.7 [-58.8, -161.1]**	-0.9 [4.2, -6.2]
	S2	-21.3 [2.9, -51.6]	**3.8 [7.4, 0]**	**-61.2 [-29.3, -101.1]**	3.6 [7.7, -0.6]	**-32.9 [-6.5, -65.9]**	-0.1 [4.4, -4.9]
	RBD	**-118.4 [-68.5, -183.1]**	2.8 [7, -1.6]	**-348.1 [-246.1, -480.1]**	**5.5 [10.2, 0.7]**	**-105.2 [-58.5, -165.6]**	2.8 [7.9, -2.6]
	N	2.4 [21.9, -22]	1.2 [4.9, -2.7]	**50.6 [60.6, 38.1]**	**7.1 [11.1, 2.9]**	**49.4 [59.6, 36.6]**	**5.9 [10.3, 1.4]**
IgM	S	**-104.9 [-71.5, -144.8]**	-2.2 [0.9, -5.4]	**-124.9 [-87.8, -169.5]**	-3.3 [0.3, -7]	-9.8 [8.2, -31.3]	-1.1 [2.6, -5]
	S1	**-80.1 [-46.5, -121.5]**	-3.1 [0.5, -6.8]	**-113 [-73, -162.4]**	-2.3 [1.7, -6.6]	-18.3 [3.9, -45.5]	0.7 [5, -3.7]
	S2	-11 [3.1, -27.2]	0.5 [2.8, -1.8]	**-17 [-1.9, -34.3]**	**3 [5.6, 0.3]**	-5.4 [8.1, -20.8]	2.5 [5.2, -0.4]
	RBD	**-54 [-30.4, -82]**	0.3 [3.1, -2.6]	**-63.6 [-38.1, -93.8]**	-0.9 [2.4, -4.3]	-6.2 [10.2, -25.6]	-1.2 [2.3, -4.8]
	N	**-19.1 [-0.2, -41.6]**	0.8 [3.7, -2.2]	2.3 [18, -16.5]	**4.5 [7.7, 1.2]**	**17.9 [31, 2.3]**	**3.8 [7.2, 0.2]**

Differences expressed in % monthly decay rates [CI] at 7-50 and > 50 days after immunization. Statistically significant differences are in bold. Abbreviations: Nucleocapsid (N), receptor-binding-domain (RBD), spike (S)

Supplementary Table 11. *Relative differences in SARS-CoV-2 VoCs antibody waning as % monthly decay rates between post-immunization intervals*

		3rd vs. 2nd dose		infection vs. 2nd dose		infection vs. 3rd dose	
		Day 7 to 50	Day >50	Day 7 to 50	Day >50	Day 7 to 50	Day >50
IgG	RBD α	**-31.3 [-0.6, -71.3]**	**-8.8 [-4, -13.9]**	**-137.7 [-82.4, -209.8]**	**-17.3 [-11.5, -23.5]**	**-81.1 [-38.9, -136.1]**	**-7.8 [-2, -13.9]**
	RBD δ	-27.1 [3.5, -67.2]	**-10 [-5, -15.3]**	**-124.7 [-71, -195.2]**	**-20.1 [-13.9, -26.6]**	**-76.8 [-34.5, -132.4]**	**-9.2 [-3.1, -15.5]**
	RBD o BA.1	-1.4 [22.2, -32.3]	3.8 [8.1, -0.7]	**-102.8 [-55.6, -164.3]**	-3.2 [2, -8.6]	**-100 [-53.3, -160.8]**	**-7.2 [-1.5, -13.3]**
	RBD o BA.2	-11.3 [13.2, -42.7]	1.3 [5.4, -3]	**-119 [-70.9, -180.5]**	**-5.7 [-0.7, -10.9]**	**-96.7 [-53.5, -152.1]**	**-7.1 [-1.7, -12.8]**
	RBD o BA.4/5	-7 [20.5, -44]	3.3 [8.1, -1.7]	**-96 [-45.6, -163.7]**	-5.6 [0.4, -11.9]	**-83.2 [-36, -146.6]**	**-9.2 [-2.7, -16.2]**
	RBD o BQ.1.1	-10.8 [17.5, -48.9]	3.3 [8, -1.7]	**-88.7 [-40.5, -153.5]**	**-7.3 [-1.3, -13.7]**	**-70.3 [-26.7, -128.9]**	**-10.9 [-4.3, -18]**
	RBD o XBB	4.5 [26.7, -24.5]	**5.6 [9.8, 1.2]**	**-83 [-40.4, -138.6]**	-1.6 [3.5, -7]	**-91.7 [-46.9, -150.1]**	**-7.7 [-1.9, -13.8]**
IgA	RBD α	**-120.6 [-70.8, -185]**	3.6 [7.7, -0.7]	**-333.3 [-236, -458.8]**	4.4 [9, -0.5]	**-96.4 [-52.2, -153.4]**	0.8 [5.9, -4.6]
	RBD δ	**-106.4 [-59.5, -166.9]**	2.8 [7, -1.5]	**-321.1 [-226.1, -443.7]**	**5.3 [9.9, 0.5]**	**-104 [-57.9, -163.7]**	2.5 [7.6, -2.8]
	RBD o BA.1	**-22.9 [-5.9, -42.7]**	1.2 [3.7, -1.4]	**-71.9 [-48.3, -99.3]**	**9.6 [12.1, 6.9]**	**-39.8 [-20.5, -62.3]**	**8.5 [11.3, 5.6]**
	RBD o BA.2	**-31.6 [-11.7, -55]**	1.4 [4.1, -1.4]	**-106.8 [-75.8, -143.3]**	**9.4 [12.2, 6.5]**	**-57.2 [-33.5, -85.1]**	**8.1 [11.2, 4.9]**
	RBD o BA.4/5	**-24.6 [-5.3, -47.4]**	1.5 [4.3, -1.3]	**-78.3 [-50.9, -110.7]**	**10.2 [13.1, 7.2]**	**-43.2 [-21.1, -69.3]**	**8.8 [11.9, 5.6]**
	RBD o BQ.1.1	**-28.4 [-9.7, -50.4]**	1.8 [4.4, -0.9]	**-73 [-47.9, -102.3]**	**10.1 [12.8, 7.3]**	**-34.7 [-15.1, -57.6]**	**8.4 [11.3, 5.3]**
	RBD o XBB	**-20.8 [-6.1, -37.5]**	1.2 [3.4, -1]	**-47.5 [-29.7, -67.8]**	**7.1 [9.4, 4.7]**	**-22.1 [-7.3, -39.1]**	**6 [8.5, 3.4]**
IgM	RBD α	**-58.7 [-35.5, -85.7]**	1 [3.6, -1.7]	**-60.8 [-37.2, -88.5]**	1.6 [4.6, -1.5]	-1.3 [13.5, -18.7]	0.6 [3.9, -2.7]
	RBD δ	**-55.6 [-30.2, -85.9]**	1.6 [4.5, -1.5]	**-62.2 [-35.7, -94]**	1 [4.4, -2.5]	-4.3 [12.7, -24.6]	-0.6 [3.1, -4.4]
	RBD o BA.1	-12.3 [1.5, -28.1]	1.2 [3.4, -1.1]	-11.9 [2.1, -27.8]	1.5 [4.1, -1.1]	0.4 [12.8, -13.6]	0.4 [3.1, -2.4]
	RBD o BA.2	**-19.3 [-6.7, -33.2]**	0.4 [2.2, -1.5]	**-13.7 [-2, -26.8]**	0 [2.1, -2.2]	4.6 [14.5, -6.4]	-0.4 [1.9, -2.8]
	RBD o BA.4/5	-12.9 [2.7, -31.1]	1.7 [4.2, -0.9]	-9.7 [5.7, -27.6]	1.7 [4.6, -1.3]	2.9 [16.4, -12.9]	0 [3.1, -3.3]
	RBD o BQ.1.1	-16.2 [0.2, -35.3]	1.8 [4.3, -0.8]	-13.3 [3.1, -32.4]	1.9 [4.9, -1.2]	2 [15.9, -14.2]	0 [3.1, -3.3]
	RBD o XBB	-11.8 [3, -28.7]	1.5 [3.8, -1]	-11.5 [3.6, -28.8]	1.7 [4.5, -1.1]	0.3 [13.5, -15]	0.3 [3.2, -2.8]

Differences expressed in % monthly decay [CI] at 7-50 and > 50 days after immunization. Statistically significant differences are in bold

Supplementary Table 12. *Relative differences in antibody waning as % monthly decay rate between post-immunization intervals excluding hybrid immunity participants after vaccine immunization intervals*

		3rd vs. 2nd dose		infection vs. 2nd dose		infection vs. 3rd dose	
		Day 7 to 50	Day >50	Day 7 to 50	Day >50	Day 7 to 50	Day >50
IgG	S	-23.2 [0.5, -52.6]	**-9.4 [-5.5, -13.5]**	**-52.9 [-23.3, -89.7]**	**-20.9 [-15.5, -26.6]**	-24.1 [0.7, -55.1]	**-10.5 [-5.2, -16]**
	S1	-28.1 [0, -64]	**-12.8 [-8.1, -17.8]**	**-87.9 [-46.5, -141.1]**	**-23.1 [-16.7, -29.9]**	**-46.7 [-13.5, -89.7]**	**-9.1 [-3, -15.5]**
	S2	-10.4 [12.7, -39.7]	0.4 [4.3, -3.8]	-17.2 [7.4, -48.4]	**-7.2 [-1.9, -12.8]**	-6.1 [16.8, -35.4]	**-7.6 [-1.9, -13.5]**
	RBD	-20 [9.2, -58.5]	**-13.8 [-8.5, -19.4]**	**-106.4 [-56.1, -172.9]**	**-22.5 [-15.3, -30]**	**-72 [-28.6, -130.1]**	**-7.6 [-1, -14.7]**
	N	1.7 [28.8, -35.8]	1.8 [7.1, -3.8]	**41.4 [58.1, 18]**	**12.4 [18.5, 5.9]**	**40.4 [57.7, 15.9]**	**10.8 [17.3, 3.9]**
	RBD α	-23.9 [5.7, -62.7]	**-12.1 [-7, -17.5]**	**-117.5 [-65.4, -185.9]**	**-22.1 [-15.2, -29.5]**	**-75.5 [-31.9, -133.5]**	**-8.9 [-2.4, -15.9]**
	RBD δ	-22.7 [7.5, -62.9]	**-12 [-6.6, -17.6]**	**-105.2 [-54.5, -172.5]**	**-22.5 [-15.3, -30.2]**	**-67.2 [-24.4, -124.6]**	**-9.4 [-2.5, -16.7]**
	RBD o BA.1	-2.8 [22.4, -36.2]	3.5 [8.1, -1.3]	**-90.6 [-43.2, -153.6]**	-3.7 [2.5, -10.2]	**-85.4 [-37.7, -149.6]**	**-7.4 [-0.7, -14.6]**
	RBD o BA.2	-11.2 [14.5, -44.6]	0 [4.4, -4.7]	**-96.4 [-50.5, -156.4]**	**-7.6 [-1.6, -13.9]**	**-76.7 [-33.9, -133.1]**	**-7.6 [-1.3, -14.3]**
	RBD o BA.4/5	-6.1 [22.5, -45.4]	3 [8.1, -2.4]	**-77.1 [-28.8, -143.4]**	-6.3 [0.7, -13.8]	**-66.8 [-19.7, -132.6]**	**-9.6 [-2, -17.8]**
	RBD o BQ.1.1	-11.3 [18.7, -52.3]	2.6 [7.7, -2.8]	**-73.5 [-26.4, -138.2]**	**-8.8 [-1.7, -16.4]**	**-56 [-12, -117.2]**	**-11.7 [-4, -20]**
	RBD o XBB	3.6 [27.4, -27.9]	**5.7 [10.1, 1]**	**-67.7 [-26.1, -123.2]**	-2.7 [3.4, -9.1]	**-74.1 [-29, -134.9]**	**-8.8 [-2, -16.1]**
IgA	S	**-74.3 [-35.6, -124]**	2.1 [6.2, -2.2]	**-200.2 [-134.1, -285.1]**	-0.6 [4.6, -6.1]	**-72.3 [-32.5, -124]**	-2.7 [2.9, -8.7]
	S1	**-131.3 [-78.4, -200]**	2 [6.2, -2.5]	**-398 [-284.5, -544.9]**	2.3 [7.5, -3.2]	**-115.3 [-64, -182.6]**	0.3 [6, -5.7]
	S2	-5.9 [16.9, -35]	4 [7.9, 0]	**-48.9 [-17, -89.4]**	4.9 [9.7, -0.1]	**-40.5 [-9, -81.3]**	0.9 [6.2, -4.6]
	RBD	**-103.7 [-55.6, -166.5]**	2 [6.4, -2.7]	**-353.2 [-245.5, -494.6]**	**6.4 [11.7, 0.7]**	**-122.5 [-68.2, -194.4]**	4.5 [10.2, -1.6]
	N	0.3 [20.7, -25.3]	0.5 [4.4, -3.5]	**55.6 [65, 43.7]**	**6.2 [10.8, 1.3]**	**55.4 [65.1, 43.1]**	**5.7 [10.6, 0.5]**
	RBD α	**-107.7 [-58.9, -171.3]**	2.8 [7.2, -1.8]	**-348.8 [-242.6, -488.1]**	**6.2 [11.5, 0.6]**	**-116.1 [-63.6, -185.6]**	3.5 [9.3, -2.7]
	RBD δ	**-93.5 [-46.8, -155.1]**	2.3 [6.8, -2.4]	**-330.2 [-226.4, -467.1]**	**6.7 [12, 1]**	**-122.3 [-66.4, -197]**	4.4 [10.3, -1.8]
	RBD o BA.1	**-27.8 [-8.6, -50.4]**	2.4 [5.1, -0.4]	**-89.5 [-60.9, -123.3]**	**11.7 [14.8, 8.6]**	**-48.3 [-24.7, -76.5]**	**9.5 [12.9, 6.1]**
	RBD o BA.2	**-37.7 [-15.4, -64.4]**	2.5 [5.4, -0.6]	**-130.8 [-93.3, -175.6]**	**12.6 [15.8, 9.2]**	**-67.6 [-39, -102.2]**	**10.4 [13.9, 6.7]**
	RBD o BA.4/5	**-31.1 [-9.3, -57.4]**	2.8 [5.8, -0.4]	**-93.2 [-60.7, -132.3]**	**12.9 [16.3, 9.4]**	**-47.3 [-21.3, -79]**	**10.5 [14.1, 6.6]**
	RBD o BQ.1.1	**-30.1 [-9.4, -54.6]**	2.9 [5.8, 0]	**-89.5 [-59.1, -125.7]**	**12.4 [15.7, 9.1]**	**-45.7 [-21.1, -75.3]**	**9.8 [13.3, 6.1]**
	RBD o XBB	**-23.5 [-7.2, -42.2]**	2.3 [4.7, -0.1]	**-60.3 [-38.9, -84.9]**	**9 [11.8, 6.2]**	**-29.8 [-11.7, -50.8]**	**6.9 [9.9, 3.8]**
IgM	S	**-104.6 [-68.6, -148.4]**	-3.2 [0.2, -6.7]	**-127.1 [-85.8, -177.4]**	**-5.6 [-1.2, -10.2]**	-10.9 [9.5, -36]	-2.3 [2.2, -7]
	S1	**-78.9 [-42.6, -124.4]**	**-4.2 [-0.2, -8.4]**	**-115.9 [-70.9, -172.6]**	-2.9 [2.1, -8.1]	-20.7 [4.9, -53.1]	1.3 [6.3, -4.1]
	S2	-12.5 [3.1, -30.5]	-0.2 [2.4, -2.8]	**-22.5 [-5.3, -42.6]**	**3.5 [6.6, 0.3]**	-8.4 [7.4, -26.8]	**3.4 [6.7, 0]**
	RBD	**-57.7 [-31.3, -89.5]**	-1.1 [2.1, -4.4]	**-70.8 [-41.4, -106.3]**	-2.1 [2, -6.3]	-8.3 [10.7, -31.3]	-0.9 [3.3, -5.3]

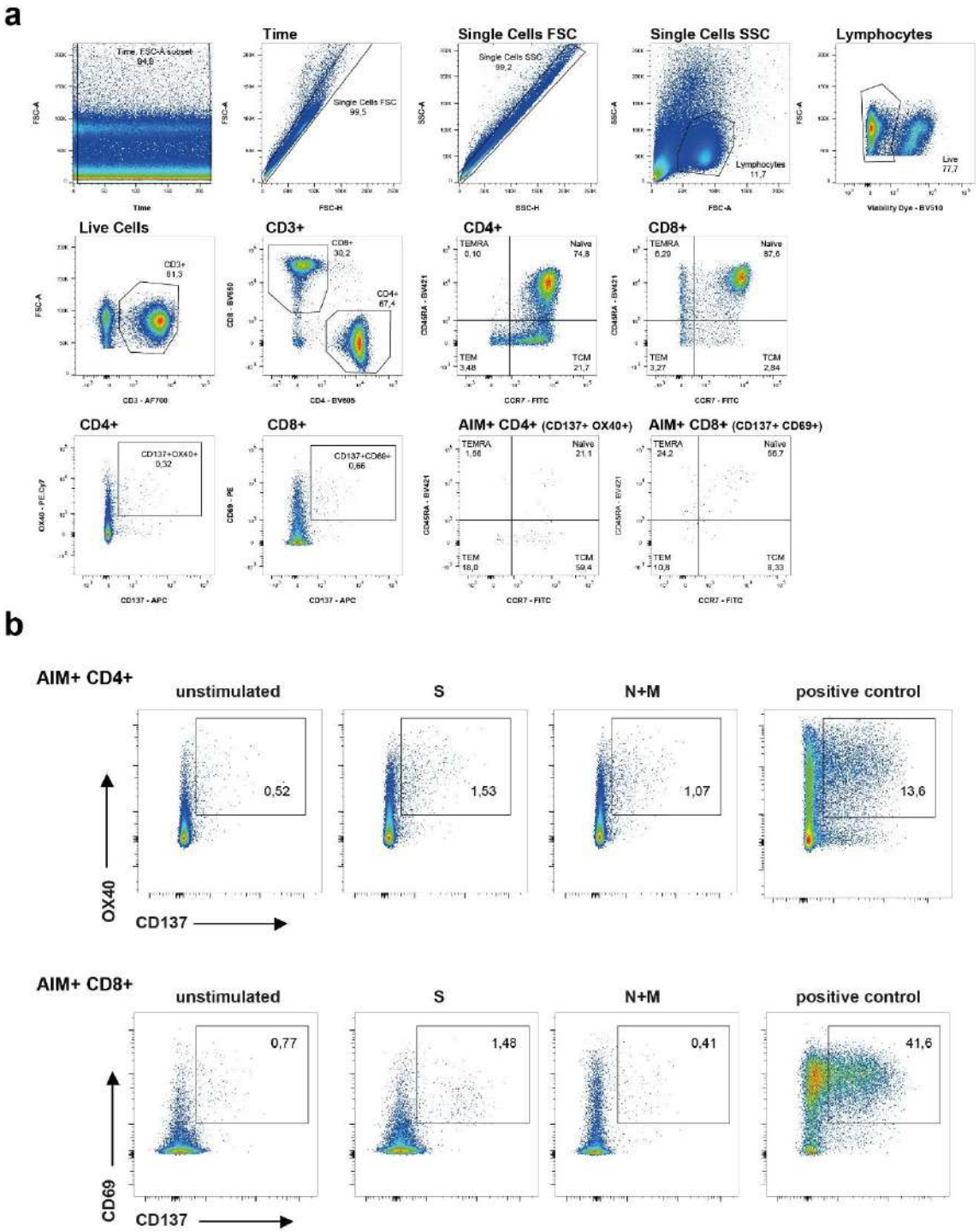
N	-17.1 [3.3, -41.9]	0.2 [3.5, -3.1]	3.1 [20.5, -18.1]	**4.8 [8.7, 0.7]**	17.3 [32.5, -1.4]	**4.6 [8.7, 0.2]**
RBD α	**-60.2 [-34.9, -90.3]**	0.1 [3, -2.9]	**-66.3 [-39.6, -98.1]**	1.8 [5.5, -1.9]	-3.8 [13.3, -24.2]	1.7 [5.6, -2.2]
RBD δ	**-52 [-24.5, -85.7]**	0.5 [3.9, -3]	**-69.7 [-38.4, -108]**	1.3 [5.5, -3.1]	-11.6 [9.4, -37.6]	0.8 [5.3, -3.9]
RBD o BA.1	-9.6 [5.4, -27]	0.7 [3.2, -1.9]	-13.5 [2.5, -32.2]	1.1 [4.3, -2.2]	-3.6 [11.4, -21]	0.4 [3.8, -3]
RBD o BA.2	**-19.3 [-5.6, -34.8]**	-0.1 [2, -2.2]	**-15.5 [-2, -30.8]**	0 [2.6, -2.7]	3.2 [14.8, -10]	0.1 [2.9, -2.7]
RBD o BA.4/5	-10.6 [6.4, -30.8]	1 [3.8, -1.9]	-11.9 [5.9, -33]	1.3 [4.9, -2.4]	-1.1 [15.2, -20.7]	0.3 [4.1, -3.6]
RBD o BQ.1.1	-16.8 [1.8, -38.9]	1.7 [4.6, -1.3]	-18.9 [0.3, -41.8]	1.8 [5.4, -2]	-1.8 [15.1, -22]	0.1 [4, -4]
RBD o XBB	-11.7 [5, -31.3]	1.4 [4.1, -1.4]	-14 [3.5, -34.8]	1.8 [5.3, -1.7]	-2.1 [13.9, -21.1]	0.5 [4.2, -3.3]

Differences expressed in % monthly decay [CI] at 7-50 and > 50 days after immunization. Statistically significant differences are in bold

Supplementary Table 13. *T cellular responders six months after booster vaccination (3rd dose)*

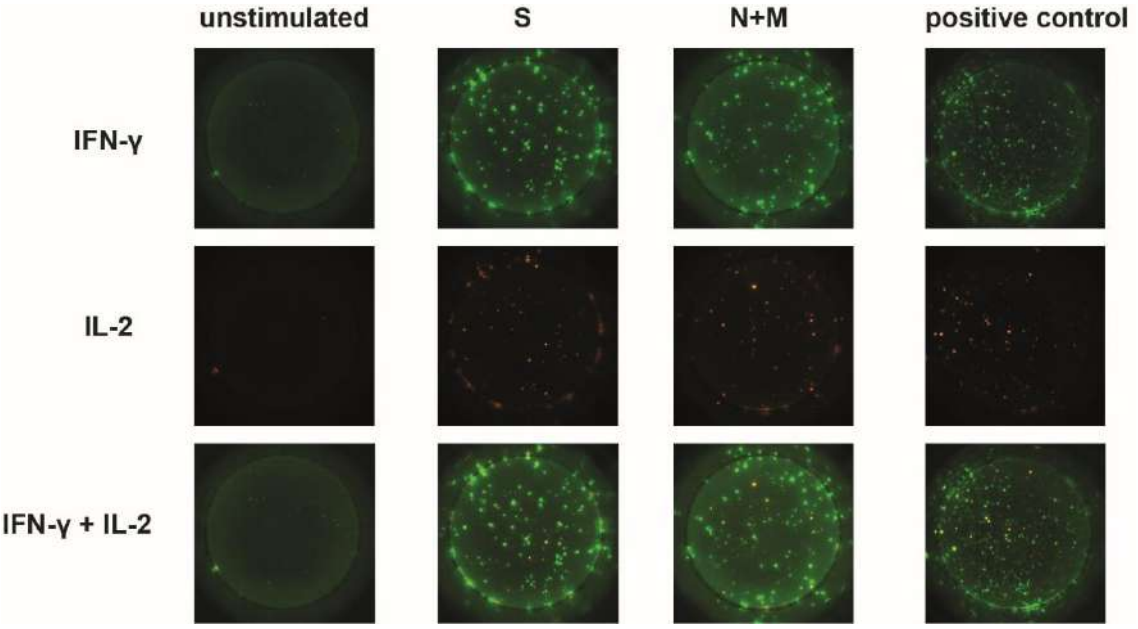
	TOTAL	Hybrid immunity	Vaccinated
AIM	**N = 25**	**N = 14**	**N = 11**
Total responders	12 (48%)	8 (57.14%)	4 (36.36%)
S	12 (48%)	8 (57.14%)	4 (36.36%)
AIM+CD4+	12 (48%)	8 (57.14%)	4 (36.36%)
AIM+CD8+	5 (20%)	3 (21.43%)	2 (18.18%)
N+M	2 (8%)	2 (14.29%)	0 (0%)
AIM+CD4+	2 (8%)	2 (14.29%)	0 (0%)
AIM+CD8+	0 (0%)	0 (0%)	0 (0%)
FluoroSpot	**N = 20**	**N = 13**	**N = 7***
Total responders	20 (100%)	13 (100%)	7 (100%)
S	20 (100%)	13 (100%)	7 (100%)
IFN-γ	9 (50%)*	7 (53.85%)	2 (40%)*
IL-2	20 (100%)	13 (100%)	7 (100%)
IFN-γ+IL-2	18 (90%)	13 (100%)	5 (71.43%)
N+M	17 (85%)	13 (100%)	4 (57.14%)
IFN-γ	4 (22.22%)*	3 (20.08%)	1 (20%)*
IL-2	14 (70%)	11 (84.61%)	3 (42.86%)
IFN-γ+IL-2	15 (75%)	11 (84.61%)	4 (57.14%)

**Two participants were excluded from the analysis because they had >100 SFUs in the unstimulated condition*

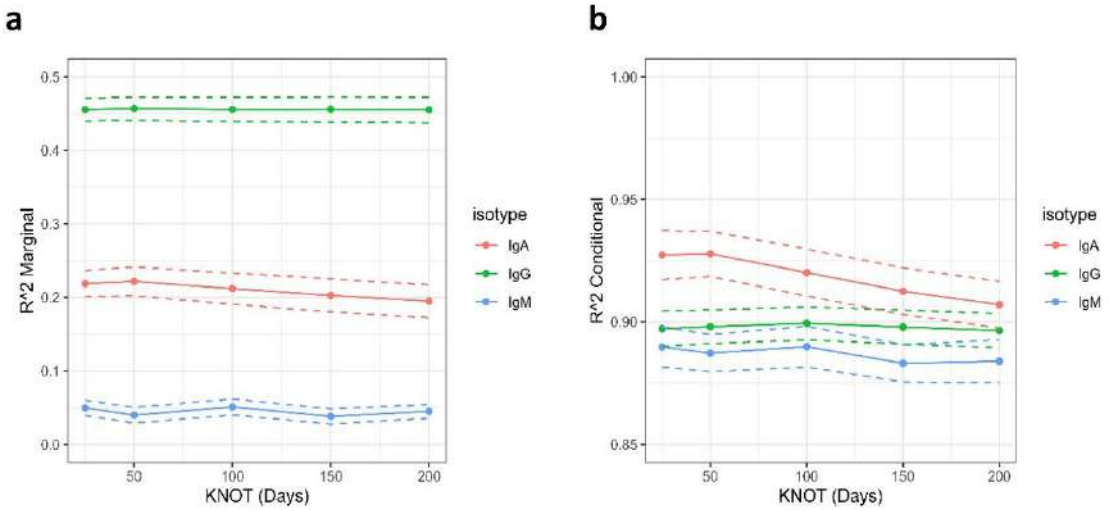


Supplementary Fig. 1. Gating strategy for flow cytometry analysis. (a) Figure depicting the gating strategy applied in AIM assay. Gates were previously defined using fluorescence minus one (FMO) controls. The time vs SSC-A gate was used to exclude any irregularities at the beginning and end of acquisition. SSC-H and SSC-A as well as FSC-H and FSC-A were used to discriminate singlets and FSC-A vs SSC-A to select lymphocytes. Monocytes, B cells, and dead cells were excluded by selecting the $CD14^-$, $CD19^-$, and $eFluor506^-$ cells, respectively. Within live cells, $CD3^+$ cells were identified, with further identification of $CD4^+$ and $CD8^+$ T cells. Activated $CD4^+$ and $CD8^+$ were defined by $CD137^+$ $OX40^+$ and $CD137^+$ $CD69^+$ activation-induced markers (AIM+), respectively. Within AIM+ $CD4^+$ and AIM+ $CD8^+$ cells, memory subsets were identified through CCR7 and CD45RA expression: T central memory ($CD45RA^-$ $CCR7^+$), T effector memory ($CD45RA^-$ $CCR7^-$), terminally differentiated cells ($CD45RA^+$ $CCR7^-$) and naïve cells ($CD45RA^+$

CCR7$^+$). **(b)** Representative example of flow cytometry plots. CD4$^+$ and CD8$^+$ T cells expressing activation-induced markers for each condition: unstimulated, S, N+M, and positive control (PHA). Spike (S), nucleocapsid (N); membrane (M).

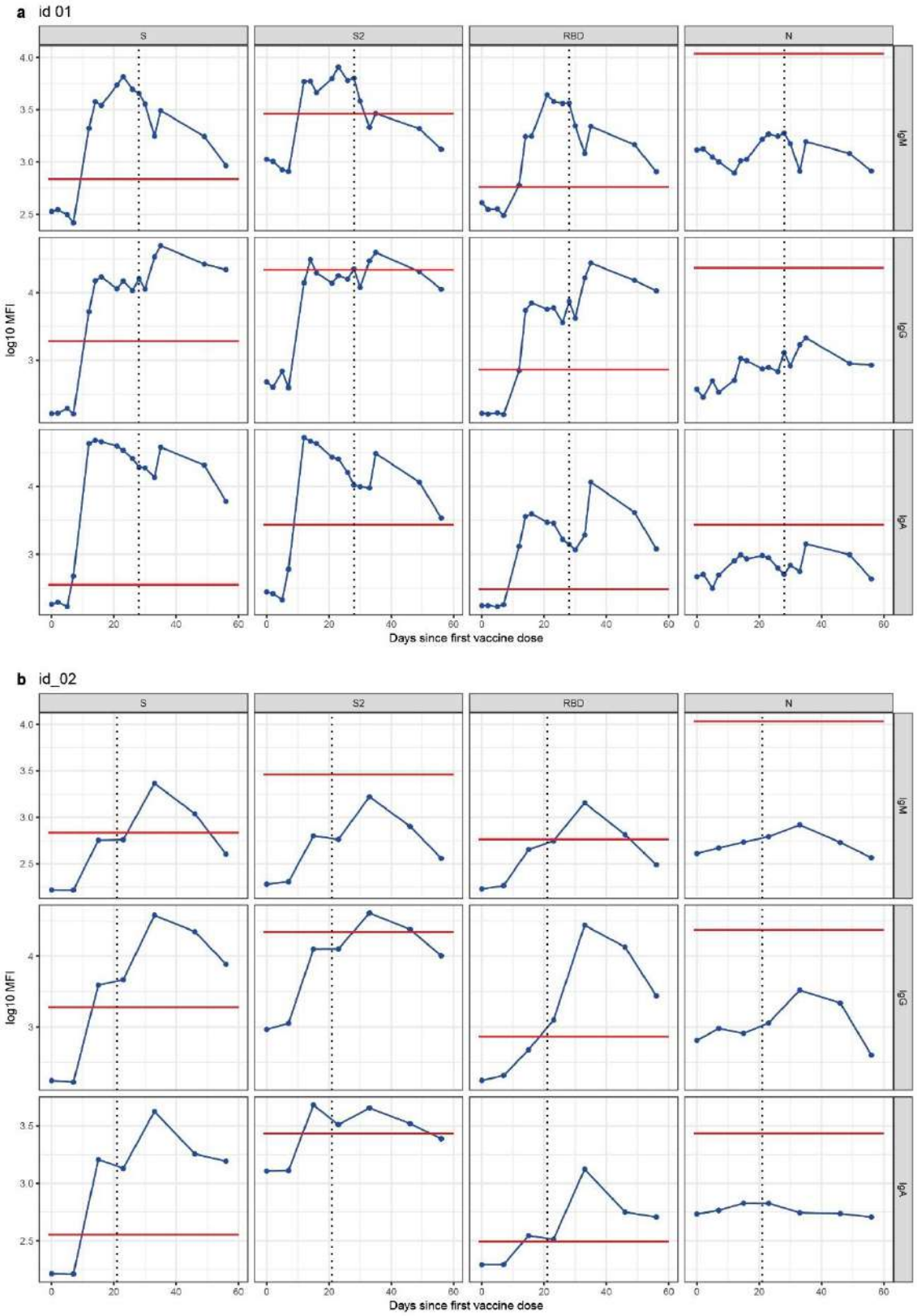


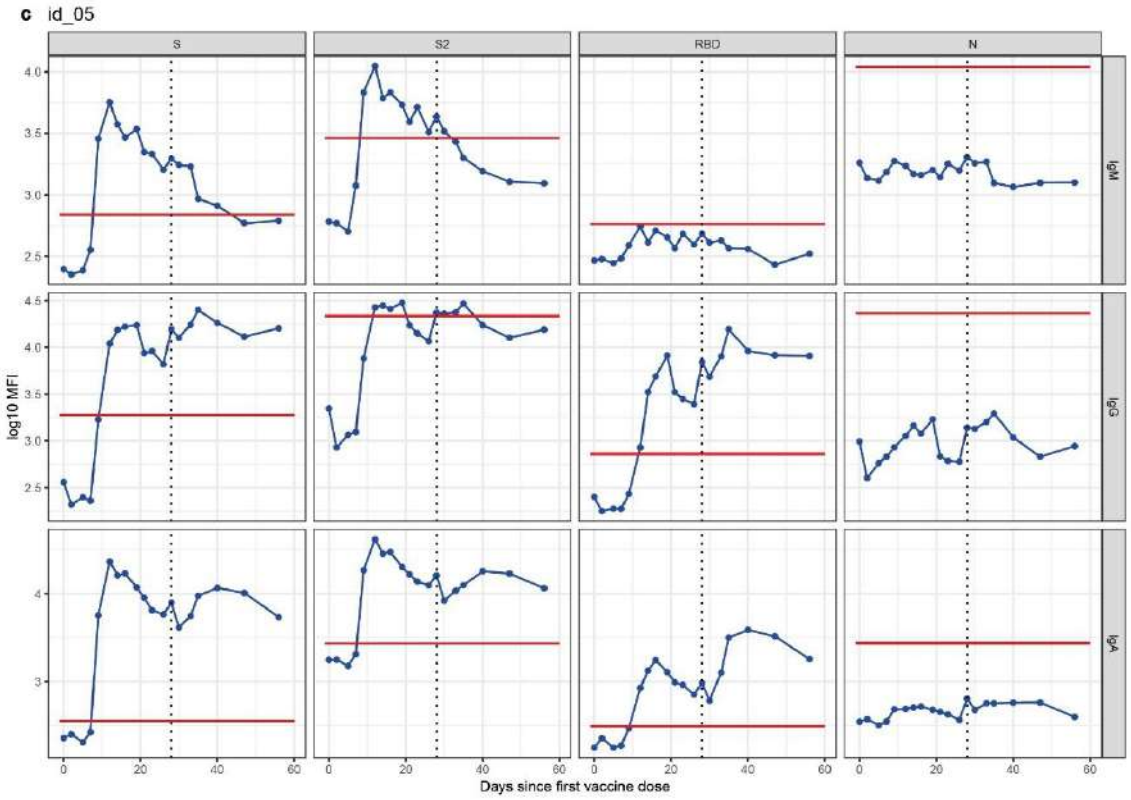
Supplementary Fig. 2. Representative images of wells from IFN-γ/IL-2 FluoroSpot assay. Spots from T cells secreting IFN-γ, IL-2 and both (IFN-γ + IL-2) for each condition: unstimulated, S, N+M and positive control (PHA). Spike (S), nucleocapsid (N); membrane (M).

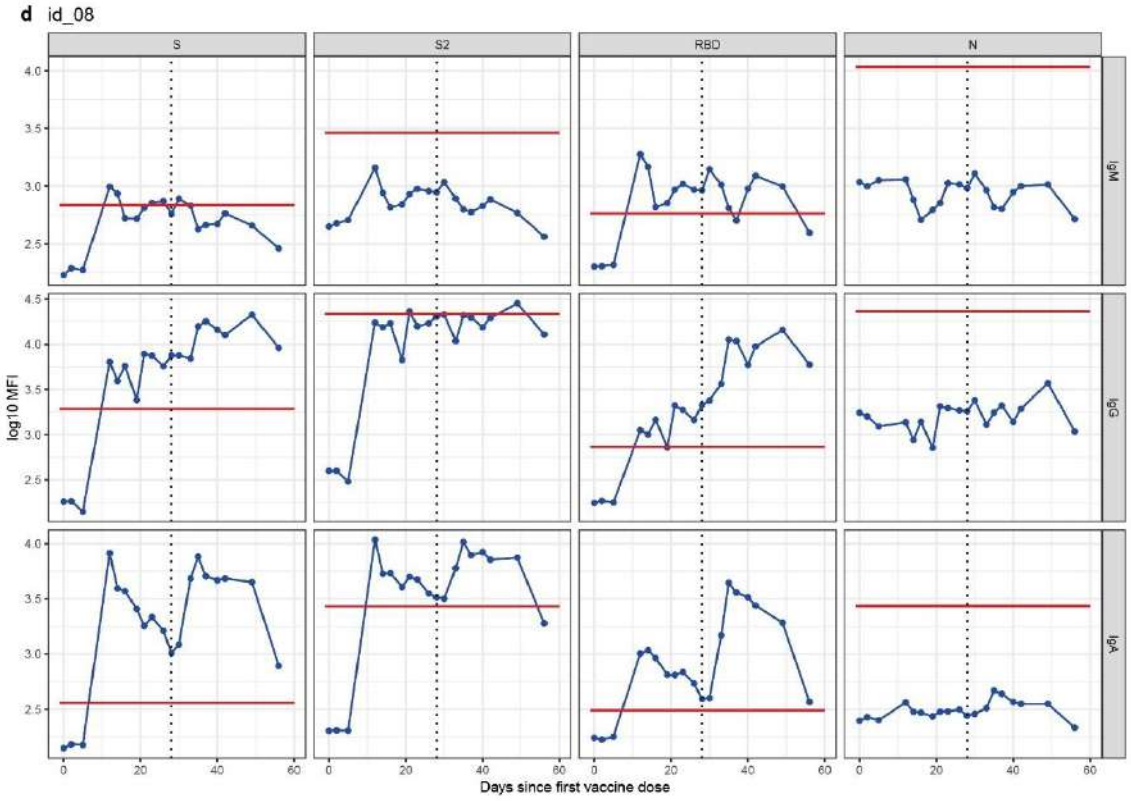


Supplementary Fig. 3. Breakpoint determination in piecewise linear mixed-effects models. Evaluation of variance explained by the models using R^2marginal **(a)** and R^2conditional **(b)** for different knots as days after immunization for each isotype.

Supplementary Fig. 4. SARS-CoV-2 antibody kinetics following the first vaccine dose for representative participants (a-l).




c id_05
S
S2
RBD
N
IgM
IgG
IgA
log10 MFI
Days since first vaccine dose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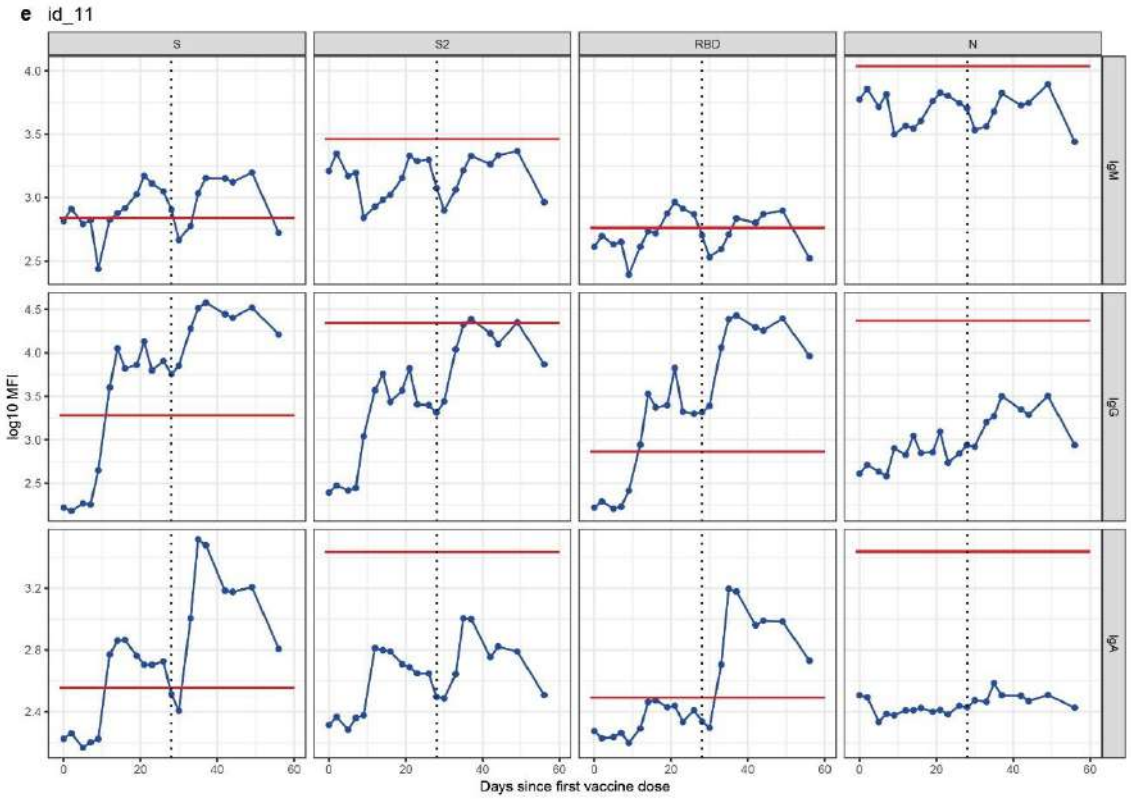
S2
RBD
N
IgM
IgG
IgA
log10 MFI
Days since first vaccine dose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S2
RBD
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IgM
IgG
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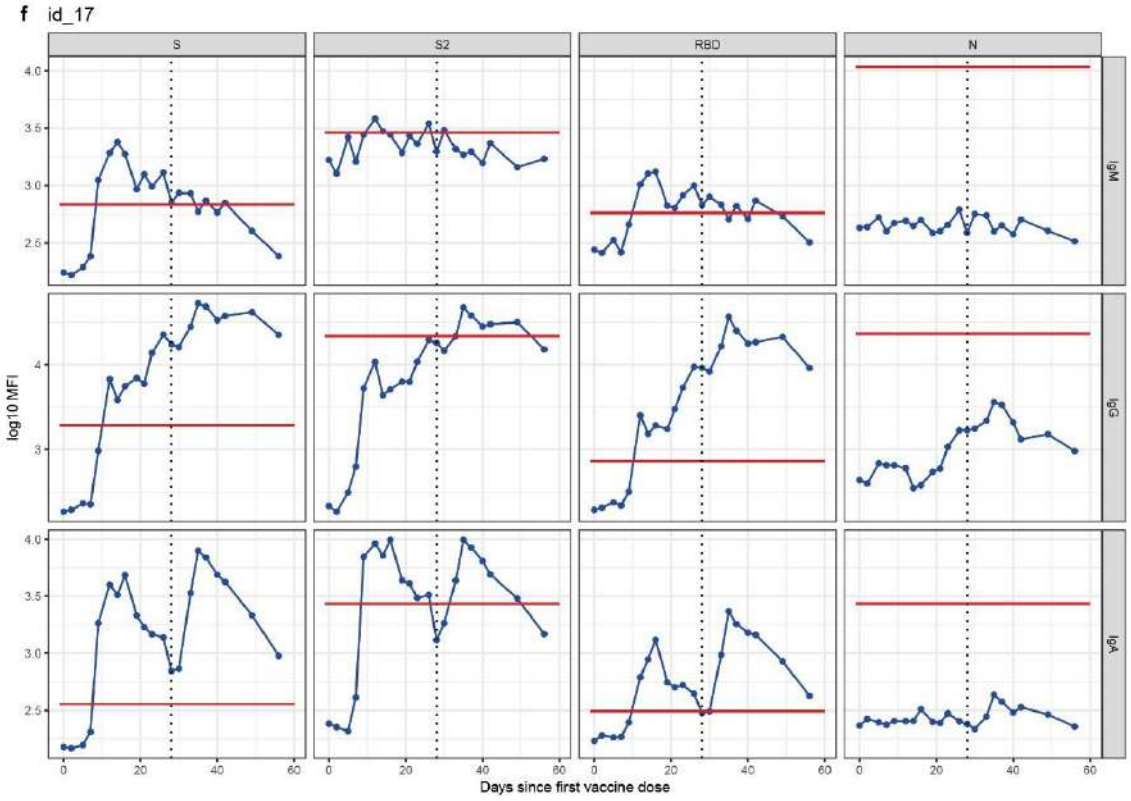
Days since first vaccine dose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S
S2
RBD
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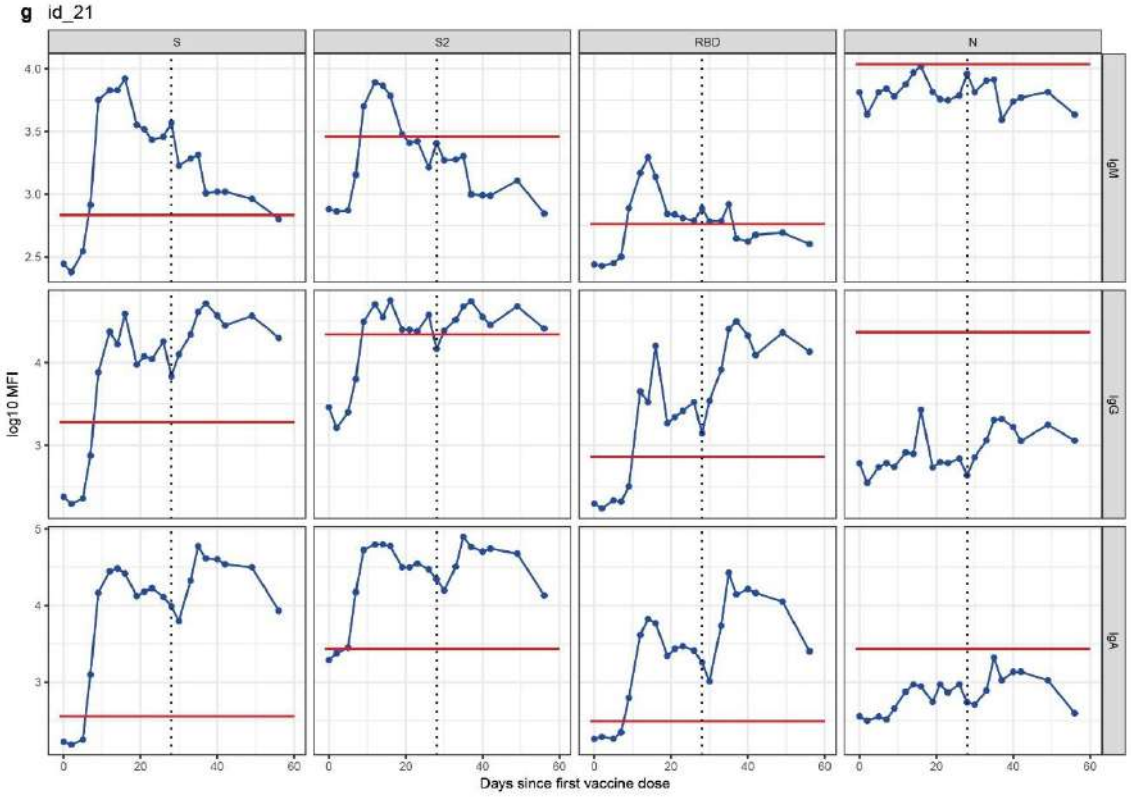
IgM
IgG
IgA
log10 MFI
Days since first vaccine dose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S
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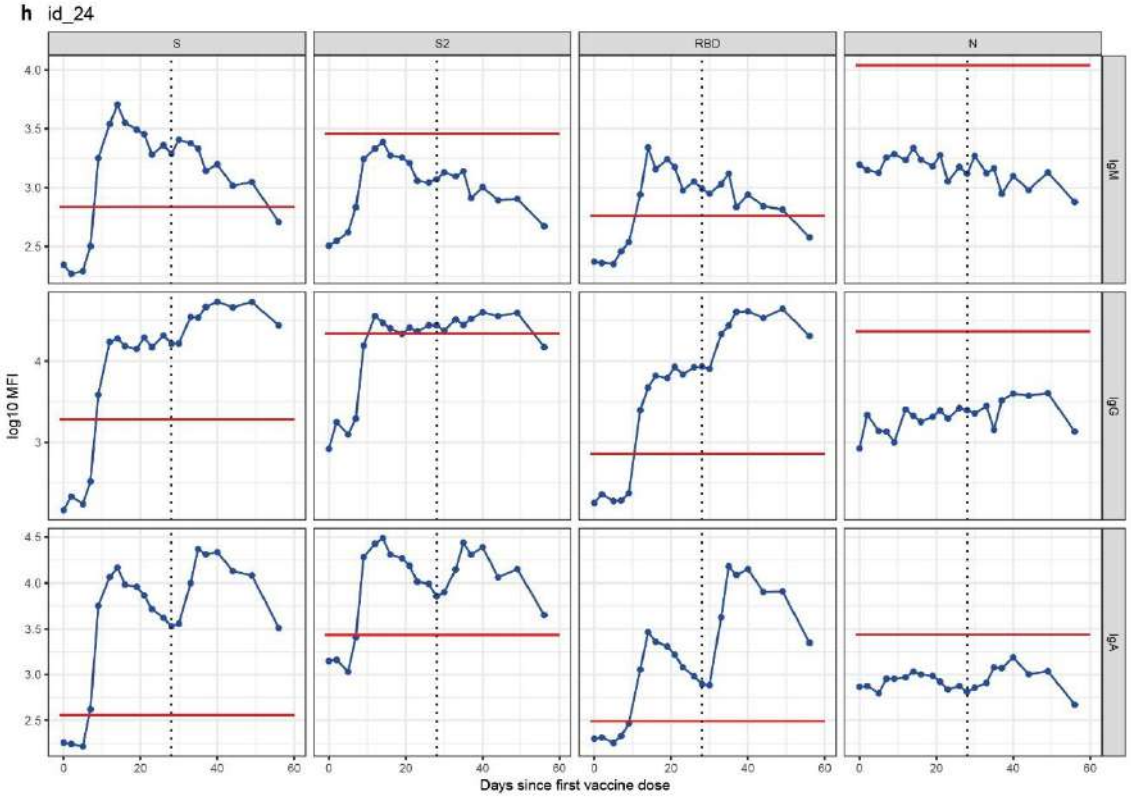
RBD
N
IgM
IgG
IgA
log10 MFI
Days since first vaccine dose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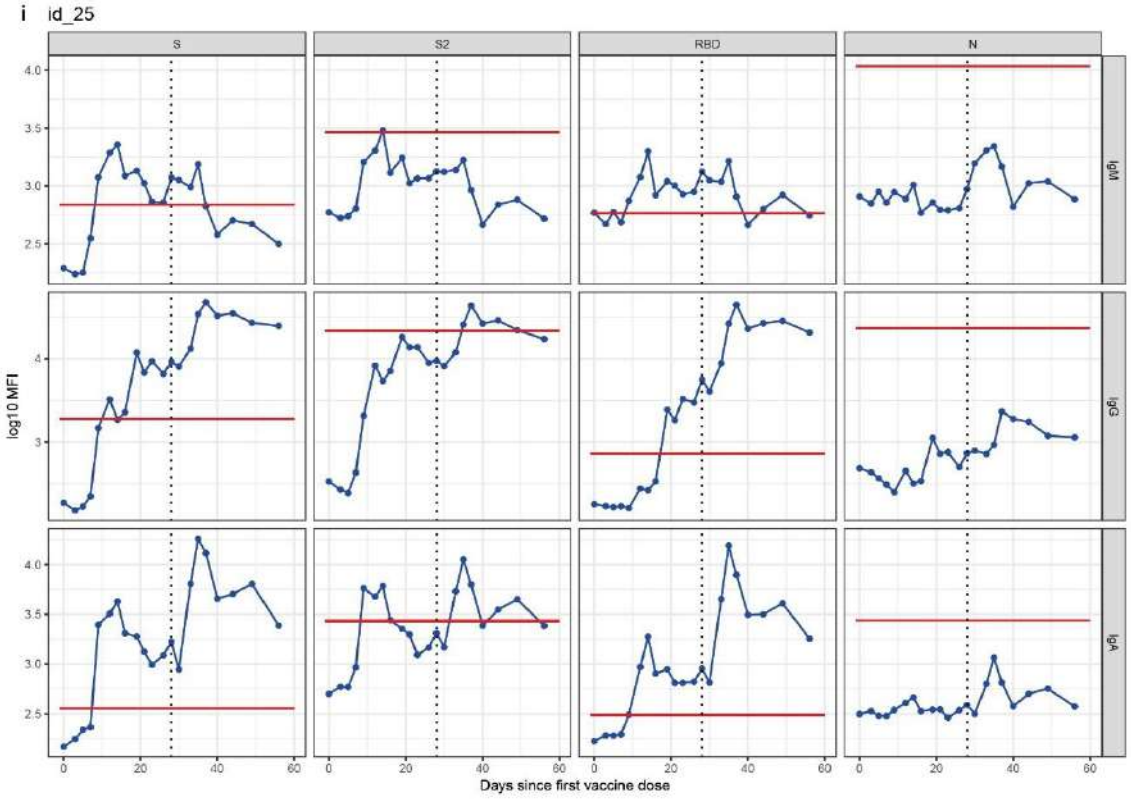
S
S2
RBD
N
IgM
IgG
IgA
log10 MFI
Days since first vaccine dose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S
S2
RBD
N
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IgG
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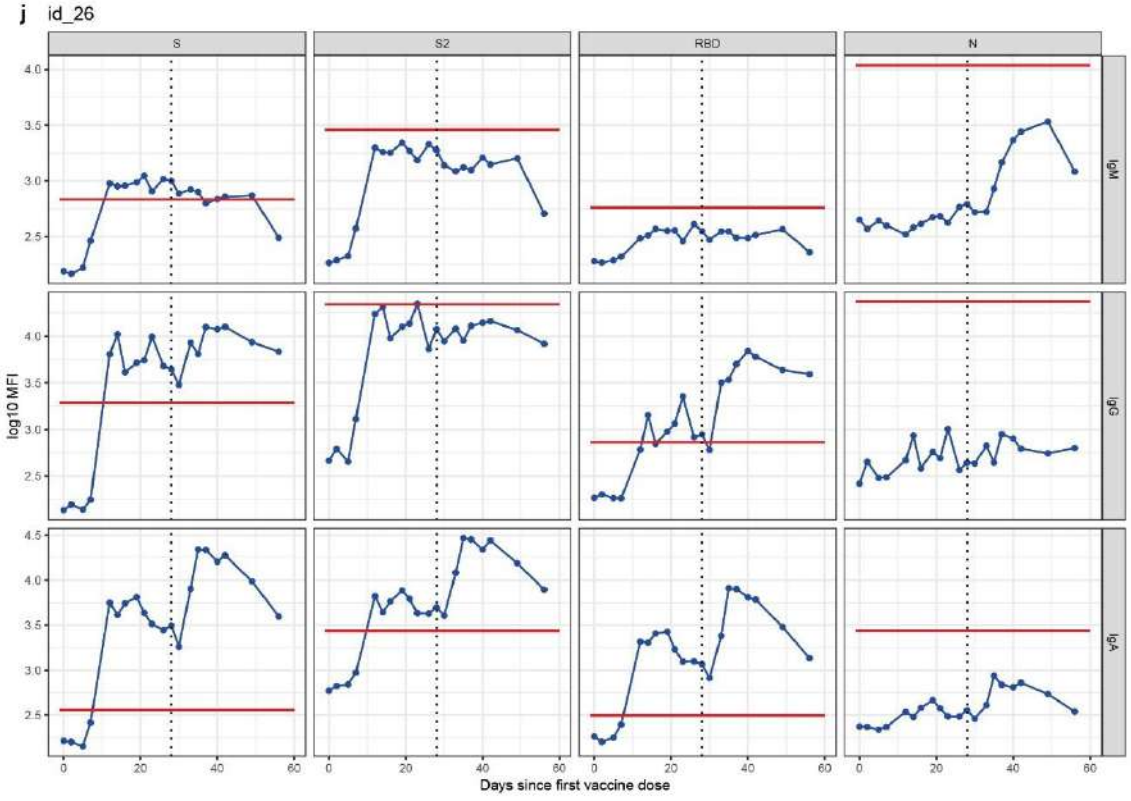
Days since first vaccine dose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S
S2
RBD
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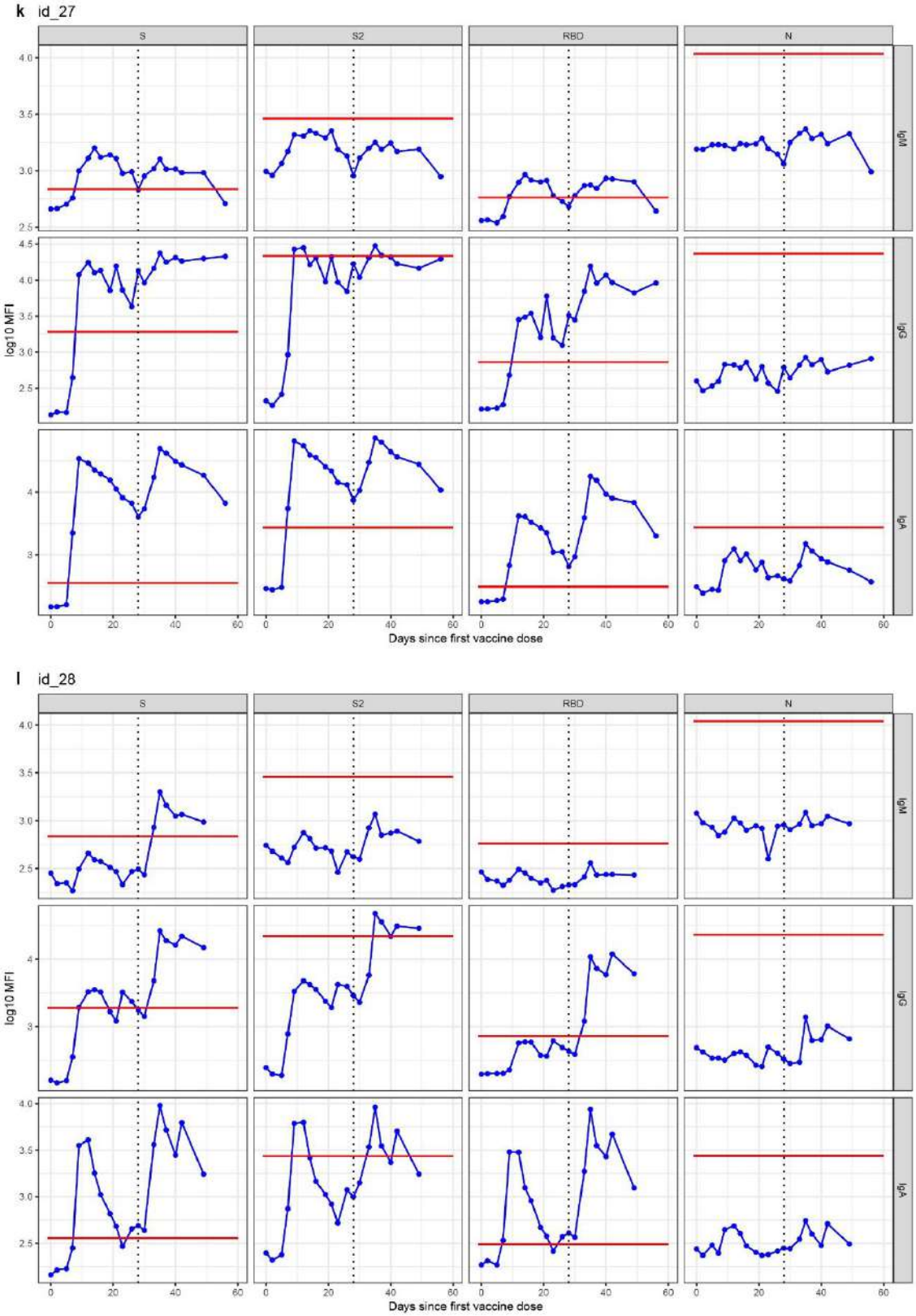
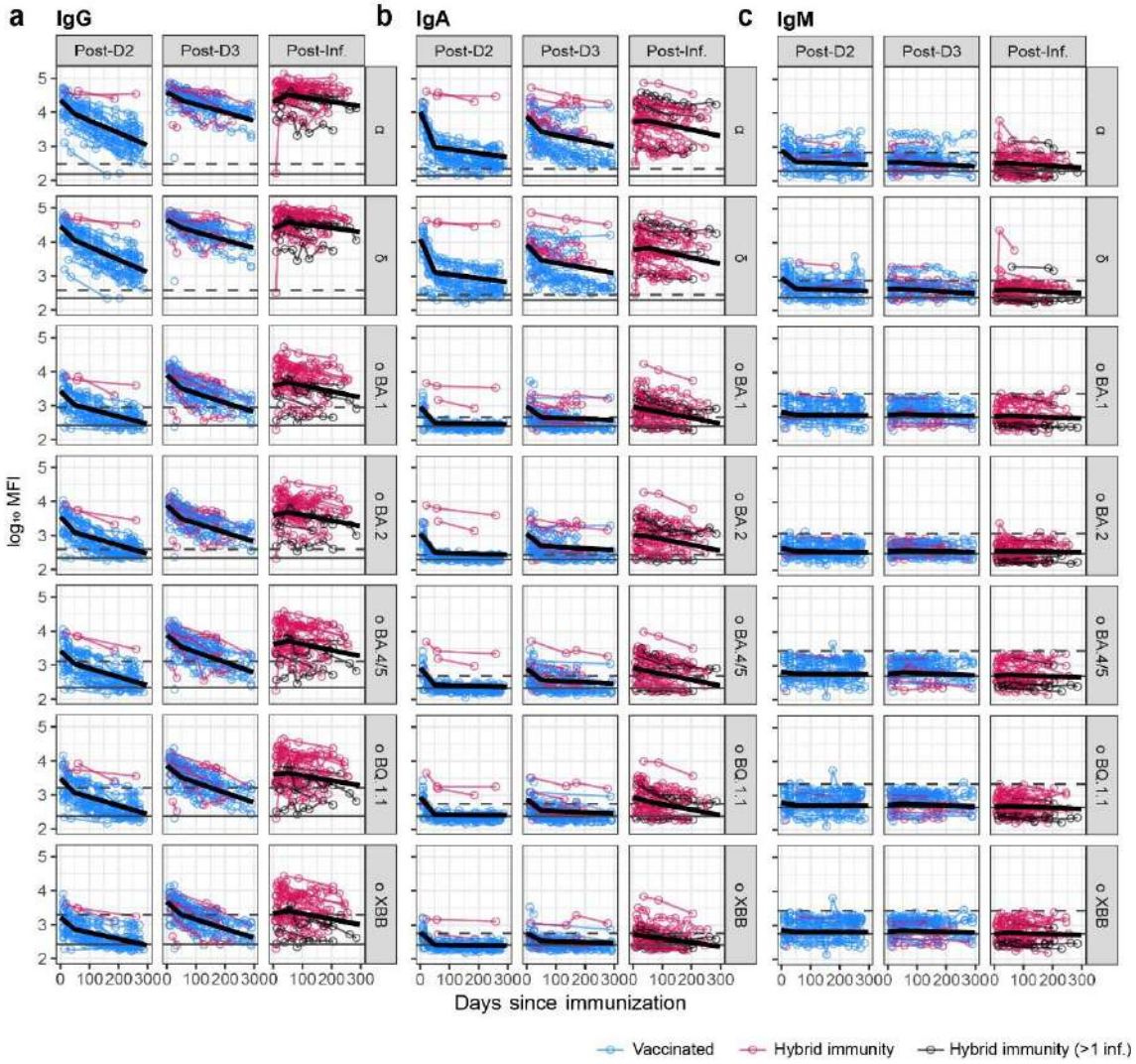
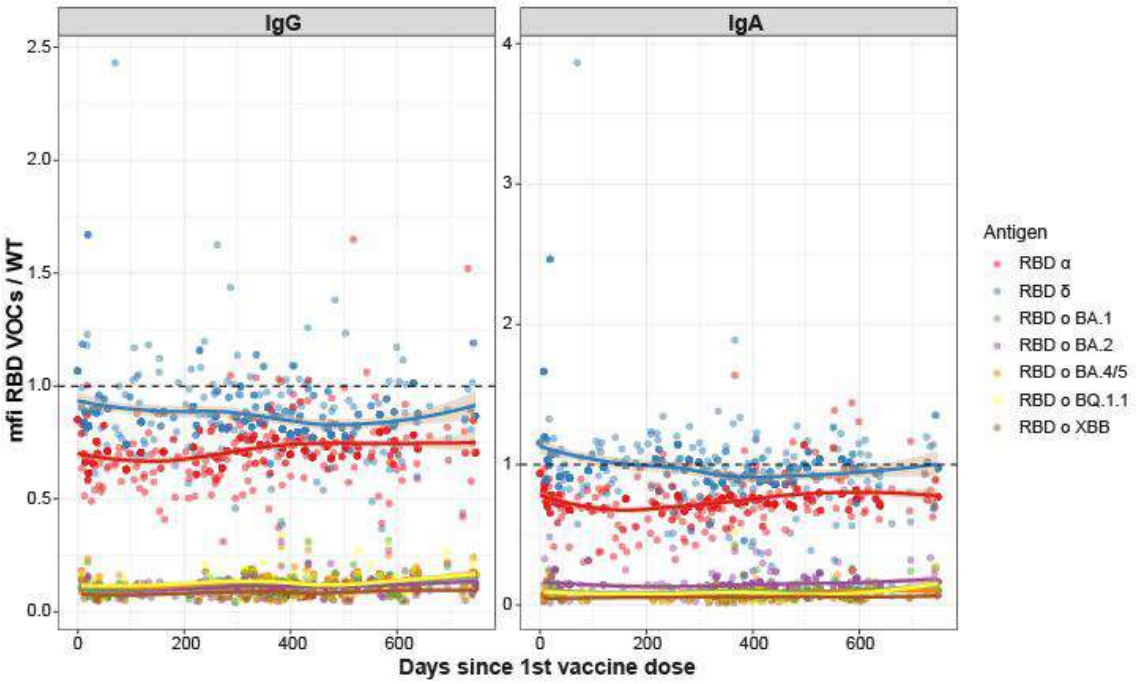
IgM
IgG
IgA
log10 MFI
Days since first vaccine dose



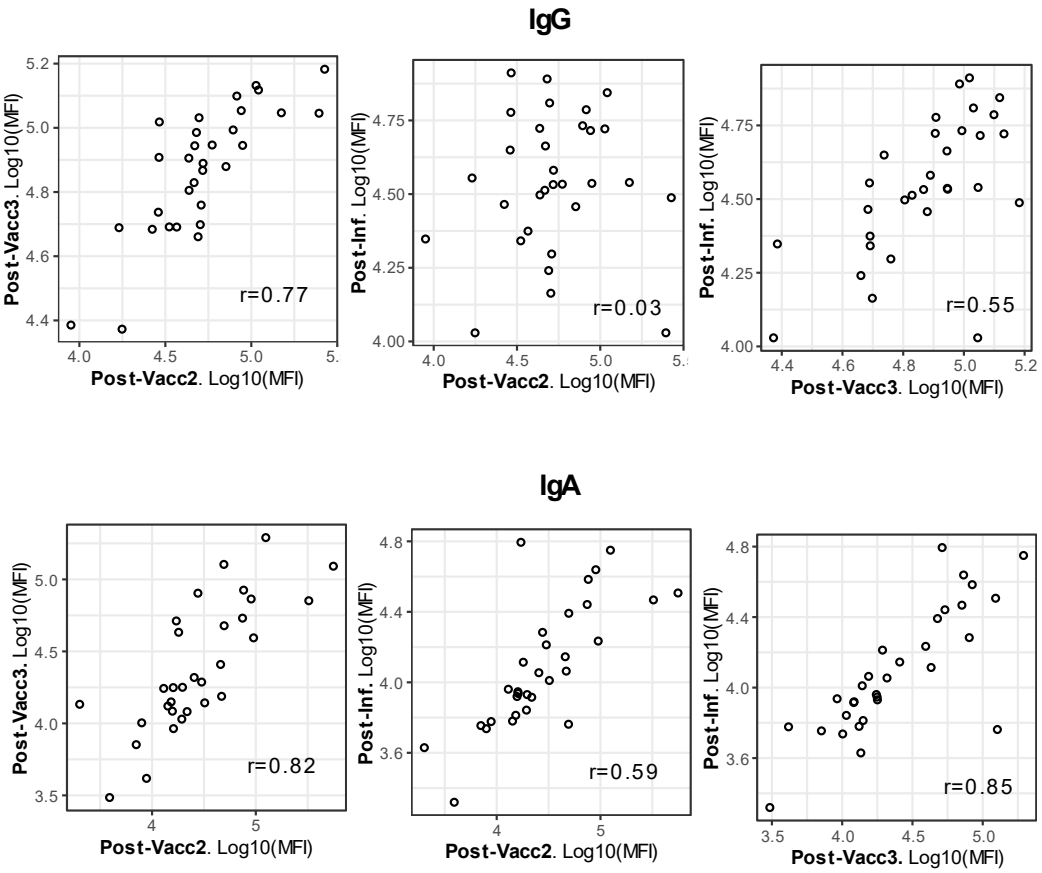
Blue lines connect repeated measures from the same representative participants before and after COVID-19 first vaccine dose. Solid red horizontal lines represent the seropositivity cutoff. Dashed black vertical lines represent 2nd vaccine dose. Nucleocapsid (N), receptor-binding-domain (RBD), spike (S).



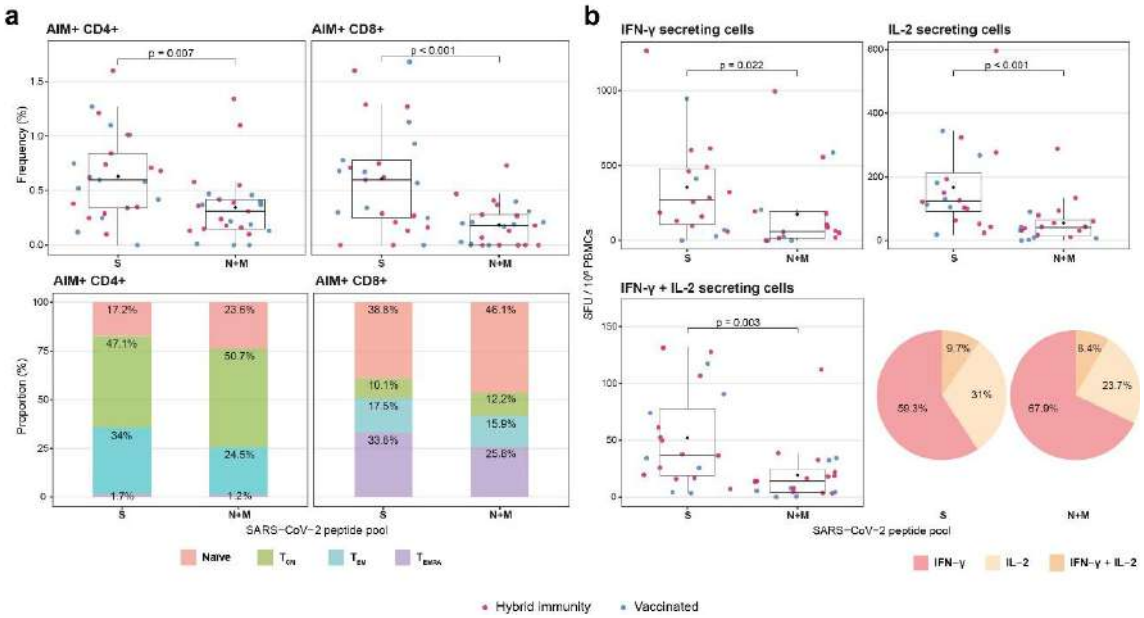
Supplementary Fig. 5. SARS-CoV-2 VoCs antibody kinetics following vaccine doses and infections. Thick black lines correspond to the group geometric mean levels predicted by the piecewise linear mixed model (LMM) fitted for IgG **(a)**, IgA **(b)** and IgM **(c)**, each antigen and immunization interval, with the random intercept set to zero (fix effect prediction). Thin lines connect repeated measures from the same participant in the same immunization interval, red ones corresponding to hybrid immunity (at least one natural infection prior to the vaccine dose). For post-infection data, reinfections are indicated as black trajectories. Dashed black horizontal lines represent the seropositivity cutoff and solid black horizontal lines the geometric mean level for the seronegative population. Nucleocapsid (N), receptor-binding-domain (RBD), spike (S), post-2nd dose (Post-D2), post-3rd dose (Post-D3) and post-infection (Post-Inf.).



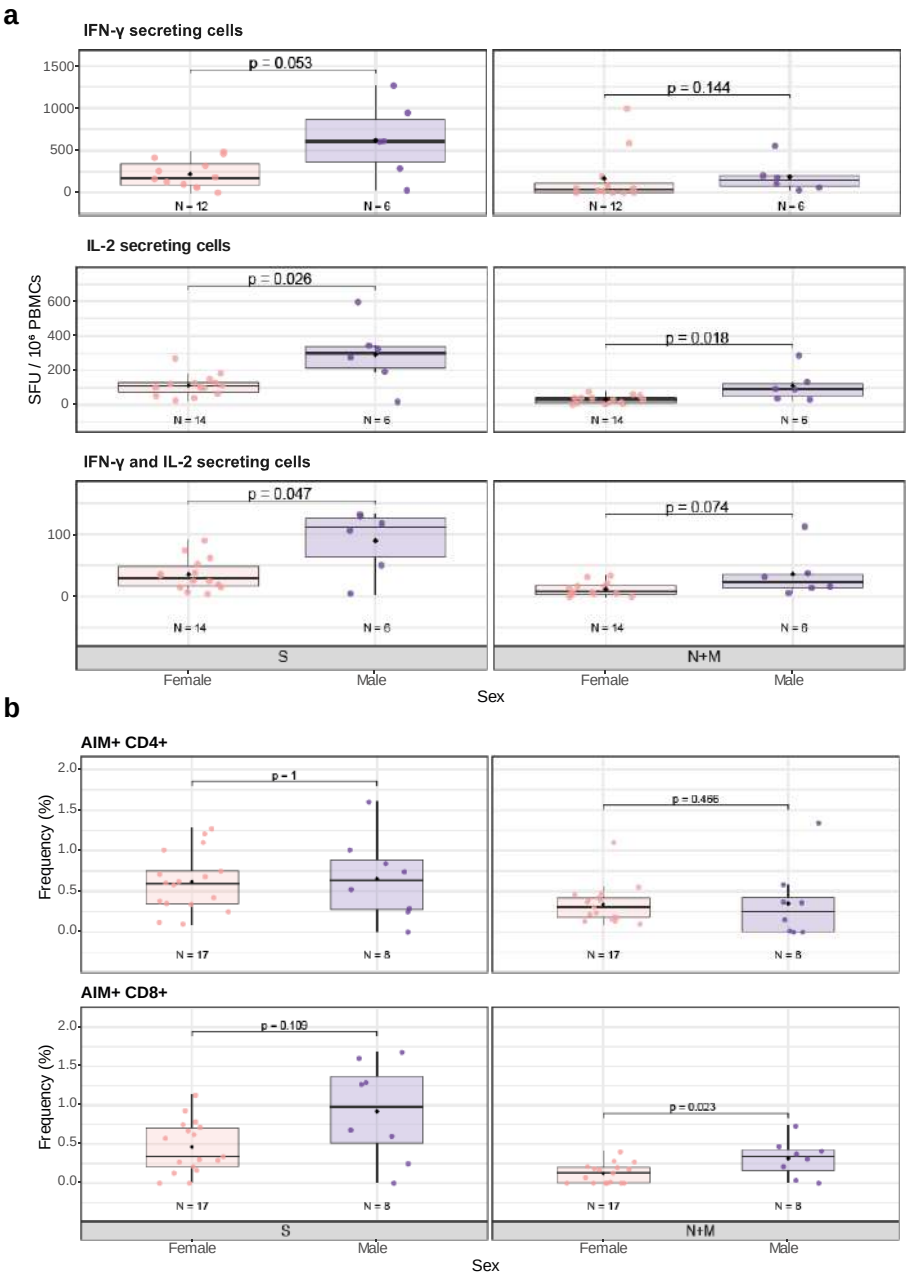
Supplementary Fig. 6. IgG and IgA levels to RBD from SARS-CoV-2 VoCs relative to Wuhan over time. Scatterplot representing the mean of the fold-change of anti-RBD from VoCs vs. anti-RBD from wild-type for each timepoint with smooth curves (loess) and standard error as a confidence interval (shaded areas). Horizontal dashed line corresponds to the same antibody levels for wild type and VoCs.



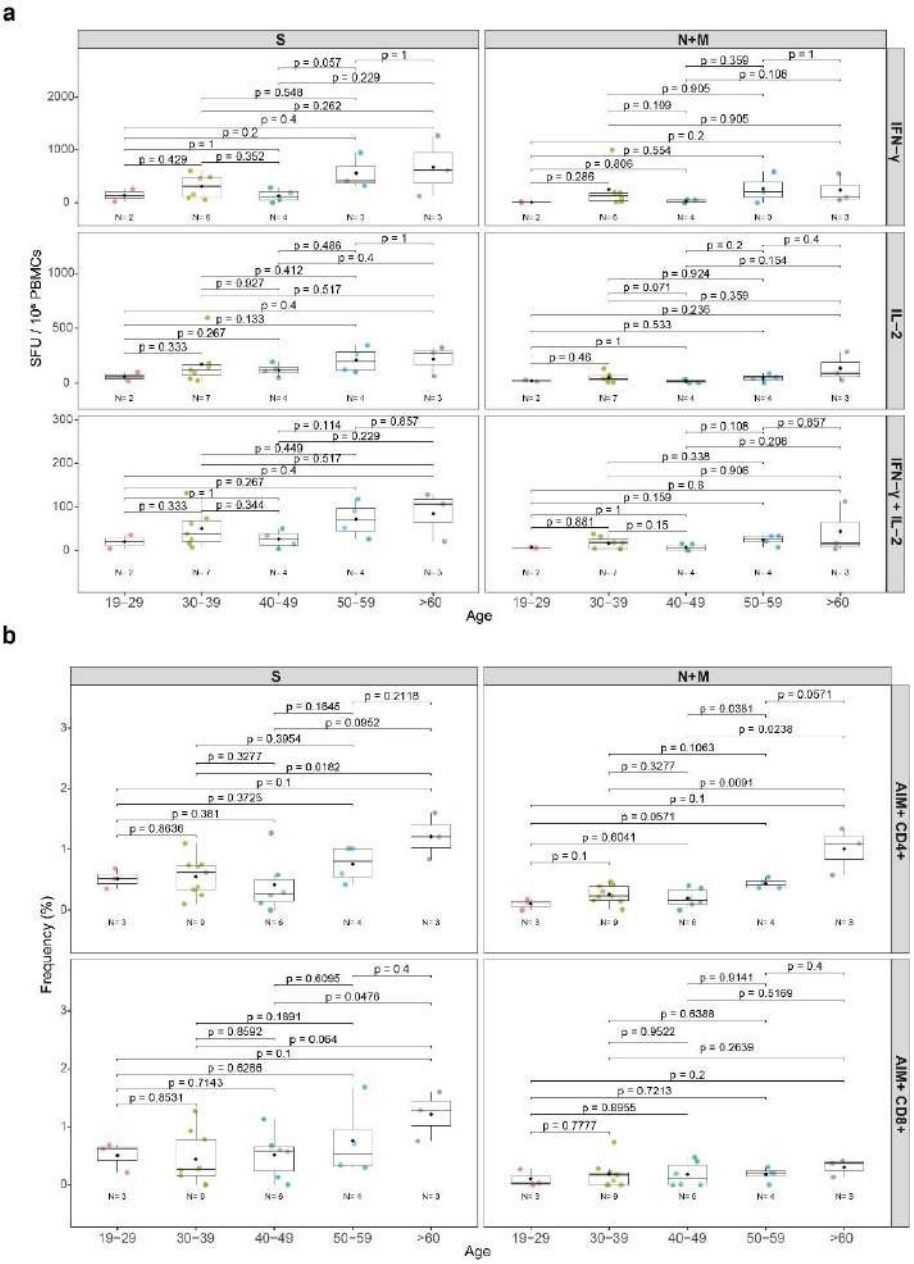
Supplementary Fig. 7. Correlation of estimated peak IgG and IgA levels (day 7) against the S antigen between different immunization intervals. Correlation is derived from the estimated covariance matrix of the random effects intercepts in the linear mixed models.



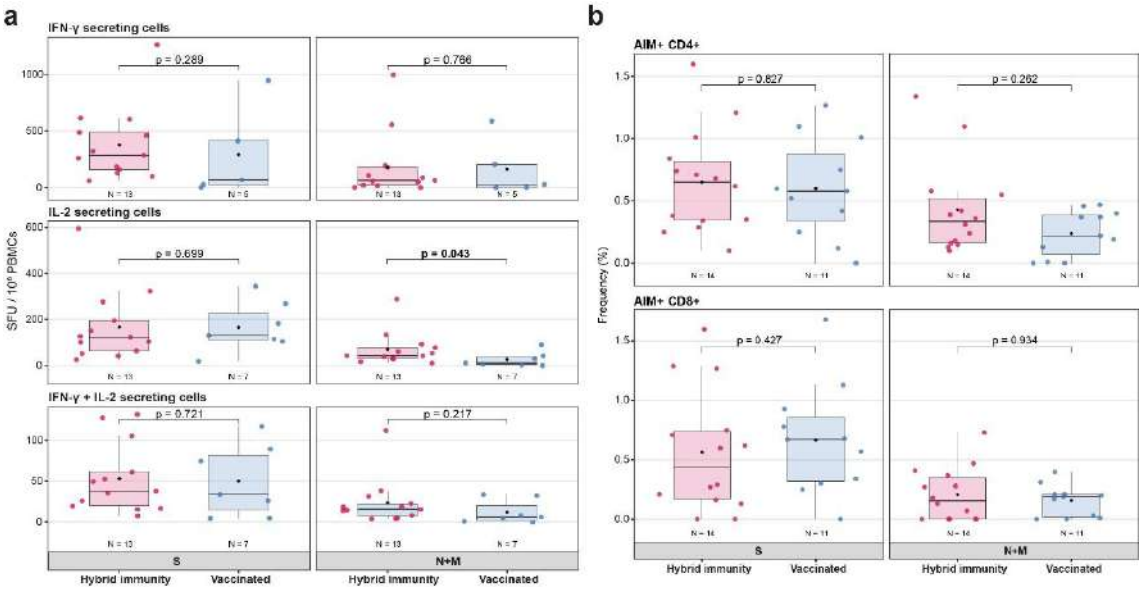
Supplementary Fig. 8. SARS-CoV-2 T cell responses six months after COVID-19 booster vaccination (3rd dose) by peptide pool stimulation. **(a)** SARS-CoV-2 AIM+ $CD4^+$ and AIM+ $CD8^+$ T cell responses. Frequencies (%) of activated $CD4^+$ and $CD8^+$ T and stacked bar chart representing the proportion (%) of AIM+ $CD4^+$ and AIM+ $CD8^+$ T cell memory subsets upon S or N+M stimuli. Naïve (red), T_{CM} (green), T_{EM} (blue), and T_{EMRA} (purple). **(b)** Magnitude of the SARS-CoV-2 Th1 response. Total number of cells, expressed as spots-forming units per million PBMCs (SFU / 10^6 PBMCs) that responded to SARS-CoV-2 S or N+M peptide pools by secreting IFN-γ, IL-2, or both (IFN-γ + IL-2) and pie charts showing the average proportion (%) of secreting T cells by cytokines: IFN-γ (red), IL-2 (beige), or both (orange). Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Groups were compared by the Wilcoxon rank-sum test without adjusting p-values. Activation-induced markers (AIM), interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMCs), spike (S), spots-forming units (SFU), T central memory (T_{CM}), T effector memory (T_{EM}), terminally differentiated effector memory cells re-expressing CD45RA (T_{EMRA}).



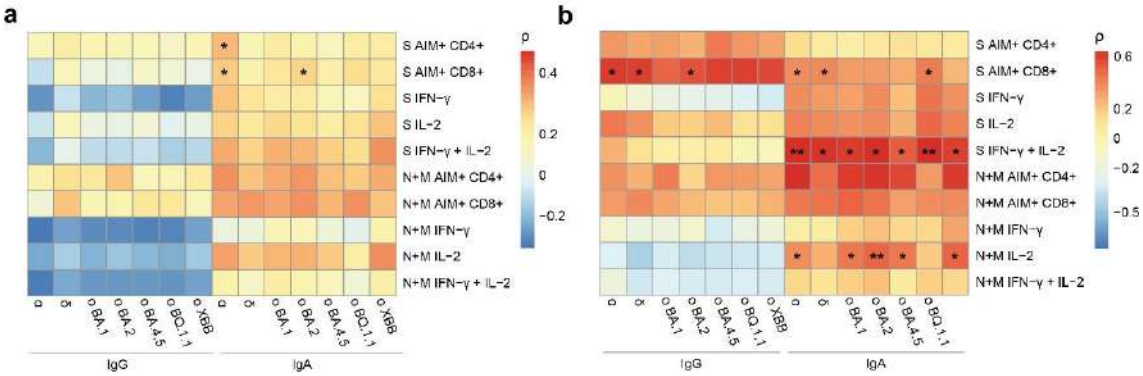
Supplementary Fig. 9. SARS-CoV-2 cellular responses 6 months after COVID-19 booster vaccination (3rd dose) by sex. (a) Magnitude of the SARS-CoV-2 Th1 response. Total number of cells, expressed as spots-forming units per million PBMCs (SFU/10^6 PBMCs) that responded to SARS-CoV-2 S or N+M peptide. **(b)** Frequencies (%) of activated $CD4^+$ and $CD8^+$ T upon S or N+M stimuli. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Sex groups were compared by the Wilcoxon rank-sum test without adjusting p-values. Activation-induced markers (AIM), interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMC), spike (S), spots-forming units (SFU).



Supplementary Fig. 10. SARS-CoV-2 cellular responses 6 months after COVID-19 booster vaccination (3rd dose) by age. (a) Magnitude of the SARS-CoV-2 Th1 response. Total number of cells, expressed as spots-forming units per million PBMCs (SFU/10^6 PBMCs) that responded to SARS-CoV-2 S or N+M peptide. **(b)** Frequencies (%) of activated $CD4^+$ and $CD8^+$ T upon S or N+M stimuli. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Sex groups were compared by the Wilcoxon rank-sum test without adjusting p-values. Activation-induced markers (AIM), interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMC), spike (S), spots-forming units (SFU).



Supplementary Fig. 11. SARS-CoV-2 cellular responses 6 months after COVID-19 booster vaccination (3rd dose) by immunity groups. (a) Magnitude of the SARS-CoV-2 Th1 response. Total number of cells, expressed as spots-forming units per million PBMCs (SFU/10^6 PBMCs) that responded to SARS-CoV-2 S or N+M peptide. **(b)** Frequencies (%) of activated $CD4^+$ and $CD8^+$ T upon S or N+M stimuli. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Hybrid immunity and vaccinated groups were compared by the Wilcoxon rank-sum test without adjusting p-values. Statistically significant differences are indicated as *p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001. Activation-induced markers (AIM), interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMC), spike (S), spots-forming units (SFU).



Supplementary Fig. 12. Correlations between cellular responses and subsequent antibody responses to SARS-CoV-2 VoCs: after one month **(a)** and one month after subsequent immunization **(b)**. Heatmaps illustrate the Spearman's correlation coefficient (ρ) between cellular responses, including frequencies of SARS-CoV-2-specific AIM+$CD4^+$ and AIM+$CD8^+$ T cells, and the magnitude of Th1 response to SARS-CoV-2 (spots-forming units per million PBMCs (SFU/10^6 PBMCs) that secrete IFN-γ, IL-2 and IFN-γ + IL-2), with IgG and IgA (log_{10} median fluorescence intensity, MFI) responses to RBD from VoCs. *p-value ≤ 0.05, **p-value ≤ 0.01 and ***p-value ≤ 0.001. Activation-induced markers (AIM), interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral blood mononuclear cells (PBMC), receptor-binding-domain (RBD).

Chapter 4

Influence of Initial SARS-CoV-2 Exposure via Vaccination or Natural Infection on Antibody and Cellular Responses to Omicron Variants

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Under review

Influence of Initial SARS-CoV-2 Exposure via Vaccination or Natural Infection on Antibody and Cellular Responses to Omicron Variants

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Keywords: SARS-CoV-2, vaccine, antibody, cellular, immune

Word count: 4,844

Abstract (181/200)

The comparison between vaccine-induced and infection-acquired adaptive immunity, and their co-occurrence —referred to as “hybrid immunity”— is of great interest and remains an area with significant knowledge gaps. Given that most of the population already has hybrid immunity to COVID-19, a key question is whether the order of infection-acquired and vaccine-induced immunity affects the immune response. Here, we analyzed the humoral and T-cell responses in a well-characterized cohort with longitudinal blood sampling spanning 2020-2023. We observed higher anti-RBD antibody levels against Omicron in individuals initially exposed to SARS-CoV-2 antigens via vaccination compared to those first exposed through natural infection. This difference diminished with an increasing number of exposures. The dynamics of antibody levels over time correlated with clinical protection: those first-infected had higher protection early on, whereas those first-vaccinated showed greater protection later, especially with the arrival of the Omicron variant. This phenomenon may reflect immune imprinting. In contrast to humoral response, T-cell response was higher in individuals first exposed through infection. Our study provides valuable insights into the impact of initial antigen exposure on humoral and cellular responses to SARS-CoV-2.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been a major challenge to society since December 2019. After the pandemic has eased, SARS-CoV-2 still appears as a main cause of morbidity and mortality, with ongoing waves driven by new variants.[1,2]

Extensive research has been conducted on the immune response to SARS-CoV-2, including the role of the innate response, the antibodies targeting the trimeric spike (S) protein, and the cellular response mediated by T cells.[3] Among the antibodies targeting the S protein, those against the receptor-binding protein domain (RBD) are crucial for neutralizing activity and protection against the disease.[4–6]

The comparison between vaccine-induced and infection-acquired adaptive immunity, and their co-occurrence ("hybrid immunity"), is of great interest and remains an area with knowledge gaps.[5,7,8] Regarding antibody dynamics, initial studies showed a higher antibody titer peak for infection-acquired antibodies compared to vaccine-induced antibodies. Additionally, the cellular and humoral responses after a vaccine shot appear to be broader and more sustained in those individuals with a previous infection compared to those with only a previous vaccine dose.[5,9] Nevertheless, the difference in the humoral response seems to disappear over time since the primo-exposure (i.e., via vaccine or infection)[10] and with additional exposures, such as after a third exposure to SARS-CoV-2 antigens.[7,11,12] With the advent of the Omicron variant and its descendants, the viral escape from neutralizing and binding antibody titers was significant,[11,13] increasing the number of breakthrough infections and raising new questions about the differences between vaccine-induced and infection-acquired immune responses.[7,11,14]

Given that most of the population already has hybrid immunity to COVID-19, one knowledge gap is whether the order of infection-acquired and vaccine-induced immunity affects the resulting immune response. Indeed, infection-acquired immunity to SARS-CoV-2 exhibits great variability,[11] including low-responder profiles, failure to mount long-lasting immunity, and delayed response to vaccination in a subset of individuals.[15–17] These effects are likely to occur during the first-ever exposure to the SARS-CoV-2 antigens. Whether they impact long-term and broad immunity in the scenario of multiple previous vaccine doses and infections, where Omicron is the predominant variant, is unknown.

We leveraged a well-characterized longitudinal cohort of health-care workers (CovidCatCentral)[18,19] to evaluate whether a first exposure to SARS-CoV-2 antigens via infection or vaccination is associated with a better anti-RBD antibody response to Omicron variants in individuals with hybrid immunity. Subsequently, we explored whether the dynamics of the anti-RBD antibody response to the ancestral Wuhan strain differed by first exposure groups, as well as their T-cell response and clinical protection.

Methods

Study design, population, and setting

The CoviCatCentral cohort consists of health-care workers in primary care centers from three counties in Barcelona province, Spain, who were offered COVID-19 vaccination starting December 2020. The follow-up was conducted via eleven cross-sectional surveys, herefter called timepoints. A first group was recruited during the first wave of the COVID-19 pandemic (March–April 2020, n = 247) with symptomatic SARS-CoV-2 infection confirmed by reverse transcriptase polymerase chain reaction (RT-qPCR) and/or antigen rapid diagnostic test (RDT). A second group was recruited from March–April 2021, at timepoint 5, after complete primary series vaccination (n = 200) and without previous SARS-CoV-2 infection, characterized by absence of a clinical episode of COVID-19, and any positive RT-qPCR, RDT, or serology tests. The second group was recruited to obtain similar characteristics (e.g, age, sex, professional category, smoking habits) to the first group. A detailed scheme for the surveys' recruitment is shown in **Supplementary eFigure 1**.

Analyses in this study were performed with samples collected in T10 (Nov 2022) and T11 (May 2023). Out of 405 participants with blood samples available at T10 and/or T11, 51 were excluded for not having hybrid immunity to SARS-CoV-2, resulting in 361 eligible participants. We excluded three individuals with less than three exposures before sample collection and one individual with inconsistent data, remaining 357 individuals.

The study protocols were approved by the IRB Comitè Ètic d'Investigació Clínica IDIAP Jordi Gol (20/162-PCV). Written informed consent was obtained from all study participants prior to study initiation. Demographic and clinical data for each participant were collected at baseline and during follow-up visits through telephone interviews and electronic standardized questionnaires by study physicians and nurses as described elsewhere.[18,19]

Quantification of antibodies to SARS-CoV-2

We measured IgA and IgG plasma levels (median fluorescence intensity, MFI) to the full-length (FL) SARS-CoV-2 nucleocapsid (N) and Spike (S) antigens, and the Receptor Binding Domain (RBD) that lies within the S1 region (RBD ancestral and variants Delta, BA.1, BA.2, BA.4/5, BQ.1.1, and XBB), by quantitative suspension array technology assays (xMAP, Luminex), following a previously described protocol.[20–22] A nucleotide fragment encoding the ancestral N FL, followed by a 6xHis-tag, was cloned into pET22b expression vector, transformed in *E. coli* BL21 DE3, induced with IPTG, and purified by affinity chromatography using HisTrap columns, and controlled for purity by SDS-PAGE and Coomassie staining.[23] The ancestral S FL and the RBD proteins were fused with C-terminal 6xHis and StrepTag sequences and purified from the supernatant of lentiviral-transduced CHO-S cells cultured under a fed-batch system.[21] Codon-optimized nucleotide fragments encoding the RBD variants (Delta, BA.1, BA.2, BA.4/5, BQ.1.1, and XBB) were synthesized and cloned into pcDNA3.1/Zeo (+) expression vector (Thermo Fisher Scientific).[24] Recombinant proteins were produced by transient transfection of exponentially growing Freestyle TM 293-F suspension cells (Thermo Fisher Scientific) using the polyethylenimine (PEI)-precipitation method. Proteins were purified from culture supernatants by high-performance chromatography using the Ni Sepharose® Excel Resin (GE Healthcare), according to manufacturer's instructions, dialyzed against PBS using Slide-A-Lyzer® dialysis cassettes (Thermo Fisher Scientific), quantified using NanoDrop TM One instrument (Thermo Fisher Scientific), and controlled for purity by SDS-PAGE using NuPAGE 3-12% Bis-tris gels (Life Technologies).

Plasma samples were tested at 1:500 dilution for IgA and IgG, and additionally at 1:5000 for IgG to avoid saturated anti-S IgG levels. To quantify IgA, samples and controls were pretreated with anti-human IgG (Gullsorb) at 1:10 dilution, to avoid IgG interferences.

Optimal testing dilutions were previously assessed to assure samples were within the quantitative range of the assay. For this analysis, we used the dilution factor 1:500 for IgA and 1:5000 for IgG. To allow the comparison of antibody levels between T10 and T11 and correct any batch effect, we normalized the MFI values using the following formula:

$$normalized\ MFI \quad = \quad \frac{\text{sample MFI}}{\text{PC MFI}} * \text{Mean MFI of T10 and T11 PCs}$$

Where PC MFI is the positive control MFI value, and Mean MFI of T10 and T11 PCs is the mean of T10 and T11 positive controls for a given isotype and antigen combination. The operators conducted the assays in a blinded manner. The results are presented as log_{10}-transformed MFI.

Cellular Assay

The magnitude of T-cell responses to ancestral SARS-CoV-2 FL S and N membrane (M) peptide pools [PepTivator® SARS-CoV-2 Prot_S Complete, Prot_N, Prot_M (Miltenyi)] was measured using the human IFN-γ/IL-2 FluoroSpot kit (Mabtech)[25,26] in a random subset of 49 individuals with hybrid immunity and available peripheral blood mononuclear cells (PBMCs) (21 first-infected and 28 first-vaccinated). We combined the stimuli according to vaccine or non-vaccine antigens, S and N+M, respectively.

PBMCs were isolated from venous blood samples by density-gradient centrifugation using Ficoll-Paque (Merck), cryopreserved in heat-inactivated fetal bovine serum (HI-FBS) (Thermo Fisher Scientific) with 10 % dimethyl sulfoxide (Merck), and stored in liquid nitrogen until use. After blocking the pre-coated FluoroSpot plates with culture medium-10% HI-FBS, $2x10^5$ thawed PBMCs were added to wells containing the stimulus (1 μg/mL of peptide concentration) or the negative control (only culture medium [TexMACS Medium (Miltenyi)-1% penicillin/streptomycin (Thermo Fisher Scientific)], and $5x10^4$ PBMCs were added to the wells containing the positive control (phytohemagglutinin (Merk), 5 μg/ml). PBMCs were incubated at 37 °C and 5 % CO2 for 20 h. All conditions were performed in duplicate.

Cells secreting IFN-γ and/or IL-2 were detected and counted as spot-forming units (SFU). Seven participants with ≥100 SFU in unstimulated wells for IFN-γ were excluded from the analysis. SFU counts in the unstimulated wells were subtracted from those in the stimulated wells to account for background responses, and negative values were set to zero. The results were expressed as SFUs/10^6 PBMCs.

Definitions

Asymptomatic infections were defined as an increase in the IgG or IgA levels between consecutive timepoints in individuals without COVID-19 symptoms and without any positive RT-qPCR or RDT or vaccination during the time interval evaluated. For those vaccinated between timepoint intervals, an individual was considered infected if the fold change was greater than 4 for IgG or IgA against N. For those not vaccinated between timepoint intervals, for the T1 – T2 interval up to the T7 – T8 interval, an individual was considered

infected if at least two different IgG or IgA antibodies against nucleocapsid or spike antigens exhibited a fold-change greater than 4. In later intervals, an individual was considered infected if at least one of the measured IgG or IgA antibodies had a fold-change greater than 3. The lower fold-change threshold in these later intervals was applied since individuals diagnosed with an infection during these intervals exhibited smaller fold-change increases compared to earlier intervals. This reduction in fold-change increase is attributed to elevated antibody levels at later timepoints.

Symptomatic infections were defined by individuals with COVID-19 symptoms and a positive RT-qPCR or RDT.

Chronic comorbidities were self-reported and divided into the following categories: chronic respiratory diseases (chronic obstructive pulmonary disease, asthma), cardio-metabolic (dyslipidemia, hypertension, diabetes, other cardiovascular diseases), obesity, neurologic, gastrointestinal, chronic renal disease, immunosuppressed status (autoimmune disease, cancer, other immunosuppression), hypothyroidism, depression, pregnancy, and history of allergy. Additionally, we assessed the tobacco smoking status as active smoker, former smoker and never smoker.

The definitions of previous infections and vaccinations always followed the logic of what happened before the evaluated timepoint.

Data analysis

We described variables as mean ± SD, median [p25-p75], and counts (%) as appropriate. We evaluated the linear correlation for the $\log_{10}$ anti-RBD MFI values between isotypes, the seven RBD variants, and T10 and T11 with the Pearson correlation coefficient.

We compared the anti-RBD antibody levels between the first-vaccinated individuals with the first-infected ones (reference group) at T10 and T11 using linear mixed models. The models accounted for the repeated sampling with random intercepts per individual. We defined a priori a set of potential confounding variables and performed a sequential adjustment as follows: M0, without adjustment; M1, M0 plus age (restricted cubic spline with 3df) and sex; M2, M1 plus number of chronic comorbidities and tobacco smoking status; M3, M2 plus number of non-Omicron and Omicron symptomatic infections (as factors), and number of non-Omicron and Omicron asymptomatic infections (as factors); M4, M3 plus number of non-bivalent and bivalent vaccines (as factors); and finally, M5 (main model), M4 plus days

from last infection (restricted cubic spline with 3df) and days from last vaccine (restricted cubic spline with 3df).

We ran two sensitivity analyses: MS1, M4 but expanding the number of chronic comorbidities as three binary factors (cardio-metabolic, immunosuppressed and previous allergy); and MS2, running M4 in those individuals without any history of asymptomatic infections.

To evaluate whether the difference between first-infected and first-vaccinated depends on the total number of previous exposures, we fitted the main model (M5) with an interaction term between the exposure (first-vaccinated x first-infected) and the number of previous exposures (as factor). We evaluated a potential statistical interaction with a likelihood ratio test comparing the model with and without the interaction term. This analysis was performed after excluding three individuals with >6 exposures, because this occurred only in the group of those first-infected.

To evaluate whether the difference between first-infected and first-vaccinated occurs mainly due to specific previous vaccination and infection histories, we fitted a model as M5 but using the exact history instead of number of previous infections and vaccinations. To create this history, we considered asymptomatic and symptomatic infections as equal previous infections and non-bivalent and bivalent vaccines as equal previous vaccines, to deal with sparse data and improve power. We fitted the model with a categorical variable containing the 9 most common history combinations (i.e., combinations with at least 20 samples, n=212 individuals), while collapsing those below 20 samples as “others” (n=145 individuals). Among the 9 most common history combinations, we chose those with a meaningful contrast to understand the first exposure effecy (i.e., those with the same number of exposures, but differing on the primo-exposure), resulting in four comparisons. The confidence intervals for these contrasts accounted for Bonferroni corrections.

The results from the linear mixed models are presented as a transformed beta value with the formula: ([10^beta]-1)*100, giving the difference (in percentage) in antibody levels when comparing first-vaccinated to the first-infected (reference group). We estimated the 95% confidence intervals (CI) for the estimates from the model.

To explore the observed difference between first-vaccinated and first-infected, we evaluated their IgA and IgG antibody dynamics (anti-S, anti-N and anti-RBD_{Wuhan}) using the

data available across the eleven timepoints. We plotted the MFI levels against each timepoint and fitted a LOWESS curve.

To investigate the association between the first exposure group and the risk of SARS-CoV-2 breakthrough infections, we conducted a time to event analysis using Cox Proportional hazards regression modeling. We used three sub-cohorts starting at timepoints T8, T9 and T10, with individuals being followed-up up to the starting point of the next timepoint (i.e, T9, T10 and T11). Individuals were followed-up until the first episode of SARS-CoV-2 breakthrough infection (outcome of interest), receipt of an additional vaccine dose, or the last day of study follow-up (end of T9, T10, or T11, respectively). The main model included both asymptomatic and symptomatic infections as outcome of interest. The date of asymptomatic infections was inferred by taking the midpoint between the timepoints defining the period. To avoid misclassification, participants who had both an asymptomatic infection and a vaccination within the same timepoint interval were excluded from the analysis. As a sensitivity analysis, we run the same Cox model considering only symptomatic infections. Models were adjusted by age, sex, number of comorbidities, smoking status, number of previous exposures, and time since last exposure. The Cox proportional hazards assumption was evaluated by examining Schoenfeld residuals.

Nonparametric tests were used to analyze T-cell data. We compared responses to S and N+M by paired Wilcoxon Signed-Rank tests. The proportions (%) of secreting T cells induced by S vs. N+M antigens were compared using the chi-square test. Comparisons of magnitude of T-cell responses between first-infected/first-vaccinated groups were performed by the Wilcoxon rank-sum test.

A P-value of ≤0.05 was considered statistically significant for all estimates, except for the interaction when we considered a P ≤0.10. We performed the statistical analysis in the software R version 4.2.1.

Results

Population characteristics and history of exposures

The population characteristics are shown in **Table 1**. The majority of individuals had available samples from both study timepoints, T10 and T11, accounting for 81% of the total samples. Individuals who were first-infected were older, had more cardio-metabolic

comorbidities, more common history of allergy, and less frequently active smokers compared with the first-vaccinated group.

The temporal distribution of the number of previous asymptomatic and symptomatic infections and vaccine doses are shown in **Figure 1** and **Table 2**. Overall, the number of previous exposures was higher in the first-infected group compared with the first-vaccinated group. The number of infections was higher in the first-infected group when considering any infection, mainly driven by non-Omicron infections. The number of asymptomatic infections was comparable between groups in terms of number of exposures and for non-Omicron and Omicron periods. Overall, the number of previous vaccinations was higher in the first-vaccinated group and the number of bivalent vaccines was similar between groups. The time from the last exposure relative to the sample collection was 173 [27-299] days for T10 and 224 [164-380] days for T11; and it was shorter for the first-vaccinated group compared to the first-infected group for T10 and similar for T11.

Anti-RBDs antibody correlations

We measured IgA and IgG in 646 plasma samples (337 from T10 and 309 from T11). The correlations between anti-RBD IgA and IgG levels to Wuhan and six different Omicron lineages for each timepoint are shown in **Supplementary eFigure 2**. The correlations between anti-RBD-Wuhan and anti-RBD-Delta responses were higher than between anti-RBD-Wuhan and the other anti-RBDs targeting Omicron lineages. The majority of correlation coefficient values < 0.80 occurred when contrasting RBD-XBB with others. Overall, the correlations between IgA and IgG were <0.40 for all timepoints and different RBDs. The correlations of anti-RBDs antibody levels between T10 and T11 were positive and close to 0.70.

Association between antibody titers at T10 and T11 and type of first exposure

The unadjusted average MFI values, accounting for the number of previous exposures, for both IgA and IgG across the seven RBDs were higher for those first-vaccinated than first-infected (35%, 95% CI, 20 to 51, for IgA and 53%, 95% CI, 33 to 76, for IgG against RBD-XBB), although this difference was less clear for IgA and RBD-Wuhan and RBD-Delta (21%, 95% CI, -2 to 48, for IgA and 54%, 95% CI, 35 to 76, for IgG against RBD-Wuhan, **Figure 2-M0** and **Supplementary eFigure 3**). When adjusting for the sequential set of potential confounders, this difference remained showing greater MFI values for first-vaccinated than first-infected

(35%, 95% CI, 15 to 58, for IgA and 70%, 95% CI, 42 to 105, for IgG against XBB, **Figure 2, main model**, **M5**). For example, in the two sensitivity analyses, the pattern of association remained similar.

In the main model (M5), the covariates associated with an increased IgG response were previous number of Omicron infections for some RBDs and, remarkably, previous number of non-bivalent vaccines (e.g., three previous for non-Bivalent vaccines compared with one previous non-bivalent for RBD-XBB: 44%, 95% CI, 14 to 82, **Supplementary eTable 1**) and having received at least one bivalent vaccine for the majority of RBDs (e.g., RBD-XBB, 64%, 95% CI, 40 to 91). For IgA response, the covariates did not have a clear pattern associated with increased antibody response, except for the bivalent vaccine (RBD-BA4.5, 25%, 95% CI, 7 to 46, **Supplementary eTable 1**).

We further explored whether the observed differences between first-vaccinated and first-infected groups varied according to the number of previous exposures (infectons or vaccinations). We observed that the greater antibody response for first-vaccinated groups was mainly driven by the individuals with a maximum of three previous exposures and that this difference decreased as the individuals had been exposed to more exposures (**Figure 3**). This pattern was almost linear and consistently observed for IgA across all RBDs and for IgG for RBD-Wuhan and RBD-Delta. When evaluating IgG against Omicron lineages, the pattern of decreasing the difference between first-vaccinated and first-infected was present, and seems to have a threshold, being the interaction mainly driven by the difference occurring before and after four exposures. This pattern was no evidence regarding the first-exposure group for IgG against RBD-XBB, which was almost flat over the number of exposures.

We compared the differences between first-vaccinated and first-infected individuals considering their actual previous history and sequence of exposures (**Figure 4**). The histories with consistent increased IgG response against RBD for the first-vaccinated compared with first-infected groups were the sequence of two vaccines doses followed by an infection compared with an initial infection followed by one vaccination and one infection (VacVacInf vs InfVacInf), and the history of three vaccine doses followed by an infection (VacVacVacInf vs InfVacVacInf). The history VacVacVacInf vs. InfVacVacInf showed increased response for IgA against Omicron RBDs.

Kinetics of antibodies against N, RBD and S Wuhan since March 2021 (T5) by first exposure

When we looked at the temporal evolution of IgA and IgG to N, S and RBD from its first assessments in 2021, we observed that anti-N antibody levels, for both IgA and IgG, were overall lower for the first-vaccinated individuals compared with the first-infected individuals across all timepoints, with greater difference after T9. Regarding anti-S and anti-RBD levels, both IgA and IgG, were lower for the first-vaccinated individuals during the first timepoints (T5 to T8), and then reversed after the T9 (**Figure 5**).

Association between magnitude of T-cell responses at T11 and type of first exposure

We analyzed the magnitude of T-cell responses in a subset of 49 individuals (21 from the first-infected and 28 from the first-vaccinated groups) at T11. All individuals tested had detectable T-cell responses against S or N+M from Wuhan. S-specific T-cell responses were more robust than those to N+M (**Supplementary** e**Figure 4A**) although they were correlated (Spearman rho range 0.69-0.87, $p < 0.05$, **Supplementary** e**Figure 4B**). Most S-specific T cells secreted IFN-γ, while most N+M-specific T cells secreted IL-2. Within S-specific T cells, the first-vaccinated group had higher proportion of IL-2 secreting T cells (37.8%) compared to the first-infected group (21.8%) (**Supplementary** e**Figure 4A**). In contrast to the pattern observed for antibodies, the first-infected group showed significantly increased magnitude of T-cell responses compared to the first-vaccinated group three years later to both S (3.5 and 1.9 times higher for IFN-γ and IFN-γ + IL-2, respectively) and N+M (3.2, 2.7 and 2.7 times higher for IFN-γ, IL-2 and IFN-γ + IL-2, respectively) (**Figure 6A**). In the first-vaccinated group no correlations were found between the magnitude of T-cell responses and antibody levels, except for S-specific IFN-γ secreting T cells with IgG levels anti-RBD from Delta variant (Spearman rho 0.44, < 0.05) (**Figure 6B**). In contrast, within the first-infected group, the magnitude of T-cell responses to N+M was moderately correlated with IgG levels against Wuhan and other variants (Spearman rho range: 0.42 – 0.66, $p < 0.05$), while a negative correlation was observed between T-cell responses and IgA levels, particularly against RBD from Omicron variants (Spearman rho ranges -0.46 – -0.58, < 0.05, **Figure 6B**).

Association between protection against breakthrough infection and first exposure

Having an initial vaccination as opposed to being first-infected was associated with an increased risk of symptomatic and asymptomatic breakthrough infections during the T8 - T9 period (HR 3.48; 95% CI, 1.05-11.5). However, there was a shift in this risk for T9-T10 (HR

0.50; 95% CI, 0.31-0.82) and T10-T11 (HR 0.71, 95% CI, 0.43 to 1.17) periods, when being first-vaccinated was associated with a decreased risk of infection (**Supplementary eTable 2**).

Discussion

In a longitudinal evaluation of 357 individuals with hybrid immunity to SARS-CoV-2, the first exposure to SARS-CoV-2 antigens via a natural infection resulted in lower IgA and IgG levels against Omicron variant RBDs compared with those first exposed via vaccination after an average of 420 days since their first exposure. This difference appeared to decrease as the total number of previous exposures increased. When evaluating a long series of antibody levels against Wuhan antigens, first-vaccinated individuals had lower levels of anti-RBD and anti-S antibodies at the beginning of follow-up, as previously described,[10,27] which shifted after the appearance of Omicron. In contrast, T-cell responses against Wuhan antigens were of greater magnitude in those first-infected compared to those first-vaccinated.

The importance of the first exposure to SARS-CoV-2 antigens was studied early in the pandemic.[10,27] However, few studies have evaluated its impact in a scenario mimicking the current situation, characterized by hybrid immunity and the circulation of the Omicron variant. Srivastava et al.,[7] in a cohort of 496 individuals in New York City, observed similar antibody dynamics between groups. They reported higher anti-S levels in the first-infected group up to the third vaccine dose, after which the levels became comparable. Contrasting with our results, Srivastava et al. found the first-infected vs first-vaccinated difference disappeared after the third dose, while we observed this difference shifted. This discrepancy may be due to different antibody types and assays, in addition to differences in the adjustment for potential confounders. Importantly, the difference on anti-RBD antibody levels decrease as many exposures occurred, except for anti-RBD XBB. To the best of our knowledge, this is the first study showing this phenomenon.

Regarding protection against breakthrough infections, we observed a parallelism between anti-RBD antibodies and clinical protection, i.e., when first-infected individuals had higher anti-RBD antibody levels, they also had less risk of breakthrough infections compared to the first-vaccinated group, and this shifted when first-vaccinated group had higher anti-RBD antibody levels. Srivastava et al. found a similar pattern regarding this protection against breakthrough infections; nevertheless they observed similar anti-S levels.[7] This indirectly

reinforces the role of anti-RBD antibodies as being closer to neutralizing functions than anti-S.

In contrast with the previous findings regarding humoral and clinical protection assessment, the T-cell responses against Wuhan were more robust in those first-infected compared with the first-vaccinated at T11, even though all individuals had hybrid immunity before T10 and T11. The literature shows that T-cell response is a marker for protection against progression to severe disease, a fact that explains the protection observed against severe disease even when the neutralizing antibody levels against Omicron are relatively low.[28] Discordance between T-cell response and neutralizing antibodies has also been described, particularly in mild infections.[29] More robust cellular responses in those first-infected compared with first-vaccinated have been reported,[30–33] such as a durable transcriptional and epigenetic signature of inflammation in S-specific memory $CD4^+$ T cells after two years of the first exposure.[32] The first contact via infection exposes the immune system to a wider repertoire of antigens, includes mucosal antigen contact, exposure to different viral loads and a broader involvement of the innate immune response, likely providing a broader and more sustained T-cell response. Interestingly, we did not observe correlations between T-cell responses and IgG levels among those first-vaccinated, despite they having higher anti-RBD levels; while there were consistent positive correlations between T-cell responses and IgG levels among those in the first-infected group.

Some studies provided some insights on the potential mechanisms of the differences between first-infected and first-vaccinated, beyond the difference in T-cell response magnitude. Tejedor Vaquero et al. observed differences between previously infected and non-infected individuals in the recall of IgG subclasses to RBD after mRNA vaccination.[34] After the first dose, those previously infected had higher levels of anti-RBD IgG for all subclasses, except IgG3 for Wuhan and Beta, Gamma and Delta variants. In the second dose, those previously infected had a less robust response, and those without previous infection showed an increased recall for IgG1 against the variants of concerns. Pérez-Alós et al. also showed that primary infection before Omicron was essential for a robust IgA response, which might also explain why the difference between first-infected and first-vaccinated is less clear for IgA in our results.[33]

We observed a main change in the antibody dynamics after the emergence of Omicron. Reynolds et al. evaluated a cohort of healthcare workers with three COVID-19 vaccines and

different histories of infections to SARS-CoV-2 variants before Omicron.[35] Evaluating different aspects of humoral and cellular immune response, they observed there was a boost response upon an Omicron infection, but this boosting was much less robust in those with a prior Wuhan infection, a phenomenon defined by them as "hybrid immune damping". This could explain our main findings of a greater anti-RBD antibody response in those first-vaccinated compared with those first-infected (all with Wuhan). This imprinting for Omicron responses was also observed in other cohorts.[33] Interestingly, this imprinting wanes as the number of repeated Omicron exposures increases,[36] which might also reflect our findings between first-infected vs first-vaccinated when considering the number of exposures.

Our study has some strengths. We report a well-characterized cohort of healthcare workers with close surveillance and repeated blood sampling. We also considered symptomatic and asymptomatic infections, made possible by repeated serological multiplex evaluation, which is not always possible in cohort studies evaluating humoral and cellular immune response, and clinical protection. These factors allowed us to adjust the estimates by the full history of previous SARS-CoV-2 antigen exposure in the cohort. Finally, we evaluated five Omicron antigens, which gives a broad evaluation of humoral response.

We also have several limitations. First, we did not evaluate the mucosal response, which has a significant role on protection and might differ between first-infected and fist-vaccinated groups.[11] Second, we could not evaluate more exposure histories than the four reported and different types of vaccine used because of sample size. However, we do not expect any major differences by type of vaccine since 99% of them were mRNA. Third, we did not evaluate neutralizing antibodies, although there is a high correlation between anti-RBD IgG levels and neutralizing antibodies.[37,38] Fourth, we adjusted for several confounding factors, including number of previous infections and vaccinations, time from last exposure and demographic factors, but residual confounding could be present. Finally, we analyzed a cohort of healthcare workers from the primary care sector, which allowed us to have a well-characterized cohort, but decreased our generalizability, including the absence of severe infections and the low proportion of males.

To conclude, we identified a higher anti-RBD antibody response against Omicron in individuals first exposed to SARS-CoV-2 antigens via vaccination than through natural infection. This difference decreased as the number of exposures increased. The antibody

dynamics over time reflected in clinical protection, when first-infected had higher protection early on, while having lower protection than first-vaccinated upon Omicron arrival. In contrast to humoral response, T-cell response against Wuhan was higher in those first exposed through infection. Our study reinforces the role of the vaccination campaigns against SARS-CoV-2, guaranteeing the first exposure to SARS-CoV-2 antigens via vaccination and resulting in an overall better immune response against Omicron.

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Competing interests

P. Santamaria is founder, scientific officer and stock holder of Parvus Therapeutics and receives funding from the company. He also has a consulting agreement with Sanofi. The other authors declare no competing interests.

Authors contributions

G.M., A.R-C, A.R-M., J.V-A, and C.D. designed the cohort study. A.R-M, J.V-A and A.R-C recruited participants, collected data and obtained samples. A.J., M.V., D.B., M.C., R.R., I.C. processed the samples, developed and/or performed the antibody binding assays and data preprocessing. R.R., M.C. and C.T. performed cellular immunology analyses. L.I, L.M-A, R.R. and P.S contributed with key reagents/antigens and expertise. O.R. and C.M.P. analyzed and/or managed the data. G.M., R.A. and C.D. supervised the antibody assays and data analyses. O.R. wrote the first draft of the paper with contributions from R.R., C.M.P., G.M. and C.D. All authors reviewed and approved the final version as submitted to the journal.

Data availability

In accordance with the current European and national law, the data used in this study are only available for the researchers participating in this study. Thus, we are not allowed to distribute or make publicly available the data to other parties. However, researchers can request data from investigators if they comply with certain requirements.

Tables and Figures

Table 1. Characteristics of cohort stratified by the first exposure being an infection or vaccination

	First-infected (n=197)	**First-vaccinated** (n=160)	**p-value**
Timepoint (%)			0.813
T10	28 (14.2)	20 (12.5)	
T10 and T11	159 (80.7)	130 (81.2)	
T11	10 (5.1)	10 (6.2)	
Age, mean (SD), years	50.2 (11)	47.1 (10)	0.006
Female, n (%)	165 (83.8)	138 (86.2)	0.613
Number of chronic comorbidities			
mean (SD)	0.78 (1.01)	0.46 (0.73)	
median [IQR]	0 [0, 1]	0 [0, 1]	0.002
Chronic respiratory disease, n (%)*	8 (4.1)	12 (7.5)	0.24
COPD, n (%)	0 (0.0)	3 (1.9)	0.178
Asthma, n (%)	8 (4.1)	10 (6.2)	0.486
Tobacco smoking status, n (%)			<0.001
No	137 (69.5)	92 (57.5)	
Previous smoker	49 (24.9)	38 (23.8)	
Active smoker	11 (5.6)	30 (18.8)	
Cardio-metabolic, n (%)*	31 (15.7)	11 (6.9)	0.016
Dyslipidemia, n (%)	14 (7.1)	4 (2.5)	0.083
Hypertension, n (%)	13 (6.6)	6 (3.8)	0.339
Diabetes, n (%)	7 (3.6)	0 (0.0)	0.043
Cardiovascular diseases, n (%)	2 (1.0)	2 (1.2)	>0.99
Obesity, n (%)	23 (11.7)	9 (5.6)	0.071
Neurologic, n (%)	2 (1.0)	2 (1.2)	>0.99
Gastrointestinal, n (%)	9 (4.6)	4 (2.5)	0.451
Chronic renal disease, n (%)	0 (0.0)	1 (0.6)	0.917
Immunosuppressed status, n (%)*	20 (10.2)	8 (5.0)	0.109
Autoimmune disease, n (%)	13 (6.6)	6 (3.8)	0.339
Cancer, n (%)	6 (3.0)	2 (1.2)	0.435
Other immunosuppression, n (%)	2 (1.0)	0 (0.0)	0.572
Hypothyroidism, n (%)	17 (8.6)	10 (6.2)	0.519
Depression, n (%)	5 (2.5)	2 (1.2)	0.625
Pregnancy, n (%)	3 (1.5)	1 (0.6)	0.767
History of allergy, n (%)	33 (16.8)	13 (8.1)	0.024

* The individuals can have more than one comorbidity within the category, thus the combined variable does not necessary is the sum of each comorbidity. COPD represents chronic obstructive pulmonary disease.

Table 2. Infections and Vaccination history before T10 and T11 timepoints

	T10			T11		
	First-infected (n=197)	**First-vaccinated (n=160)**	**p-value**	**First-infected (n=197)**	**First-vaccinated (n=160)**	**p-value**
Number of previous exposures,[#] mean (SD)	4.50 (1.00)	4.31 (0.66)	0.034	4.70 (1.06)	4.44 (0.77)	0.012
Number of previous exposures,[#] n (%)			<0.001			0.001
3	33 (16.8)	13 (8.1)		25 (12.7)	12 (7.5)	
4	68 (34.5)	90 (56.2)		63 (32.0)	80 (50.0)	
5	64 (32.5)	52 (32.5)		66 (33.5)	53 (33.1)	
6	29 (14.7)	5 (3.1)		34 (17.3)	15 (9.4)	
7	2 (1.0)	0 (0.0)		8 (4.1)	0 (0.0)	
8	1 (0.5)	0 (0.0)		1 (0.5)	0 (0.0)	
Infections						
Number of previous infections,* mean (SD)	1.95 (0.69)	1.33 (0.52)	<0.001	2.04 (0.71)	1.38 (0.56)	<0.001
Number of previous infections,* n (%)			<0.001			<0.001
1	49 (24.9)	111 (69.4)		41 (20.8)	105 (65.6)	
2	110 (55.8)	45 (28.1)		111 (56.3)	49 (30.6)	
3	36 (18.3)	4 (2.5)		41 (20.8)	6 (3.8)	
4	2 (1.0)	0 (0.0)		4 (2.0)	0 (0.0)	
Number of previous asymptomatic infections, mean (SD)	0.41 (0.56)	0.42 (0.55)	0.831	0.41 (0.56)	0.42 (0.55)	0.831
Number of previous asymptomatic infections, n (%)			0.903			0.903
0	124 (62.9)	98 (61.3)		124 (62.9)	98 (61.3)	
1	66 (33.5)	57 (35.6)		66 (33.5)	57 (35.6)	
2	7 (3.6)	5 (3.1)		7 (3.6)	5 (3.1)	
Number of previous non-Omicrons infections,* mean (SD)	1.22 (0.46)	0.26 (0.47)	<0.001	1.22 (0.46)	0.26 (0.47)	<0.001
Number of previous non-Omicrons infections,* n (%)			<0.001			<0.001
0	0 (0.0)	121 (75.6)		0 (0.0)	121 (75.6)	
1	158 (80.2)	37 (23.1)		158 (80.2)	37 (23.1)	
2	35 (17.8)	2 (1.2)		35 (17.8)	2 (1.2)	
3	4 (2.0)	0 (0.0)		4 (2.0)	0 (0.0)	
Number of previous Omicrons infections,* mean (SD)	0.74 (0.61)	1.07 (0.52)	<0.001	0.82 (0.62)	1.12 (0.55)	<0.001
Number of previous Omicrons infections,* n (%)			<0.001			<0.001
0	69 (35.0)	16 (10.0)		58 (29.4)	15 (9.4)	
1	111 (56.3)	116 (72.5)		116 (58.9)	110 (68.8)	
2	17 (8.6)	28 (17.5)		23 (11.7)	35 (21.9)	
Vaccines						
Number of previous vaccines, mean (SD)	2.55 (0.82)	2.98 (0.55)	<0.001	2.65 (0.89)	3.06 (0.62)	<0.001
Number of previous vaccines, n (%)			<0.001			<0.001
1	23 (11.7)	0 (0.0)		22 (11.2)	0 (0.0)	
2	61 (31.0)	26 (16.2)		57 (28.9)	26 (16.2)	
3	95 (48.2)	112 (70.0)		85 (43.1)	98 (61.3)	
4	18 (9.1)	22 (13.8)		33 (16.8)	36 (22.5)	
Previous bivalent vaccine, n (%)	1 (0.5)	3 (1.9)	0.475	17 (8.6)	13 (8.1)	>0.99
Times						
Days from closest exposure, median [IQR]	189 [44, 319]	153 [21, 224]	0.005	227 [165, 379]	211 [162, 382]	0.514
Days from closest infection, median [IQR]	222 [140, 695]	184 [110, 295]	<0.001	357 [183, 548]	326 [173, 404]	<0.001
Days from closest vaccine dose, median [IQR]	340 [303, 356]	347 [331, 360]	0.114	504 [293, 533]	514 [339, 530]	0.437

[#] Includes vaccine doses, symptomatic and asymptomatic infections. * includes symptomatic and asymptomatic infections

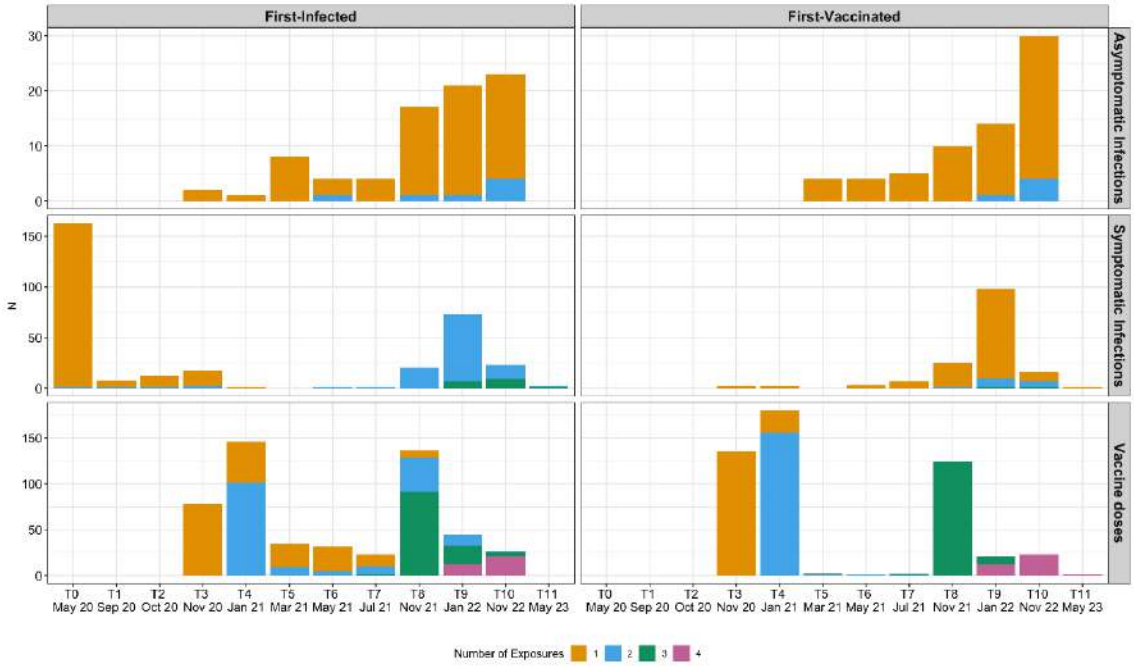

Figure 1. Temporal distribution of infections and vaccinations stratified by first exposure type. Omicron infections started between T8 and T9.

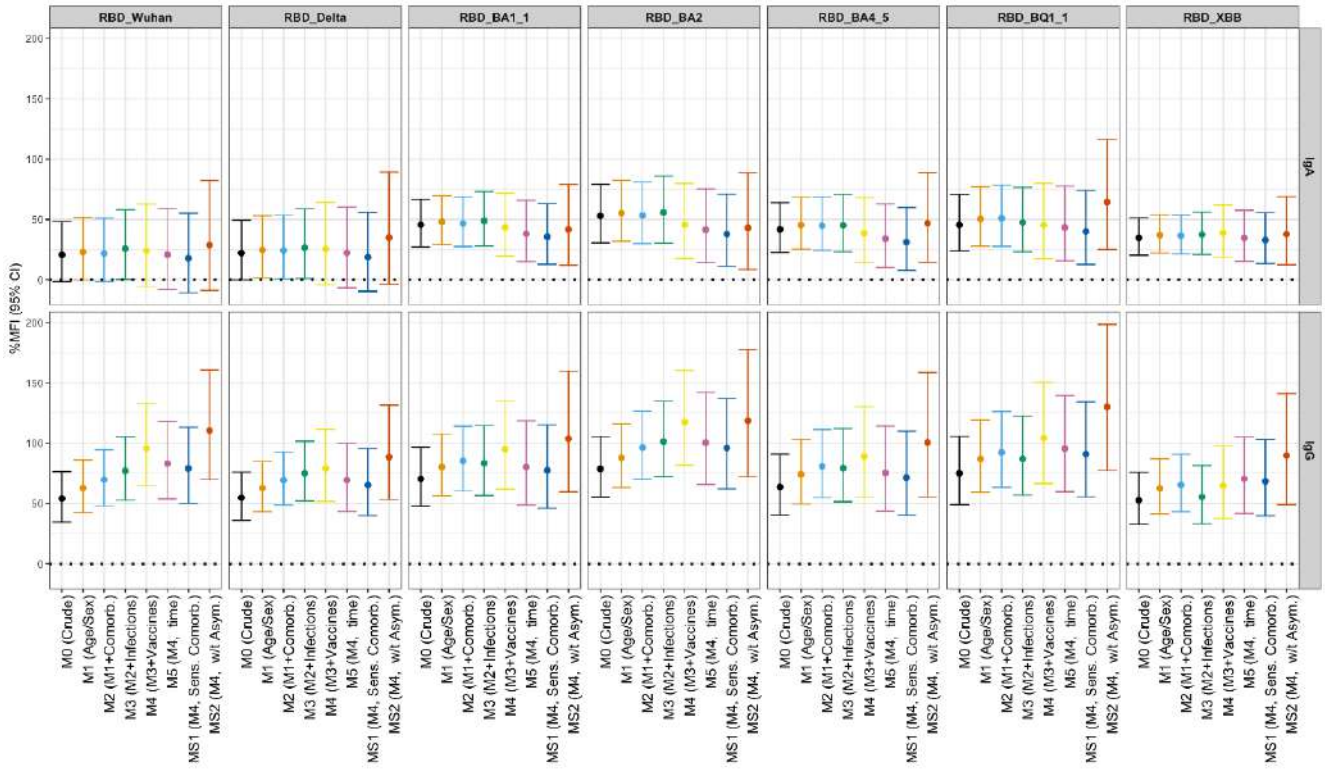

Figure 2. Difference on IgA and IgG antibody responses to RBD at T10 and T11 between first-vaccinated and with first-infected groups. Estimates from linear mixed models estimating the %MFI increase in those first-vaccinated compared to the first-infected group (reference). The models accounted for repeated measurements in the same individual with a random intercept per individual. The models were adjusted as follows: M0, crude; M1, M0 + age (restricted cubic spline with 3df) and sex; M2, M1 + number of chronic

comorbidities and tobacco smoking status; M3, M2 + number of non-Omicron and Omicron symptomatic infections (as factors), and number of non-Omicron and Omicron asymptomatic infections (as factors); M4, M3 + number of non-bivalent and bivalent vaccines (as factors); and finally, M5 (main model), M4 + days from last infection (restricted cubic spline with 3df) and days from last vaccine (restricted cubic spline with 3df). MS1, M4 but expanding the number of chronic comorbidities as three binary factors (cardio-metabolic, immunosuppressed and previous allergy); MS2, running M4 in those individuals without any history of asymptomatic infections. Receptor binding domain (RBD).

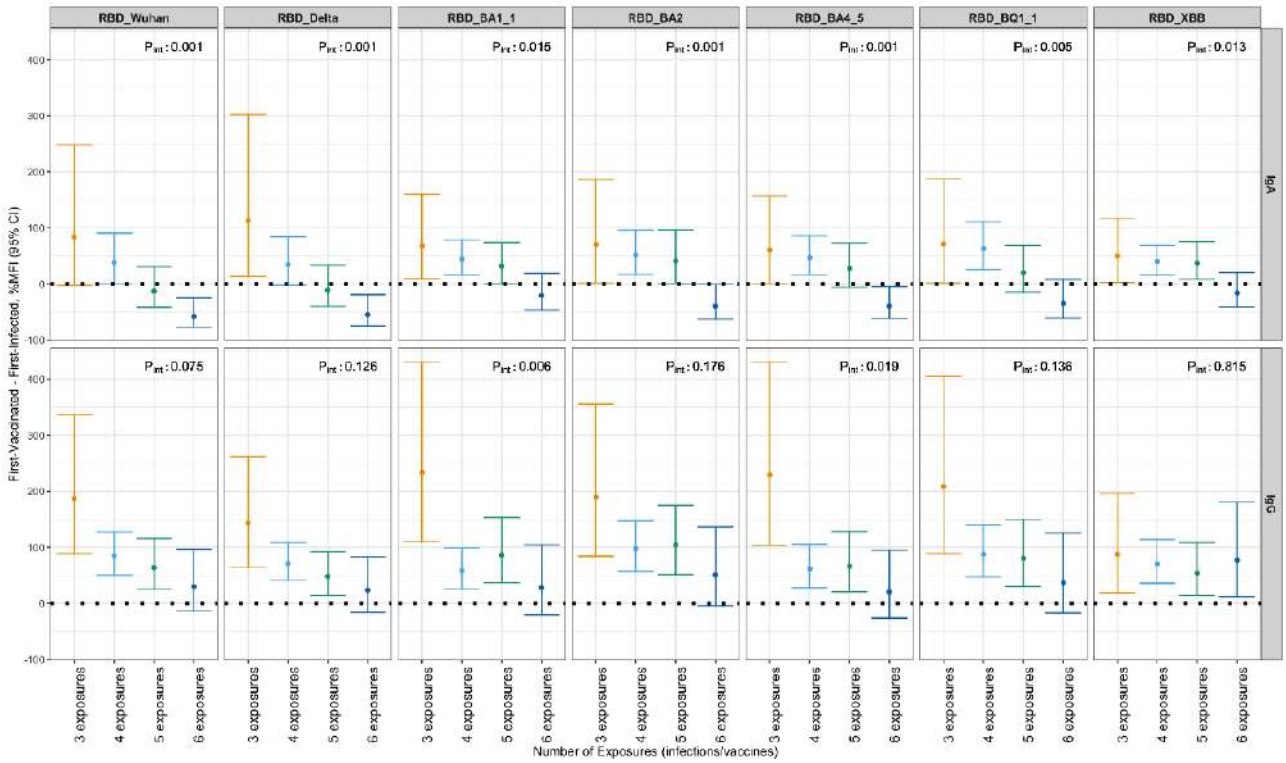

Figure 3. Interaction between number of previous exposures and the difference in antibody levels of first-vaccinated minus first-infected at T10 and T11. Estimates from linear mixed models estimating the %MFI increase in those first-vaccinated compared to the first-infected group (reference). The models accounted for repeated measurements in the same individual with a random intercept per individual. The model was adjusted as the main model (M5): age (restricted cubic spline with 3df) + sex + number of chronic comorbidities and tobacco smoking status + number of non-Omicron and Omicron symptomatic infections (as factors) + number of non-Omicron and Omicron asymptomatic infections (as factors) + number of non-bivalent and bivalent vaccines (as factors) + days from last infection (restricted cubic spline with 3df) + days from last vaccine (restricted cubic spline with 3df), with an interaction term between the main exposure and number of previous exposures (as a factor). P_{int} represents the p-value from the likelihood ration test for the interaction term. Receptor binding domain (RBD).

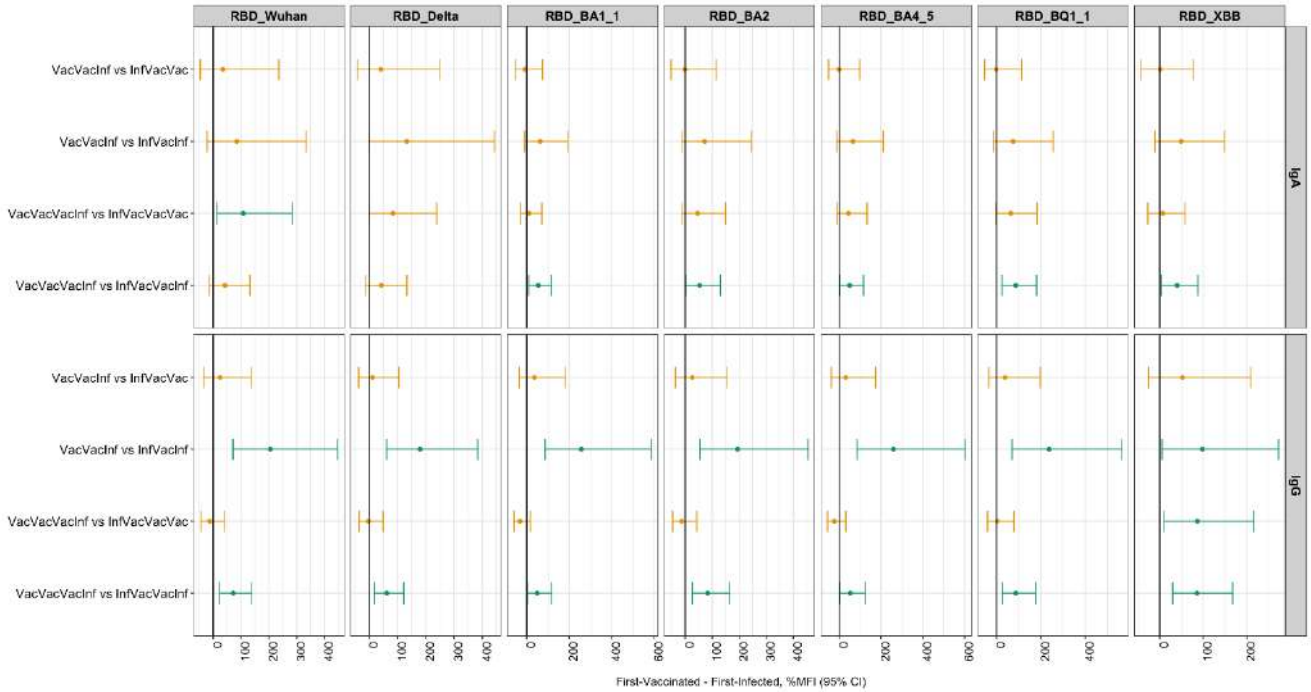

Figure 4. Sequence of previous exposures contrasts between first-vaccinated and first-infected. Estimates from linear mixed models estimating the %MFI increase in different exposure histories. The models accounted for repeated measurements in the same individual with a random intercept per individual. The model was adjusted adapting the main model (M5), i.e, excluding the number of previous infections and vaccines and using the history per se: age (restricted cubic spline with 3df) + sex + number of chronic comorbidities and tobacco smoking status + days from last infection (restricted cubic spline with 3df) + days from last vaccine (restricted cubic spline with 3df) and a factor with the history of the most common combinations. The error bars represent the 95% confidence interval corrected by multiple comparisons with Bonferroni method. Green color represents confidence intervals that do not includes zero. Receptor binding domain (RBD).

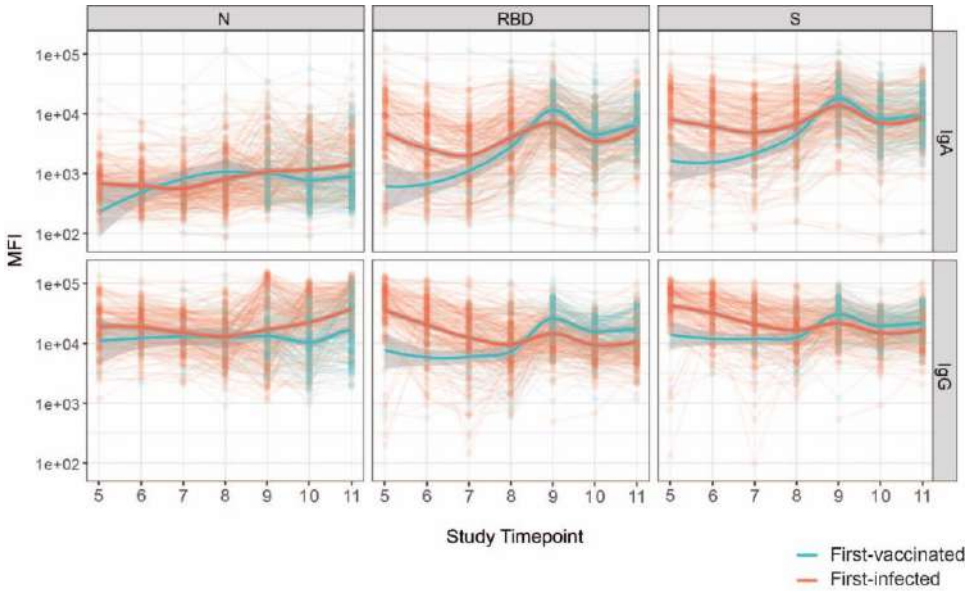

Figure 5. Longitudinal antibody levels against nucleocapsid (N), receptor-binding domain (RBD), and spike (S) proteins overtime. The blue and red solid lines represent the fitted curve calculated using the LOWESS (locally estimated scatterplot smoothing) method. Shaded areas represent 95% confidence intervals.

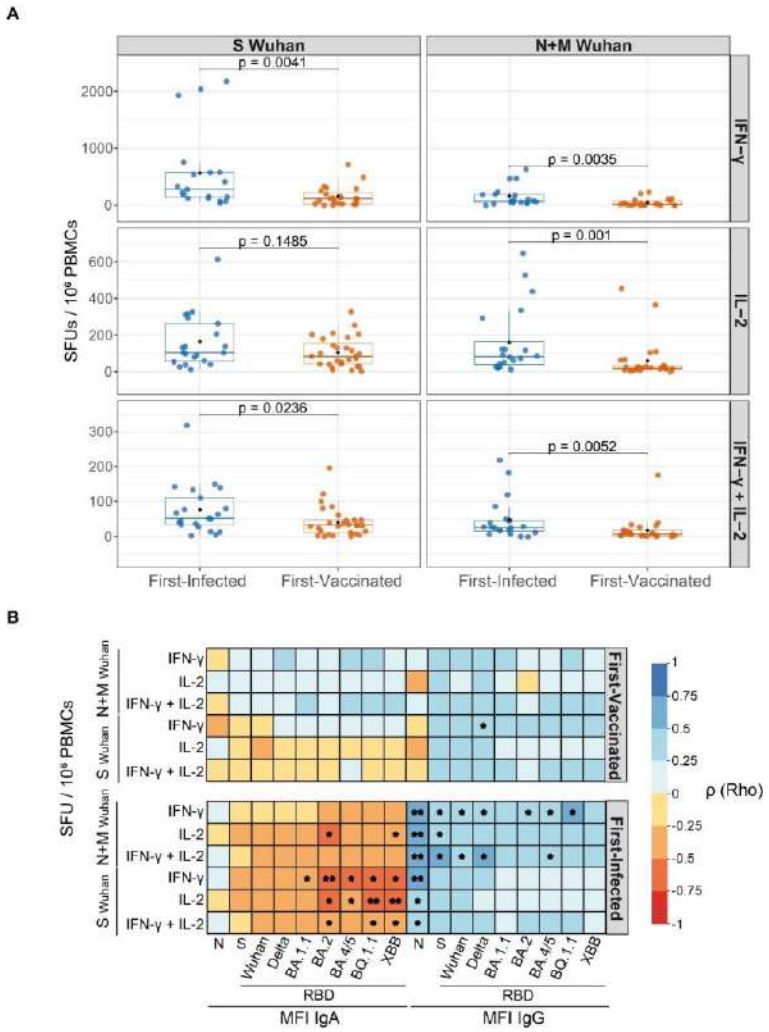

Figure 6. Magnitude of T-cell responses at T11 by first exposure groups and correlations with antibody responses. (**A**) Boxplots representing T-cell responses as SFUs / 10^6 peripheral blood mononuclear cells (PBMCs) secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) to S or N+M from Wuhan by first exposure groups. Responses were compared by Wilcoxon Signed-Rank test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). (**B**) Heatmaps illustrating the Spearman's correlation coefficient ρ (Rho) between the SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) to S and N+M with the antibody responses (median fluorescence intensity (MFI) of IgA and IgG). p-values: * ≤ 0.05, ** ≤ 0.01. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), receptor binding domain (RBD), spike (S), spot-forming units (SFU). First-infected (N = 21), First-vaccinated (N = 28).

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Supplementary material for

Influence of Initial SARS-CoV-2 Exposure via Vaccination or Natural Infection on Antibody and Cellular Responses to Omicron Variants

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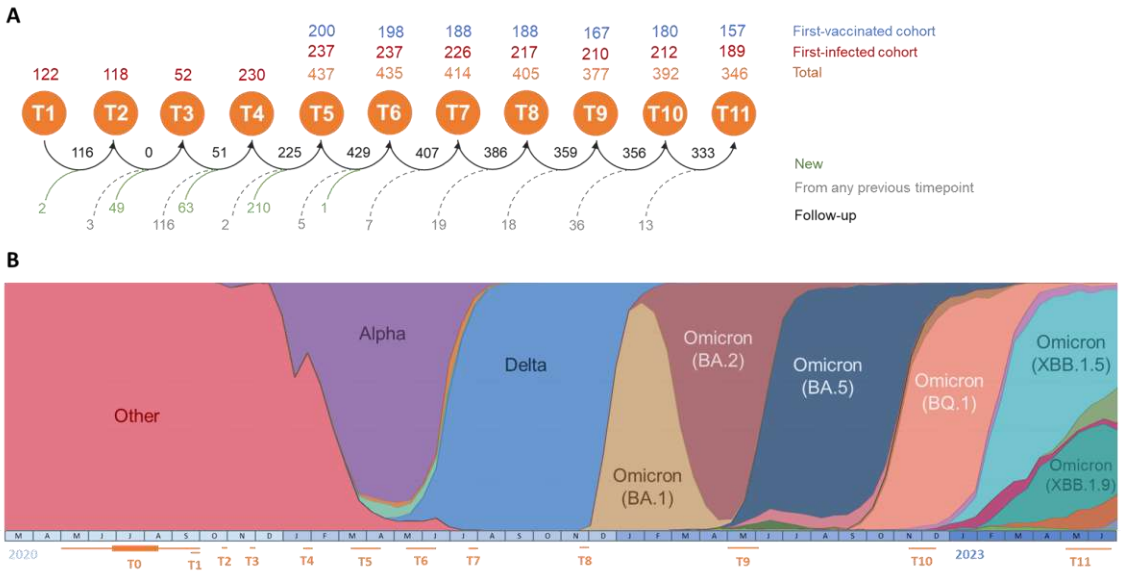
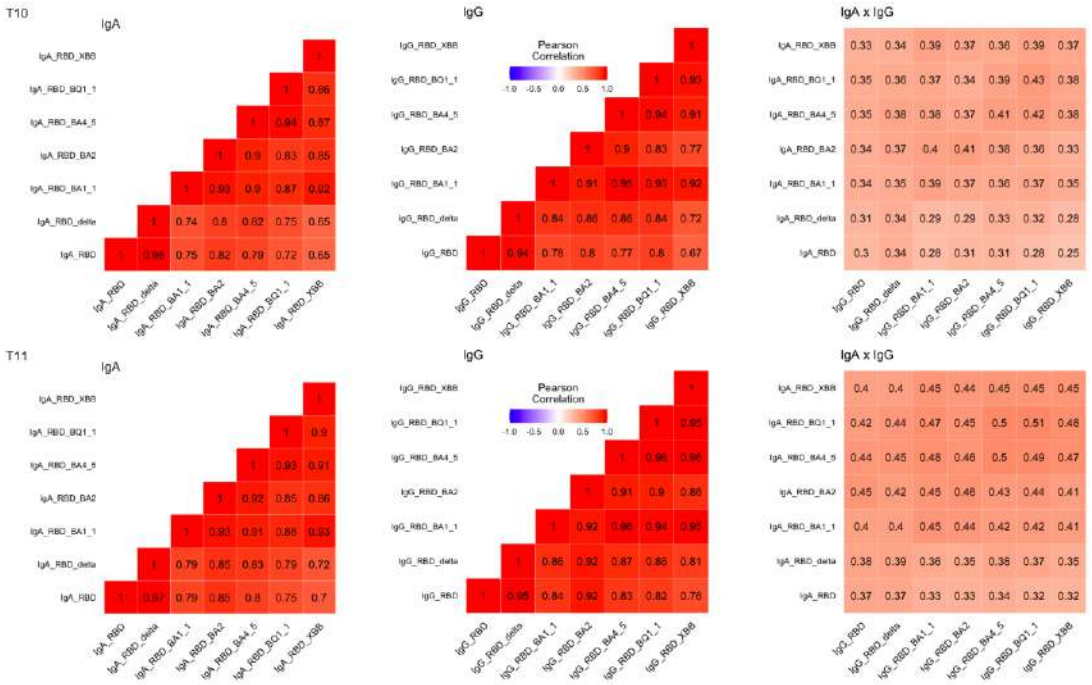


Supplementary eFigure 1. Timeline of the CovidCatCentral cohort.
(**A**). Number of individuals in each of the study timepoints for the first-vaccinated and first-infected cohorts. The blue numbers represent the first-vaccinated cohort, red numbers represent the first-infected cohort, and the orange numbers show the total number of individuals at each time point. Green numbers indicate new individuals entering the study, grey numbers indicate individuals from any previous timepoints but not consecutive, and black numbers indicate individuals who followed up from one consecutive timepoint to the next. (**B**) Prevalence of the different COVID-19 variants circulating in Spain from 2020 to 2023 and related to the timepoints.

A



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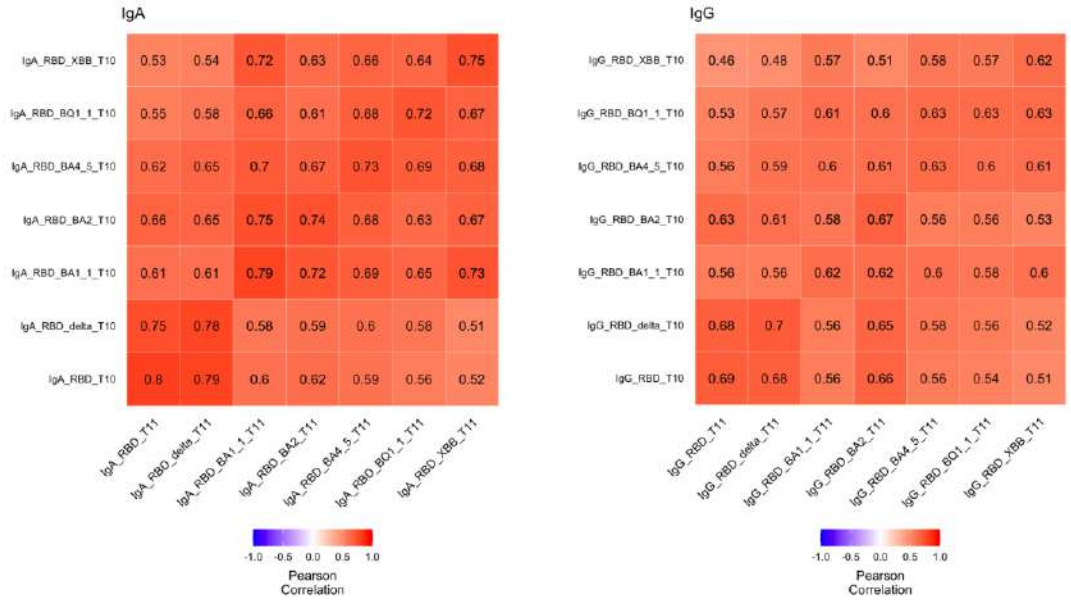
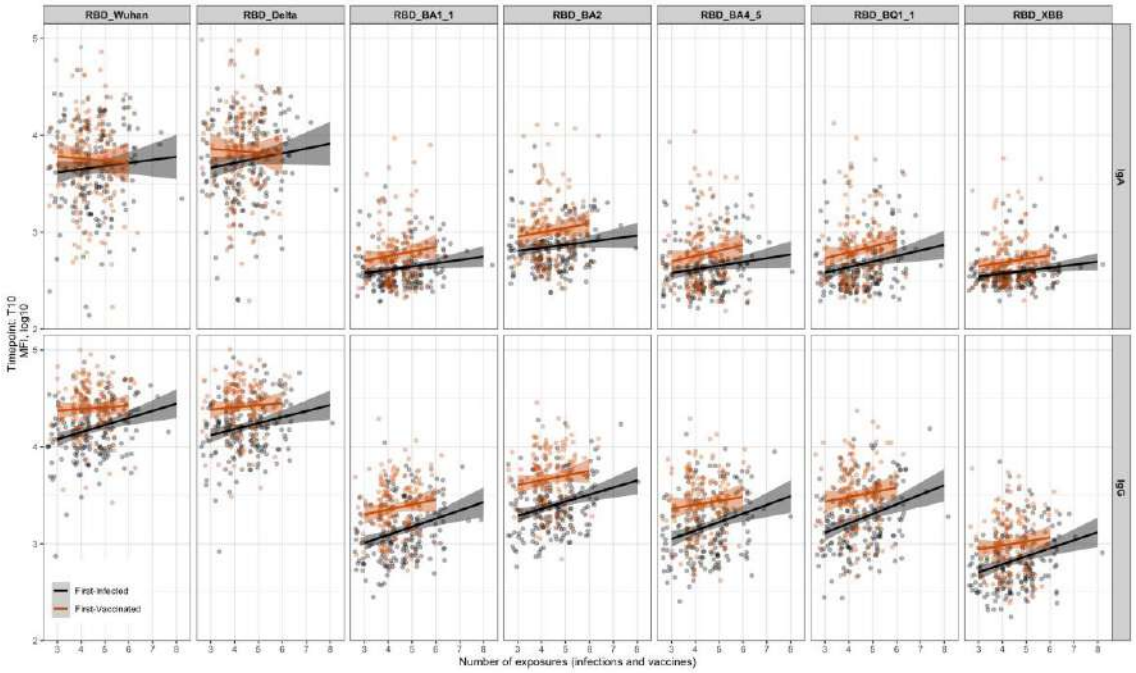


Supplementary eFigure 2. **Correlations for IgA and IgG to Receptor binding domain (RBD) from different variants.** (**A**) Between IgA, IgG and IgA x IgG at T10 and T11. (**B**) Between T10 and T11 for IgA and IgG.

A



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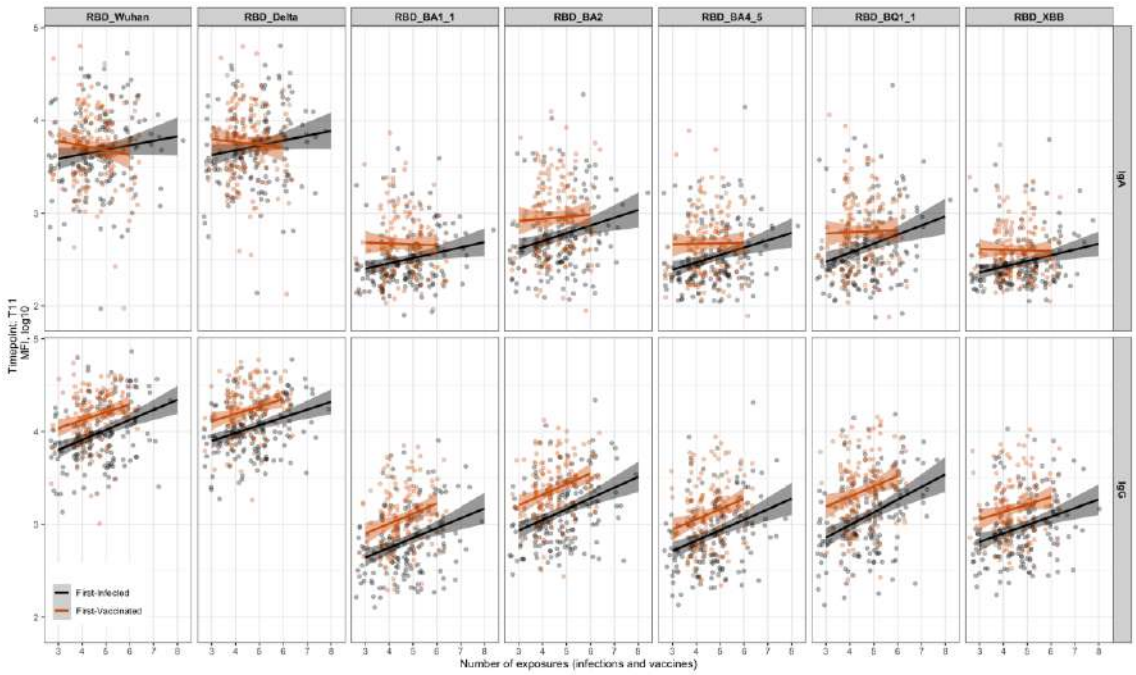
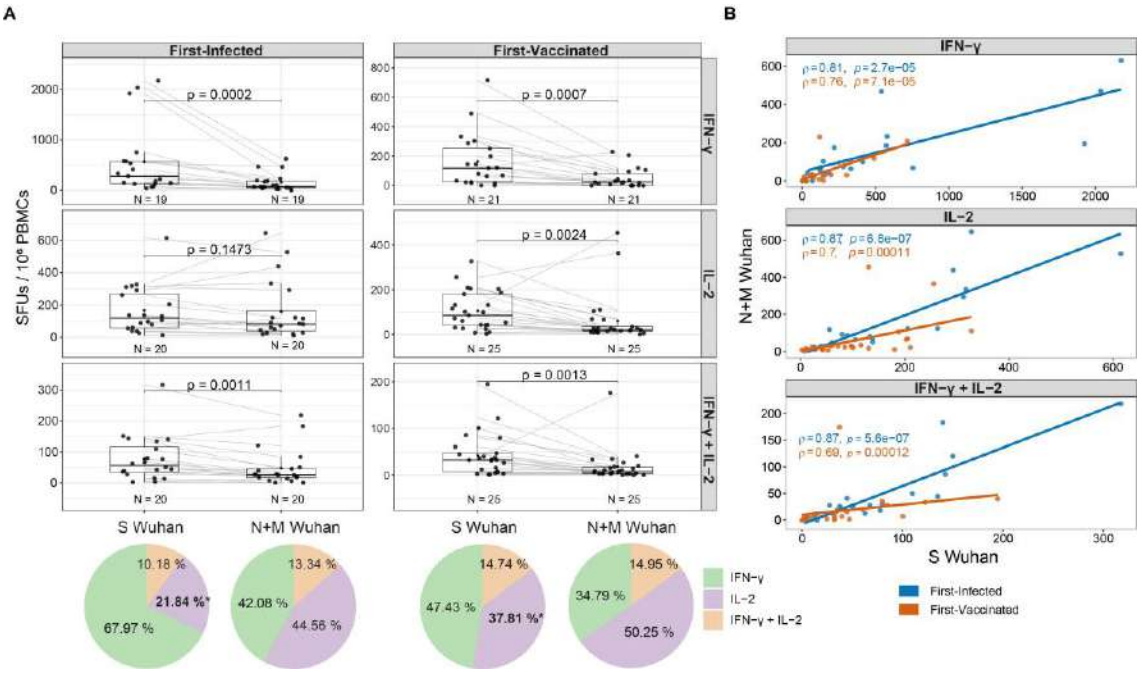


Supplementary eFigure 3. **Simple linear fit between number of exposures and antibody levels (Log_{10}MFI), stratified by the first exposure (infection/vaccine) at T10 (A) and T11 (B).**
Shaded ribbons represent 95% confidence intervals.



Supplementary eFigure 4. Magnitude of T-cell responses at T11 to S and N+M Wuhan antigens by first exposure groups. (**A**) Boxplots representing T-cell responses as SFUs / 10^6 peripheral blood mononuclear cells (PBMCs) secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and pie charts showing the average proportion (%) of secreting T cells by cytokine: IFN-γ (green), IL-2 (purple), or both (orange) to S or N+M from Wuhan by first exposure groups. Responses were compared by paired Wilcoxon Signed-Rank test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Proportions were compared by Chi-square test, statistically significant differences p-values: * ≤ 0.05. (**B**) Spearman's correlation coefficient ρ (Rho) between the magnitude of T-cell responses to S and N+M antigens. Seven participants with ≥100 SFU in unstimulated wells for IFN-γ were excluded from the analysis. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), spike (S), spot-forming units (SFU).

Supplementary eTable1. Mean difference on antibodies levels a T10 and T11 in the first-vaccinated vs the first-infected for infection and vaccine covariates from the main model (Model 5).

For IgA

Isotype	Variable domain	Covariate	RBD	RBD-Delta	RBD-BA1.1	RBD-BA.2	RBD-BA4/5	RBD-BQ1.1	RBD-XBB
IgA	Infection	One Sympt. Omicron	1 (-18 to 25)	3 (-16 to 28)	3 (-12 to 19)	5 (-12 to 25)	5 (-11 to 23)	13 (-6 to 34)	1 (-11 to 15)
		Two Sympt. Omicron	-9 (-36 to 29)	-10 (-37 to 28)	-14 (-34 to 11)	-3 (-28 to 33)	-14 (-35 to 14)	16 (-14 to 58)	-12 (-30 to 10)
		One Symp. non-Omicron	-29 (-54 to 8)	-20 (-47 to 21)	-15 (-35 to 13)	-21 (-43 to 9)	-13 (-35 to 17)	-14 (-38 to 19)	-9 (-28 to 16)
		One Asymp. Omicron	-11 (-31 to 15)	-8 (-29 to 19)	4 (-12 to 23)	8 (-12 to 31)	6 (-11 to 28)	17 (-5 to 43)	5 (-9 to 22)
		Two Asymp. Omicron	59 (-60 to 525)	61 (-58 to 522)	46 (-40 to 255)	89 (-33 to 436)	46 (-44 to 278)	109 (-27 to 497)	44 (-32 to 207)
		One Asymp. non-Omicron	-6 (-29 to 26)	-4 (-28 to 28)	9 (-10 to 32)	8 (-13 to 35)	4 (-15 to 27)	15 (-8 to 44)	6 (-10 to 24)
		Two Asymp. non-Omicron	47 (-62 to 479)	56 (-60 to 499)	-3 (-60 to 137)	24 (-56 to 251)	-21 (-70 to 104)	-28 (-75 to 106)	-19 (-62 to 72)
	Vaccine	Two non-Bivalent doses	20 (-12 to 64)	23 (-10 to 68)	**28 (4 to 57)**	**37 (8 to 75)**	**34 (8 to 67)**	23 (-3 to 57)	**20 (0 to 42)**
		Three non-Bivalent doses	18 (-17 to 68)	23 (-13 to 74)	22 (-4 to 53)	24 (-5 to 63)	26 (-2 to 62)	23 (-7 to 61)	**23 (1 to 50)**
		Four non-Bivalent doses	82 (-13 to 278)	69 (-20 to 259)	34 (-23 to 136)	86 (-5 to 265)	69 (-9 to 215)	**95 (0 to 279)**	40 (-16 to 134)
		One Bivalent dose	**27 (5 to 53)**	**26 (4 to 52)**	9 (-5 to 26)	**27 (8 to 51)**	**25 (7 to 46)**	**37 (16 to 62)**	6 (-6 to 21)

Estimates from linear mixed models estimating the %MFI increase. The model accounted for repeated measurements in the same individual with a random intercept per individual. The model was adjusted as the main model (M5) and included, first exposure, age (restricted cubic spline with 3df) + sex + number of chronic comorbidities and tobacco smoking status + number of non-Omicron and Omicron symptomatic infections (as factors) + number of non-Omicron and Omicron asymptomatic infections (as factors) + number of non-bivalent and bivalent vaccines (as factors) + days from last infection (restricted cubic spline with 3df) + days from last vaccine (restricted cubic spline with 3df).

For IgG

Isotype	Variable domain	Covariate	RBD	RBD-Delta	RBD-BA1.1	RBD-BA.2	RBD-BA4/5	RBD-BQ1.1	RBD-XBB
IgG	Infection	One Sympt. Omicron	-4 (-17 to 12)	-4 (-17 to 11)	13 (-4 to 34)	5 (-11 to 23)	9 (-8 to 30)	16 (-3 to 38)	**28 (9 to 49)**
		Two Sympt. Omicron	-12 (-33 to 16)	-9 (-30 to 18)	11 (-19 to 51)	7 (-21 to 45)	6 (-24 to 47)	37 (-1 to 90)	**85 (40 to 146)**
		One Symp. non-Omicron	-14 (-34 to 12)	-10 (-30 to 16)	-22 (-41 to 3)	-29 (-46 to -7)	-19 (-40 to 9)	-21 (-42 to 8)	-15 (-36 to 12)
		One Asymp. Omicron	2 (-14 to 19)	5 (-10 to 23)	**22 (2 to 44)**	11 (-6 to 31)	**24 (3 to 48)**	**31 (8 to 58)**	**36 (15 to 61)**
		Two Asymp. Omicron	22 (-47 to 180)	13 (-49 to 152)	3 (-57 to 147)	39 (-41 to 226)	3 (-60 to 164)	26 (-53 to 236)	10 (-55 to 165)
		One Asymp. non-Omicron	7 (-11 to 28)	5 (-12 to 25)	6 (-13 to 28)	4 (-13 to 26)	1 (-18 to 24)	5 (-15 to 30)	-4 (-20 to 17)
		Two Asymp. non-Omicron	48 (-36 to 238)	44 (-35 to 220)	-8 (-61 to 121)	2 (-57 to 140)	-22 (-69 to 101)	-26 (-72 to 97)	-18 (-66 to 98)
	Vaccine	Two non-Bivalent doses	**33 (9 to 61)**	**28 (6 to 54)**	**35 (10 to 66)**	**28 (5 to 57)**	**29 (4 to 61)**	**27 (1 to 59)**	**24 (1 to 52)**
		Three non-Bivalent doses	**64 (32 to 105)**	**38 (12 to 70)**	**55 (23 to 96)**	**53 (22 to 92)**	**48 (15 to 90)**	**55 (19 to 100)**	**44 (14 to 82)**
		Four non-Bivalent doses	**98 (5 to 274)**	46 (-19 to 164)	**116 (3 to 356)**	**108 (2 to 323)**	95 (-10 to 324)	**120 (5 to 363)**	**179 (48 to 427)**
		One Bivalent dose	**20 (3 to 40)**	**30 (12 to 50)**	19 (-1 to 42)	**28 (8 to 51)**	**32 (10 to 59)**	**43 (20 to 72)**	**64 (40 to 91)**

Estimates from linear mixed models estimating the %MFI increase. The model accounted for repeated measurements in the same individual with a random intercept per individual. The model was adjusted as the main model (M5) and included, first exposure, age (restricted cubic spline with 3df) + sex + number of chronic comorbidities and tobacco smoking status + number of non-Omicron and Omicron symptomatic infections (as factors) + number of non-Omicron and Omicron asymptomatic infections (as factors) + number of non-bivalent and bivalent vaccines (as factors) + days from last infection (restricted cubic spline with 3df) + days from last vaccine (restricted cubic spline with 3df).

Supplementary eTable2. Unadjusted and adjusted Cox Proportional Hazard model for the association between first exposure (first-vaccinated/first-infected) and breakthrough infections

	Number of events/Number at Risk	Person-years	HR (95% CI) Unadjusted	HR (95% CI) Adjusted*
Main analysis				
Period T8-T9	29/182	31.4	1.63 (0.66 - 4.01)	3.48 (1.05 - 11.5)
Period T9-T10	83/263	105.4	0.66 (0.41 - 1.08)	0.50 (0.31 - 0.82)
Period T10-T11	68/280	106.2	0.81 (0.50 - 1.33)	0.71 (0.42 - 1.17)
Sensitivity analysis, without considering asymptomatic infections				
Period T8-T9	25/178	30.4	1.89 (0.73 - 4.63)	4.97 (1.41 - 17.5)
Period T9-T10	55/235	98.1	0.53 (0.27 - 1.01)	0.46 (0.24 - 0.88)
Period T10-T11	28/240	96.6	0.54 (0.23 - 1.22)	0.44 (0.19 - 1.02)

* Model adjusted for age, sex, number of comorbidities, smoking status, number of previous exposures, and time since last exposure.

Chapter 5

Initial antigen encounter determines robust T-cell immunity against SARS-CoV-2 BA.2.86 variant three years later

Rubio R, Yavlinsky A, Escalera Zamudio M, Molinos-Albert LM, Martín Pérez C, Pradenas, Canyelles M, Torres C, Tan C, Swadling L, Ramírez-Morros A, Trinité B, Vidal-Alaball J, Aguilar R, Ruiz-Comellas A, Blanco J, van Dorp L, Balloux F, Dobaño C•✉, Moncunill G•✉

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Initial antigen encounter determines robust T-cell immunity against SARS-CoV-2 BA.2.86 variant three years later

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SUMMARY

Objectives: We aimed to evaluate the adaptive immune responses cross-recognition of the hypermutated SARS-CoV-2 BA.2.86 variant and identify the determinants influencing this recognition.
Methods: We measured BA.2.86 neutralizing antibodies and T-cell responses cross-reactivity in previously exposed participants. We investigated clinical-demographic factors and used a novel in silico analysis to assess viral genetic determinants affecting T-cell responses.
Results: Despite notable escape from neutralizing antibodies, T-cell responses remained generally preserved, albeit with a significant but small loss in T-cell cross-recognition (7.5%, 14.2%, and 10.8% average loss for IFN-γ, IL-2, and IFN-γ + IL-2, respectively, $p < 0.05$). This is consistent with the prediction of 6 out of 10 immunodominant T-cell epitopes (TCEs) altered by BA.2.86 mutations to have reduced peptide presentation. This effect is expected to be mitigated by total TCEs across the genome. Remarkably, T-cell responses and cross-recognition were 3.5 (IFN-γ), 2 (IL-2) and 2.4 (IFN-γ + IL-2) times higher when first induced by infection rather than by vaccination three years earlier, by increasing number of infections, and by ancestral/Delta than Omicron infections.
Conclusions: Our findings underscore the critical role and factors influencing T-cell immunity against evolving SARS-CoV-2 variants, such as first antigen encounter (vaccination or infection), as it is essential for developing effective control strategies.

Introduction

The evolution of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to the emergence and dominance of Omicron sublineages.[1] Currently (as of July 2024), the Omicron subvariant

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BA.2.86, also known as 'Pirola', and its immediate descendants, are the predominant variants circulating globally, accounting for over 99% of SARS-CoV-2 cases.[2] This variant has raised significant concerns due to its 63 amino acid (aa) changes compared to the ancestral SARS-CoV-2 Wuhan spike (S) protein. These changes include 51 aa substitutions, 8 aa deletions, and 4 aa insertions.[3] The BA.2.86 variant exhibits a substantial genetic divergence from its predecessor, the BA.2 variant, with 38 aa changes in the S protein. This magnitude of change is comparable to the genetic leap observed between the Delta and Omicron variants.[3,4] BA.2.86 has evolved by acquiring convergent mutational sites that optimize the host receptor angiotensin-converting enzyme 2 (ACE2) binding affinity, thereby enhancing infectivity and enabling immune evasion.[3,5,6] Despite high vaccine coverage worldwide, these mutations may reduce vaccine effectiveness against infection, significantly impacting health and socioeconomic conditions.[7]

Several recent studies have demonstrated that BA.2.86 exhibits extensive immune evasion from pre-existing humoral responses induced by vaccination, infection or any combination of both.[8–12] However, few studies have examined the ability of S-specific T cells to cross-recognize BA.2.86 in-silico[4,13] or in-vivo.[1,14]

Since it has been described that humoral responses elicited by current vaccines or SARS-CoV-2 infections are shorter-lived than T-cell responses,[15–17] understanding the potential effects of viral mutations on cellular immune evasion is crucial for our knowledge of long-term immunity against SARS-CoV-2. Hence, we aimed to investigate the ability of BA.2.86 to escape pre-existing immunity, focusing particularly on T-cell responses and their determinants. To this end, we measured the cross-reactivity of neutralizing antibodies and T-cell responses in individuals previously exposed to infection and/or mRNA vaccination. Data were analyzed in relation to clinical and sociodemographic characteristics. Additionally, we employed a novel in-silico analysis to assess potential viral genetic determinants contributing to the differential T-cell responses and their impact on peptide binding affinity.

Methods

Study design

Blood samples collected between May and June 2023 from 52 healthcare workers in the CovidCatCentral longitudinal cohort study created in 2020 in Barcelona, Spain[18–20] were used to assess the BA.2.86 evasion ability from adaptive immune responses. The BA.2.86 variant was first documented in Spain on August 22nd, 2023. Plasma and cryopreserved peripheral blood mononuclear cells (PBMCs) from venous blood samples were used for neutralization and cellular assays, respectively. Sociodemographic and clinical information were recorded at each cross-sectional visit. SARS-CoV-2 asymptomatic or undiagnosed infections were identified by serology through fold change (FC) in antibody levels between timepoints. For participants vaccinated between timepoints, an individual was considered infected when FC ≥ 4 for IgG or IgA against the nucleocapsid (N) antigen. For those not vaccinated between timepoints, an individual was considered infected when at least two antibody-antigen pairs among IgG and IgA against any of the S or N antigens had a FC ≥ 4.[21] In the absence of sequencing data, we inferred probable variant infection based on the predominant viral variant circulating in Catalonia at the date of infection.[22,23] The study protocol was approved by the IDIAP Jordi Gol Ethics committee (code 20/162-PCV), and written informed consent was obtained from all participants.

Plasma neutralizing activity

Pseudovirus-based neutralization assay was performed using HIV reporter pseudoviruses expressing SARS-CoV-2 ancestral (Wuhan-1) and BA.2.86 S proteins and Luciferase gene, as previously reported.[24] The assay was performed in duplicate. Briefly, in 96-well cell culture plates (Thermo Fisher Scientific), 200 $TCID_{50}$ (50% tissue culture infectious dose) of pseudovirus were preincubated with three-fold serial dilutions (1/60–1/14,580) of heat-inactivated plasma samples at 37 °C for 1 h. Then, 1×10^4 HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. Results were read after 48 h using the EnSight Multimode Plate Reader and BriteLite Plus Luciferase reagent (PerkinElmer, USA). The values were normalized, and the ID50 (the reciprocal dilution inhibiting 50% of the infection) was calculated by plotting and fitting the log of plasma dilution versus response to a 4-parameters equation in Prism 10 (GraphPad Software, USA).

Cellular assay

The magnitude of the T-cell responses to the S protein from Wuhan and BA.2.86, as well as to the N and membrane (M) proteins from Wuhan, was measured using the human IFN-γ/IL-2 FluoroSpot kit (Mabtech) as previously described.[25] The peptide pools used as stimulus included the full-length S, N and M proteins from ancestral [PepTivator® SARS-CoV-2 Prot_S, Prot_N, Prot_M (Miltenyi)], and full-length S from BA.2.86 [PepMix™ SARS-CoV-2 (Spike BA.2.86) (JPT)]. The peptides were 15 amino acids long with 11-amino acids overlaps and were dissolved in sterile water according to the manufacturer's instructions.

PBMCs were isolated from venous blood samples by density-gradient centrifugation using Ficoll-Paque (Merck), cryopreserved in heat-inactivated fetal bovine serum (HI-FBS) (Thermo Fisher Scientific) with 10% dimethyl sulfoxide (Merck), and stored in liquid nitrogen until use. After blocking the pre-coated FluoroSpot plates with culture medium-10% HI-FBS, 2×10^5 thawed PBMCs (with cell viability ≥ 70% after overnight resting) were added to the stimulus (1 µg/mL/peptide concentration) or unstimulated control (only culture medium [TexMACS Medium (Miltenyi)−1% penicillin/streptomycin (Thermo Fisher Scientific)] wells, and 5×10^4 PBMCs to the positive control (phytohemagglutinin (PHA) (Merk), 5 µg/mL) wells. All conditions were performed in duplicate, and were incubated at 37 °C and 5% CO_2 for 20 h.

Cells secreting IFN-γ and/or IL-2 were detected and counted as spot-forming units (SFU). Seven participants with ≥ 100 SFU in unstimulated wells for IFN-γ were excluded from the analysis. SFU counts in the unstimulated wells were subtracted from those in the stimulated wells to account for background responses, and negative values were set to zero. The results were expressed as SFU / 10^6 PBMCs. Responses were considered positive if the results were ≥ 3-fold the mean of their unstimulated wells for each cytokine and stimulus. Responders were defined as having a positive response to at least one cytokine-stimulus combination. SARS-CoV-2 non-responders showed a positive response to the positive control PHA.

Binding antibody assay

Luminex technology was used to measure binding IgM, IgG, and IgA levels (as median-fluorescence-intensity (MFI)) to the ancestral S, its subregions S2 and the RBD antigens from plasma samples as previously described.[18]

Viral genetic determinants

We employed a pioneering bioinformatic approach to assess viral genetic mutations driving T-cell responses. After filtering out genomes derived from non-human hosts, and those incomplete or with low-coverage from the complete 16.6 million SARS-CoV-2 genome sequence data available from GISAID,[26] we obtained a dataset of 15 million sequences comprising a total of 27,503 mutations within S. Data were stratified according to virus lineage, aggregating data

under the following criteria: 1) Earliest genome sequences (Wuhan-1 and those with collection dates before March 1st, 2020), 2) Pre-variant of concern (VOC) lineages (genomes predating Alpha), 3–7) All VOCs: Alpha, Beta, Gamma, Delta and Omicron (each analyzed separately), and 8) Variants of interest (VOI) Pirola (comprising the BA.2.86, JN.1, and descending sublineages). Heatmaps were generated in Python v3.8.10 using the matplotlib and seaborn packages. Each heatmap represents the normalized count score for the T-cell epitope (TCE) within a given VOC/VOI stratum.

Normalized indel count scores

For each VOC/VOI, and for each $CD4^+$ and $CD8^+$ TCE, we counted the number sequences with indels affecting one or more sites at any position within a given TCE. We then divided count values by the total number of sequences belonging to each VOC/VOI, in order to obtain a normalized VOC/VOI indel score for each TCE.

Normalized substitution count scores

For each VOC/VOI, and for each $CD4^+$ and $CD8^+$ TCE, we considered only sequences in which the TCE was unaffected by indels. For each $CD4^+$ TCE, we counted the number of sites affected by substitutions across the entire TCE. For each $CD8^+$ TCE, we restricted substitution counts to only for anchor point binding to major histocompatibility complexes (MHC)-I (corresponding to positions 1–2, 9–10 of each $CD8^+$ TCE). For each VOC/VOI-TCE combination, we divided total count by the number of sequences considered.

In-silico predictions for mutation impacting epitope function

The impact of mutations within known TCEs was assessed using NetMHCpan[27] version 4.1 and NetMHCIIpan[28] version 4.1. The predicted binding of peptides corresponding to the original epitope sequence in Wuhan-Hu-1, the epitope sequence in BA.2.86 containing lineage defining mutations (LDMs), or the epitope sequence in other lineages (e.g. JN.1) was calculated for the known MHC restriction. Where the MHC restriction was not known, human-leukocyte-antigen (HLA) supertype representatives were used and included when weak or strong binding was predicted for the Wuhan-Hu-1 peptide sequence. A threshold of rank 0.5% and 2.0% were used to define strong and weak binders for MHC-I restricted epitopes and 1% and 5.0% for MHC-II restricted epitopes. Loss of an epitope due to reduced peptide-MHC binding was estimated when a peptide went from a strong binder to a weak, or a weak binder to a non-binder. Partial loss was defined as an increase in rank for the known restricting MHC and/or several predicted restricted MHCs of > 0.5%.

Statistical analyses

Sociodemographic and clinical data were compared between groups of first antigen encounter using the CompareGroups R CRAN package.[29] For continuous normal variables, the mean and s.d. were calculated, and t-test were applied to assess differences. For continuous non-normal variables, the median and the first and third quartiles were calculated. For categorical variables, differences in proportions were calculated using chi-square test or Fisher's exact test, when applicable.

Nonparametric tests were used to analyze neutralizing antibody and T-cell data. Nominal p-values of < 0.05 were considered statistically significant. Adaptive responses to Wuhan and BA.2.86 were compared using paired Wilcoxon-Signed-Rank-test. BA.2.86 recognition was assessed by calculating the FC in adaptive BA.2.86 responses with respect to the ancestral strain (BA.2.86 / ancestral). Proportions (%) of secreting T cells induced by Wuhan vs. BA.2.86 were compared using the Chi-square test. Comparisons of T-cell responses between sociodemographic and clinical groups were performed by the Wilcoxon rank-sum test.

A multivariable linear regression model was fitted to assess the association between the magnitude of the T-cell responses to the ancestral strain and BA.2.86 as the outcome variables and first antigen encounter infection as a predictor variable. This model was adjusted for the number of vaccine doses (continuous), total infections (continuous), and probable variant of infection (categorical). For the linear regression model, we checked the linearity of the data, normality of residuals, homogeneity of residual variance, independence of the residual error terms, and multicollinearity among the predictor variables. The models performance for ancestral and BA.2.86 S had an Adjusted R^2 of 0.37 and 0.36 for IFN-γ, 0.18 and 0.33 for IL-2 and 0.25 and 0.37 for IFN-γ + IL-2, respectively. The models performance for ancestral N+M had an Adjusted R^2 of 0.23, 0.07 and 0.23 for IFN-γ, IL-2, and IFN-γ + IL-2, respectively.

Correlations were assessed using Spearman's rank correlation coefficient ρ (rho), and p-values were computed via the asymptotic t approximation. All data processing and statistical analyses were performed using R software version 4.2.3.

Data availability

All data are available from the corresponding authors upon request.

Results

Description of study population

We measured neutralizing antibodies and T-cell responses to Wuhan and BA.2.86 variant in blood samples from 52 healthcare workers participating in a prospective live COVID-19 cohort created in 2020, in blood samples collected between May and June 2023.[18–20] Clinical-demographic characteristics of participants are depicted in Table 1. The majority were female (85%) with an average age of 49 years (mean 49.17, s.d. 10.90). All participants had received three or four mRNA vaccine doses. The fourth dose was primarily (11/13) bivalent Original + Omicron BA.4/5. The median time since last vaccination was approximately 17 months. Among the participants, 49 had hybrid immunity and 3 had only vaccine-induced immunity. Forty-one individuals were likely infected with any of the Omicron subvariants due to the timing of infection, with a median time since last symptomatic infection of ~16 months. Twenty-one participants had natural infection as their first antigen encounter (first-infected) and were then vaccinated, while 31 had vaccination as first exposure (first-vaccinated) (Table 2). Half of the participants reported at least one comorbidity, including asthma (3), cardiac (2), digestive (2), autoimmune (2), mellitus diabetes (1), dyslipidaemia (3), arterial hypertension (6), hypothyroidism (4), obesity (7), and allergies (6). Most participants were non-smoker, and none had long COVID.

Memory immune responses to Wuhan and BA.2.86

The neutralizing activity of plasma antibodies and S-specific T-cell responses to both Wuhan and BA.2.86 were measured using pseudovirus neutralization and IFN-γ/IL-2 FluoroSpot assays, respectively. After the last COVID-19 exposure (median 466, IQR: 188–469 days), with 85% of participants having been infected with the Omicron variant, the plasma neutralizing activity to BA.2.86 was significantly compromised (94.25% average loss, p < 0.0001) compared to the Wuhan (Fig. 1A). In contrast, S-specific T-cell responses to BA.2.86 were significantly but only slightly reduced (7.5%, 14.2% and 10.8% average loss for IFN-γ, IL-2 and IFN-γ + IL-2, respectively, p < 0.05) than those to ancestral strain (Fig. 1B). Additionally, T-cell responses to BA.2.86 strongly correlated with those to the Wuhan (rho = 0.91, p < 0.001, Fig. S1). To quantify the BA.2.86 cross-

Table 1
Sociodemographic and clinical characteristics of study participants.

		Entire cohort	N
Sex: n (%)			52
Female		44 (84.6%)	
Age (years): mean (s.d.)		49.17 (10.90)	52
First antigen exposure: n (%)			
Infection		21 (40.4%)	
Vaccination		31 (59.6%)	
Breakthrough infections (BTIs): n (%)			52
0		7 (13.5%)	
1	1 symptomatic	27 (51.9%)	
	1 asymptomatic	6 (11.5%)	
2	2 symptomatic	2 (3.85%)	
	1 symptomatic + 1 asymptomatic[a]	8 (15.4%)	
3	2 asymptomatic + 1 symptomatic[a]	2 (3.85%)	
Total infections[b]: n (%)			52
0		3 (5.77%)	
1		25 (48.1%)	
2		19 (36.5%)	
≥3		5 (9.62%)	
Probable variant of infection n (%)			49
D614G/Delta		8 (16.3%)	
Omicron		23 (48.1%)	
D614G/Delta + Omicron		18 (36.7%)	
Vaccine doses: n (%)			52
3		39 (75.0%)	
4		13 (25.0%)	
Vaccine regimens: n (%)			52
3	Pfizer + Pfizer + Moderna	20 (38.46%)	
	Pfizer + Pfizer + Pfizer	18 (34.62%)	
	Moderna + Moderna + Moderna	1 (1.92%)	
4	Pfizer + Pfizer + Pfizer + Pfizer	9 (17.31%)	
	Pfizer + Pfizer + Moderna + Pfizer	4 (7.69%)	
Total exposures[c]: n (%)			52
4		24 (46.2%)	
5		18 (34.6%)	
≥6		10 (19.2%)	
Time since last symptomatic infection (days): median [IQR]		466 [324;663]	48
Time since last vaccine dose (days): median [IQR]		504 [343;524]	52
Time since last exposure[d] (days): median [IQR]		334 [188;469]	52
Seropositive:		52 (100%)	
Any comorbidity[e]:		25 (48.1%)	52
Smoking:			52
Active smoker		8 (15.4%)	
Previous smoker		11 (21.2%)	
Non-smoker		33 (63.5%)	
Long COVID		0 (0%)	

[a] Whatever the order.
[b] Include symptomatic and asymptomatic infections.
[c] Include symptomatic and asymptomatic infections and vaccine doses.
[d] Include symptomatic infections and vaccine doses.
[e] Include Asthma (3), Cardiac (2), Digestive (2), Autoimmune (2), Mellitus diabetes (1), Dyslipidaemia (3), Arterial hypertension (6), Hypothyroidism (4), Obesity (7), and Allergies (6).

Table 2
Sociodemographic and clinical characteristics by groups of first antigen encounter.

		Infected	Vaccinated	p-value
		N=21	N=31	
Sex: n (%)				1.000
Female		18 (85.7%)	26 (83.9%)	
Age (years): mean (s.d.)		50.29 (10.81)	48.42 (11.09)	0.588
Infection pre-1st dose		21 (100%)	0 (0%)	
Breakthrough infections (BTIs): n (%)				**0.026**
0		4 (19.05%)	3 (9.68%)	
1	1 symptomatic	7 (33.33%)	20 (64.52%)	
	1 asymptomatic	5 (23.81%)	1 (3.23%)	
2	2 symptomatic	0 (0%)	2 (6.45%)	
	1 symptomatic + 1 asymptomatic[a]	3 (14.29%)	5 (16.12%)	
3	2 asymptomatic + 1 symptomatic[a]	2 (9.52%)	0 (0%)	
Total infections[b]: n (%)				**< 0.001**
0		0 (0%)	3 (9.68%)	
1		4 (19.0%)	21 (67.7%)	
2		12 (57.1%)	7 (22.6%)	
≥3		5 (23.8%)	0 (0%)	
Probable variant of infection: n (%)				**< 0.001**
D614G/Delta		4 (19%)	4 (14.3%)	
Omicron		0 (0%)	23 (82.1%)	
D614G/Delta + Omicron		17 (81.0%)	1 (17.9%)	
Vaccine doses: n (%)				0.870
3		15 (71.4%)	24 (77.4%)	
4		6 (28.6%)	7 (22.6%)	
Vaccine regimens: n (%)				0.816
3	Pfizer + Pfizer + Moderna	7 (33.3%)	13 (41.9%)	
	Pfizer + Pfizer + Pfizer	7 (33.3%)	11 (35.5%)	
	Moderna + Moderna + Moderna	1 (4.76%)	0 (0%)	
4	Pfizer + Pfizer + Pfizer + Pfizer	4 (19.0%)	5 (16.1%)	
	Pfizer + Pfizer + Moderna + Pfizer	2 (9.52%)	2 (6.45%)	
Total exposures[c]: n (%)				**<0.001**
3		4 (19.0%)	0 (0%)	
4		7 (33.3%)	20 (64.5%)	
≥5		10 (47.6%)	11 (35.5%)	
Time since last symptomatic infection (days): median [IQR]		492 [260;1145]	460 [334;485]	0.201
Time since last vaccine dose (days): median [IQR]		504 [231;523]	508 [426;524]	0.845
Time since last exposure[d] (days): median [IQR]		233 [195;412]	343 [188;470]	0.634
Seropositive:		21 (100%)	31 (100%)	.
Any comorbidity[e]:		14 (66.7%)	11 (35.5%)	0.054
Smoking:				0.085
Active smoker		1 (4.76%)	7 (22.6%)	
Previous smoker		3 (14.3%)	8 (25.8%)	
Non-smoker		17 (81.0%)	16 (51.6%)	
LongCOVID		0 (0%)	0 (0%)	.

Bold p-values indicate statistical significance.
[a] Whatever the order.
[b] Include symptomatic and asymptomatic breaktrhough infections.
[c] Include asymptomatic and symptomatic infections and vaccine doses.
[d] Include symptomatic infections and vaccine doses.
[e] Include Asthma (3), Cardiac (2), Digestive (2), Autoimmune (2), Mellitus diabetes (1), Dyslipidaemia (3), Arterial hypertension (6), Hypothyroidism (4), Obesity (7), and Allergies (6).

recognition, we calculated the FC of BA.2.86 responses relative to the ancestral strain (Fig. 1C-D). Despite BA.2.86 effectively evading a significant proportion of neutralizing antibodies, T-cell responses remained relatively intact. Nearly all participants (92.31%) had detectable T-cell responses to Wuhan, with a slight decrease in responders (88.24%) observed for BA.2.86 (Fig. 1E). T-cell responses to Wuhan and BA.2.86 predominantly secreted IFN-γ (61% and 63%, respectively), followed by IL-2 (28% and 26%), with a minority secreting IFN-γ + IL-2 (12% and 11%), indicative of polyfunctionality (Fig. 1E). Correlations between plasma neutralizing activity and T-cell responses for Wuhan or BA.2.86 were notably weak (rho ranging -0.002 and 0.22, $p > 0.05$, Fig. S2).

Clinical-demographic factors influencing T-cell responses to BA.2.86

We aimed to elucidate the clinical-demographic factors associated to S BA.2.86 recognition by T cells. No associations in the T-cell responses to BA.2.86 or cross-recognition were found in relation to sex ($p > 0.05$, Fig. S3), presence of any comorbidity ($p > 0.05$, Fig. S4), smoking status ($p > 0.05$, Fig. S5), immunity groups (hybrid immunity vs. only vaccinated) ($p > 0.05$, Fig. S6), number of vaccine doses ($p > 0.05$, Fig. S7), total number of exposures (including both vaccine doses and infections) ($p > 0.05$, Fig. S8), nor time since last

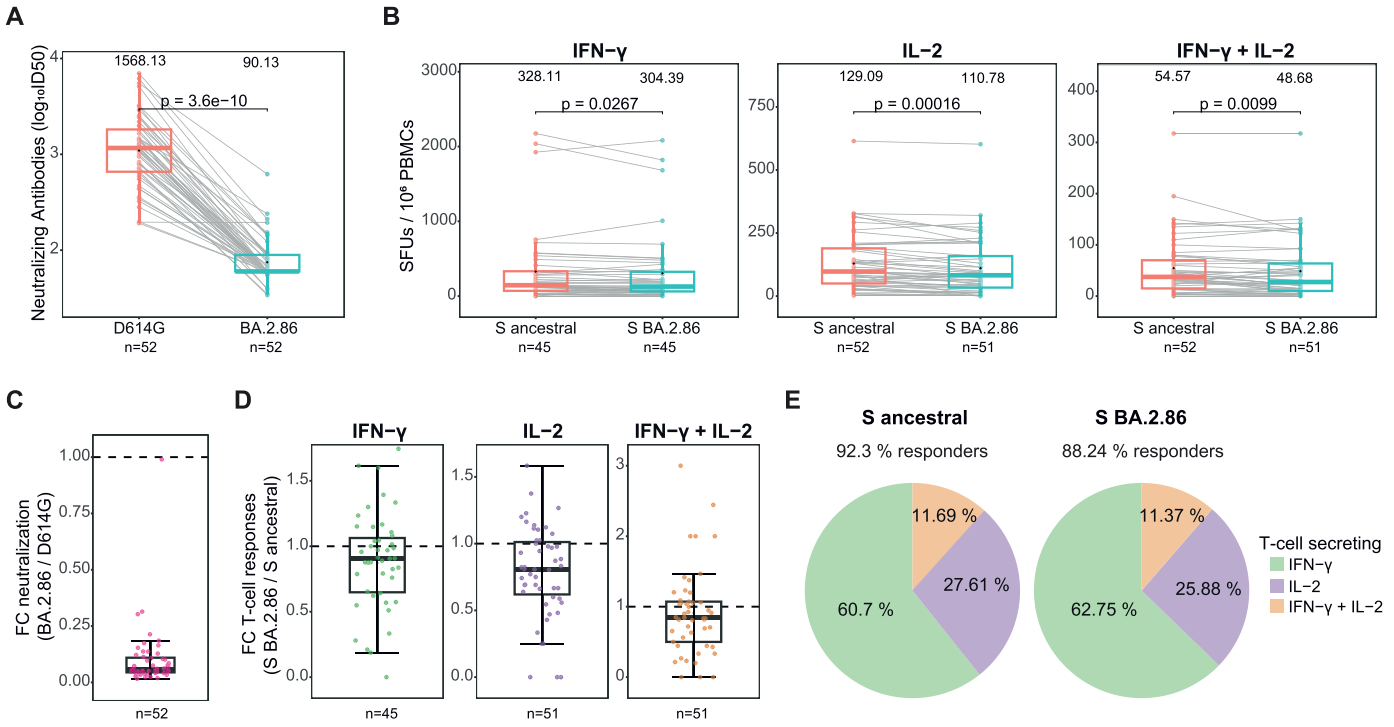

Fig. 1. Plasma neutralizing activity and T-cell responses to Wuhan and BA.2.86. Neutralizing activity as log_{10}ID50 (**A**) and S-specific T-cell responses as SFUs / 10^6 peripheral blood mononuclear cells (PBMCs) secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) (**B**) to ancestral and BA.2.86. Responses were compared by paired Wilcoxon Signed-Rank test. Mean values are on the top. Fold change in neutralizing activity (**C**) and T-cell responses (**D**) to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral). Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). (**E**) Percentage of responders and pie charts showing the average proportion (%) of secreting T cells by cytokine. Proportions were compared by Chi-square test, and there were not statistically significant differences. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), spike (S), spot-forming units (SFU), D614G (ancestral).

exposures (infection, vaccination or any of them) (rho ranging 0.03 and 0.31, p > 0.05, Fig. S9).

Decreased T-cell responses to BA.2.86 by Omicron infection

While no differences in the magnitude of T-cell responses to BA.2.86 were found based on the number of infections (p > 0.05, Fig. S10), individuals who had experienced only one infection exhibited decreased recognition of BA.2.86 by IL-2 and polyfunctional secreting T cells compared to participants infected twice (Fig. 2A). Subsequently, we investigated which variants were responsible for influencing BA.2.86 T-cell recognition. In the absence of sequencing data, we inferred probable variant infection based on the predominant viral variant circulating in Catalonia at the date of infection.[22,23] Characteristics of the cohort by probable variant of infection groups are detailed in Table S1. Participants infected by earlier variants, ancestral or Delta, exhibited a greater magnitude of T-cell responses to Wuhan and BA.2.86 compared to participants infected with Omicron variants, although differences only reached statistical significance for the Wuhan S and N+M (Fig. 2B and S11). Furthermore, individuals infected with only Omicron variants showed a decreased magnitude of T-cell responses (by IFN-γ and polyfunctional T cells) to BA.2.86 (Fig. 2B), and decreased BA.2.86 recognition by IL-2 secreting T cells (Fig. 2C) than individuals infected with both earlier strains (ancestral or Delta) and Omicron.

Increased T-cell responses to BA.2.86 by infection before vaccination three years earlier

We found that participants who had been infected before vaccination (first-infected) showed an increased magnitude of T-cell responses three years later to both S ancestral (3.5 and 1.9 times higher for IFN-γ and IFN-γ + IL-2, respectively) and BA.2.86 (3.5, 2 and 2.4 times higher for IFN-γ, IL-2 and IFN-γ + IL-2, respectively) compared to participants without infection before vaccination (first-vaccinated) (Fig. 2D). Similarly, first-infected individuals exhibited an increased magnitude of T-cell responses to N+M from the ancestral strain (3.5, 2.9, and 3 times higher for IFN-γ, IL-2 and IFN-γ + IL-2, respectively) three years later compared to those first-vaccinated (Fig. S11). Additionally, first-infected participants exhibited 1.5 higher BA.2.86 cross-recognition by IL-2 secreting T cells (Fig. 2E). After adjusting in multivariable linear regression models for the potential confounders (number of vaccine doses, total infections, and probable variant) (Table 2), infection before vaccination was still significantly associated with increased magnitude of T-cell responses to Wuhan and BA.2.86 strains three years after exposure compared to individuals who were first-vaccinated (Fig. 2F and S11).

Furthermore, in the first-infected group, the magnitude of T-cell responses to both Wuhan and BA.2.86 three years after the first SARS-CoV-2 infection was positively correlated with the binding antibody levels measured five months (mean 151.7, IQR 60.5 (days)) after first infection (Fig. 2G), especially to IgG (rho for Wuhan: IFN-γ 0.66, IL-2 0.51, IFN-γ + IL-2 0.59, p < 0.05; rho for BA.2.86: IFN-γ 0.66, IL-2 0.52, IFN-γ + IL-2 0.59, p < 0.05) and IgM anti-S antigens (rho for Wuhan: IFN-γ 0.74, IL-2 0.72, IFN-γ + IL-2 0.63, p < 0.01; rho for BA.2.86: IFN-γ 0.69, IL-2 0.67, IFN-γ + IL-2 0.57, p < 0.05). Conversely, in the first-vaccinated group, the magnitude of T-cell responses 28 months after primary vaccination (January 2021) was not correlated with any of IgG, IgA and IgM levels measured three months (mean 93.42, IQR 11 (days)) after primary vaccination (rho < 0.12, p > 0.05, Fig. 2G). Moreover, there was no correlation between the magnitude of T-cell responses to any of the two lineages and the antibody levels measured at the same time point in the first-vaccinated or first-infected group, except for IgG and IgM anti-S2 in the first-infected group (rho ≈ 0.54, p < 0.05, Fig. S12).

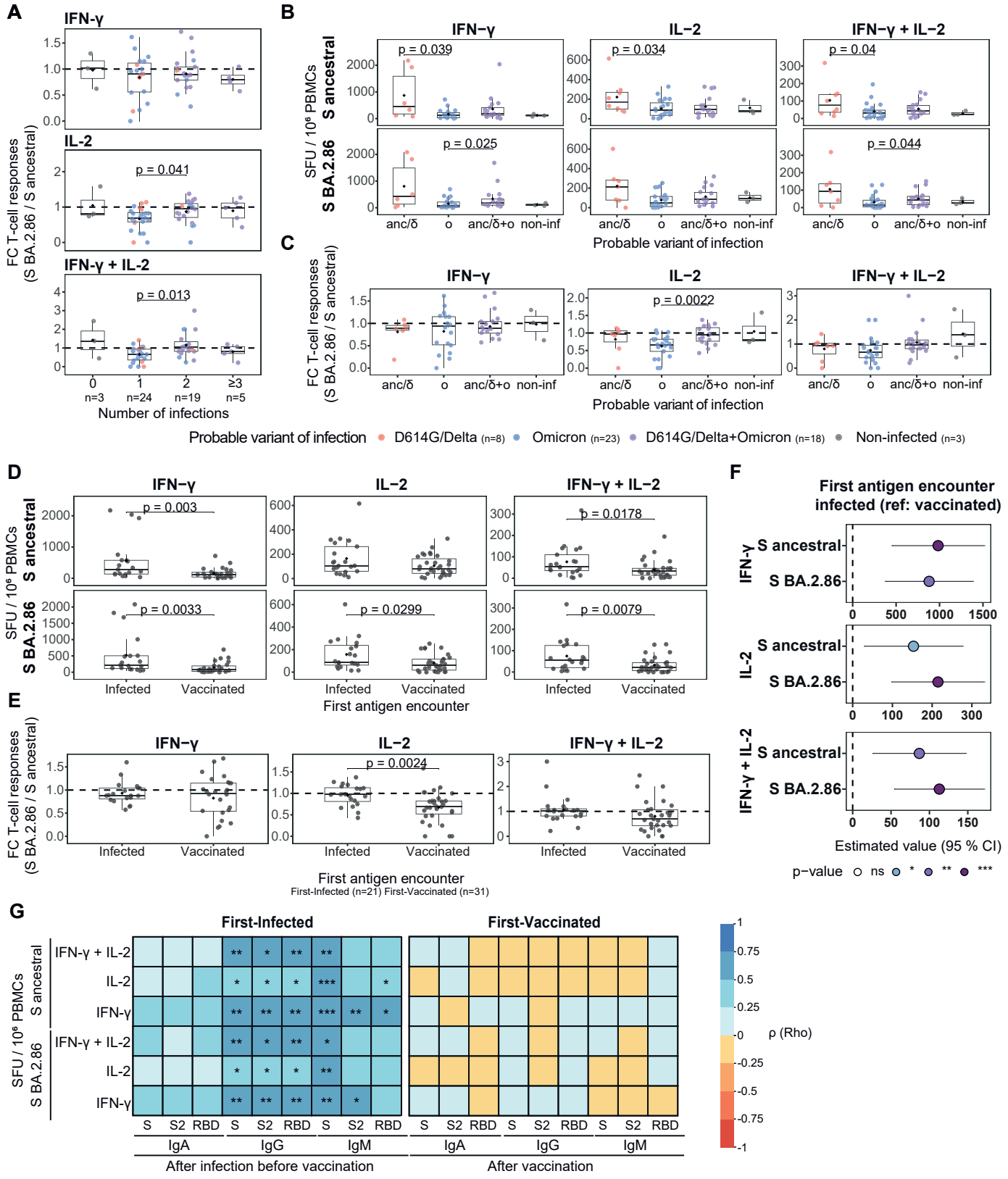

Fig. 2. Factors influencing T-cell responses to ancestral and BA.2.86 spike. (**A**) BA.2.86 recognition as fold change in BA.2.86 T-cell responses with respect to ancestral strain by number of SARS-CoV-2 infections. (**B**) Magnitude of T-cell responses as SFU / 10^6 peripheral blood mononuclear cells (PBMCs) of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**C**) BA.2.86 T-cell recognition by probable variant of infection. (**D**) Magnitude of T-cell responses and (**E**) BA.2.86 recognition by first antigen encounter. T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). (**F**) Forest plot showing the association of being infected before vaccination with magnitude of T-cell responses. The represented values and CI show the SFU / 10^6 PBMCs increase in individuals infected before vaccination compared to individuals not infected prior vaccination. Multivariable linear regression models were fitted to calculate the estimates (dots) and 95% confidence intervals (lines) and adjusted for the number of vaccine doses, total infections and the probable variant of infection. (**G**) Heatmaps illustrating the Spearman's correlation coefficient ρ (rho) between the magnitude of T-cell responses three years post-first infection or 28 months post-vaccination with the antibody responses (median fluorescence intensity (MFI) of IgA, IgG, and IgM) to S, S2 and RBD from ancestral strain 5 months post- first infection (first-infected) and 3 months post-first vaccination (first-vaccinated). p-values: * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), spike (S), receptor binding domain (RBD), spot-forming units (SFU).

Genomic correlates of T-cell responses

We then investigated potential genetic determinants as contributors to the differential T-cell responses observed to BA.2.86 compared to other virus lineages. Through a novel in-silico analysis of a comprehensive dataset comprising approximately 16 million SARS-CoV-2 genomes available from GISAID,[26,30] we computed mutation frequencies for indels (insertions or deletions) and point substitutions within the S protein, normalized by the total number of sequences per virus lineage. Assuming that aa changes within TCEs may lead to a reduction or loss of epitope binding/recognition, we mapped the mutations onto the S protein sequence to identify those falling within immunodominant $CD8^+$ and $CD4^+$ TCEs reported.[31] This list of S mutations represents the most comprehensive TCE data associated with SARS-CoV-2 available to date (Tables S2 and S3). We considered substitutions and indels separately, as the latter are expected to have a stronger impact on epitope function (e.g., full disruption of recognition sites).

Lineage-specific patterns of mutations within T-cell epitopes

Immunodominant $CD8^+$ (n=41) and $CD4^+$ (n=55) TCEs locate within the S1 and S2 protein domains (Tables S2 and S3). An apparent lineage-specific pattern for indels and substitutions was observed for both $CD8^+$ and $CD4^+$ TCEs, in which Omicron (defined as the B.1.1.529 and descending sublineages prior to the emergence of the BA.2.86) and BA.2.86 share similar profiles (Fig. 3).

For indels, $CD8^+$ TCEs 9 and 10 (Fig. 3A) and $CD4^+$ TCEs 3, 9, and 10 (Fig. 3C), all falling within the N-terminal domain (NTD) of S1 were affected. Although affected TCEs were the same between Omicron and the BA.2.86 variant, average indel counts in the BA.2.86 were approximately 3-fold higher than in Omicron (3.17-fold for epitope 9 and 3.12-fold for epitope 10 in $CD8^+$, and 2.75-fold for epitope 3 and 3.12 for epitopes 9 and 10 in $CD4^+$ TCEs).

For substitutions, a higher proportion of $CD8^+$ and $CD4^+$ TCEs were found to be affected (Fig. 3B and D), consistent with these being better tolerated at the protein function level. Again, Omicron and BA.2.86 shared a similar profile, with an increased number of substitutions observed in BA.2.86 consistent with genetic divergence. For $CD8^+$ TCEs, Omicron showed six affected epitopes, while the BA.2.86 showed eight. From these, four were overlapping between lineages, and four showed increased substitution counts in the BA.2.86, with epitopes 2, 10, 11, 20 and 21 being affected (Fig. 3B). For $CD4^+$ TCEs, Omicron showed 10 affected epitopes, while the BA.2.86 showed 18. From these, nine were overlapping between lineages, and 16 showed increased substitution counts in the BA.2.86, with epitopes 1, 2, 9–17, 20, 21 and 22 being affected (Fig. 3D). Relative to pre-VOC virus lineages, all $CD8^+$ and $CD4^+$ TCEs affected by either indels or substitutions in BA.2.86 are derived (novel), and expected to yield a loss or reduction in epitope binding/recognition by T cells.

BA.2.86-specific substitutions are predicted to affect peptide-MHC binding

When further tracking specific aa changes falling within $CD8^+$ and $CD4^+$ TCEs, we found a subset of 14 BA.2.86 (and JN.1)-specific LDMs potentially impacting epitope function: S50L, V127F, L216F, H245N, I332V, D339H, K356T, V445H, G446S, N450D, L452W, N460K, L455S and H681R. For LDMs affecting only $CD8^+$ TCEs, S50L falls in epitope 2, L216F in epitopes 10 and 11, H245N in epitope 12, G446S in epitope 20, N450D and L452W in epitope 21, L455S in epitope 21 (with L455S being unique to the JN.1) and H681R in epitope 28. For LDMs affecting only $CD4^+$ TCEs, S50L falls in epitope 1, V127F in epitope 2, L216F in epitope 11, H245N in epitopes 12, 14, and 15, I332V in epitope 16 and 17, D339H in epitopes 17 and 18, K356T in epitope 20, V445H in epitope 21, N460K in epitope 22 and L455S in epitope 21 (with L455S being unique to the JN.1). $CD4^+$ TCEs 9 and 10 and $CD8^+$ TCE 10 are affected by the non-LDM deletion at position 211. Following in-silico analyses predicting the impact of mutations on peptide binding affinity to MHCs, we identified $CD8^+$ TCEs 2, 10, 12, and 28 as potentially affected, with changes to epitopes 10, 12 and 28 being predicted to result in a significant loss of the likelihood for peptide presentation driven by specific LDM (Table 3). Due to limitations in estimating peptide binding to MHC-II or unknown restriction, for $CD4^+$ TCEs, only epitopes 16, 20, and 22 were predicted to be HLA binders of these previously described epitopes; however, BA.2.86 LDMs were predicted to strongly affect both epitopes 20 and 22 (Table 4). In summary, LDMs acquired by BA.2.86 are predicted to affect its recognition at several immunodominant $CD4^+$ and $CD8^+$ TCEs.

Discussion

Despite the notable escape of BA.2.86 from pre-existing humoral immunity, T-cell responses remained, in general, preserved in individuals previously exposed through vaccination and/or infection. Furthermore, although a considerable proportion of LDMs are expected to affect BA.2.86-specific epitope function, with at least five TCEs predicted to be (total) lost, the overall impact is expected to be mitigated due to the majority of TCEs being still unaffected by mutations, translating into the minimal effect observed in functional assays. Moreover, TCEs located in S antigen are only a subset of all TCEs distributed across the viral genome. The large number of TCEs and their higher conservation outside S precludes effective T-cell immune evasion in previously infected individuals. Nevertheless, and of interest, our analysis revealed that T-cell responses and cross-recognition of BA.2.86 were heterogeneous in our study population, and were influenced by various factors, including the number of infections, the specific variant encountered, and the nature of the first antigen exposure (vaccination or infection) despite three years had passed since then and with additional vaccine and infection exposures.

Our results confirm prior research,[8–11] in which BA.2.86 exhibited substantial immune evasion from pre-existing neutralizing antibodies. However, T-cell responses were relatively well-preserved, consistent with the findings from limited studies on the BA.2.86 cross-recognition[1,14] and other variants.[1,32–37] Our data show that T cells responding to both Wuhan and BA.2.86 predominantly secreted IFN-γ, followed by IL-2, with a minority of polyfunctional cells, as previously described for SARS-CoV-2.[38] Notably, these T-cell responses did not correlate with binding antibody levels nor neutralizing activity, indicating a discrepancy between antibodies and T cell-mediated immunity in terms of variant cross-recognition.[39,40] The consistent preservation of T-cell responses across variants suggests that most targeted epitopes are located in stable regions of the S protein, or that the mutations do not impair epitope recognition.[1,41] This preservation of S-specific T-cell responses underscores their potential importance in a context of declining neutralizing antibody responses against successively evolving variants.[1] Moreover, fewer mutations in non-S proteins compared to S proteins may enhance broader and more robust T-cell variant recognition in previously infected individuals.

Two infections, particularly if one involves an Omicron variant, would lead to greater BA.2.86 T-cell cross-recognition than a single mild-to-moderate infection.[1,42,43] While we confirmed this, we found that being infected solely by Omicron variants, regardless the number of infections, decreased the magnitude of the T-cell responses and the recognition of BA.2.86 compared to being infected by ancestral/Delta or ancestral/Delta + Omicron. Lower T-cell responses observed in individuals infected exclusively with Omicron variants, compared to those infected with ancestral or Delta variant, might be explained by the combination of accelerated antigen clearance due to pre-existing vaccine-induced immunity[44] and the

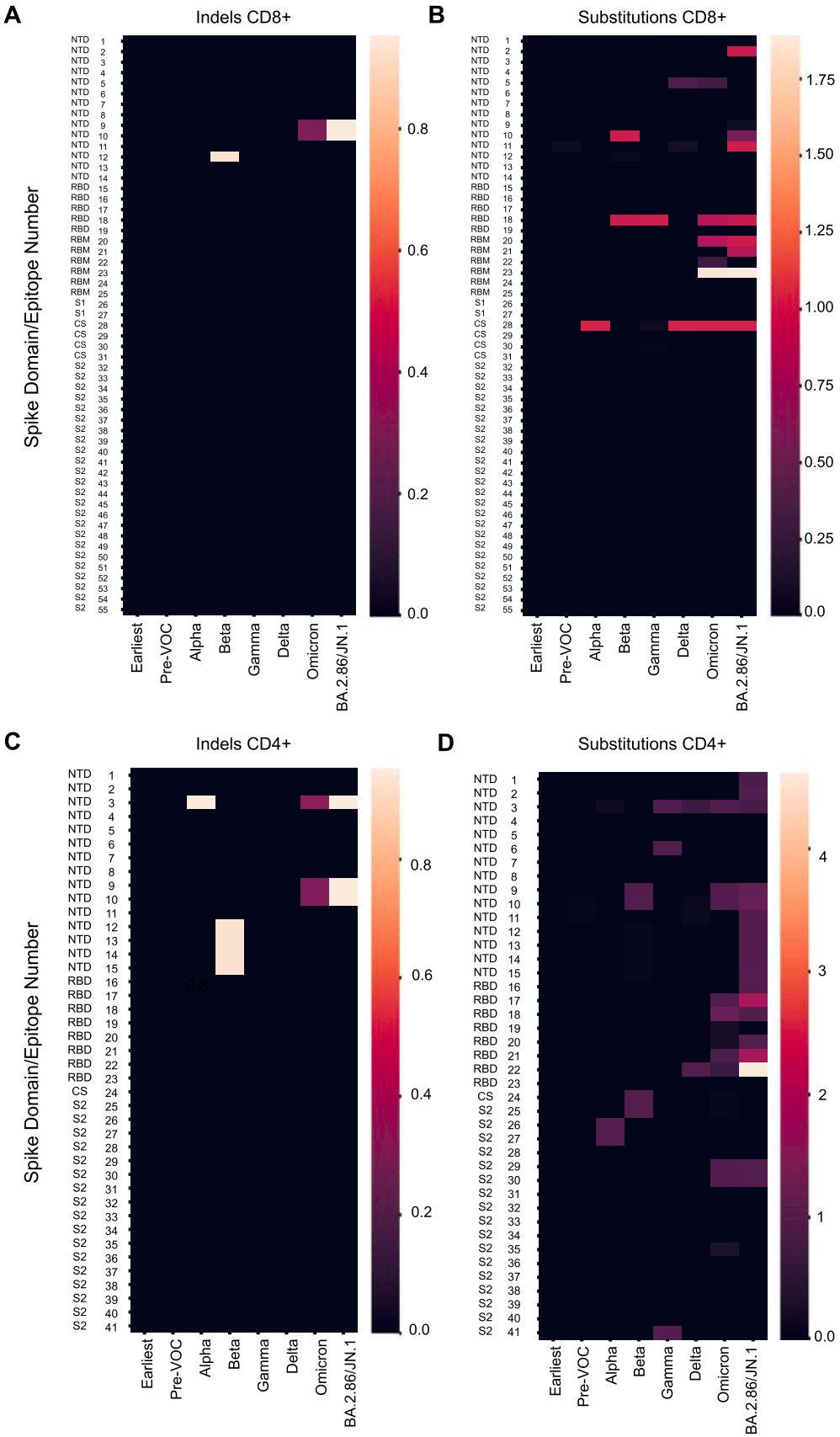

Fig. 3. T-cell epitopes (TCEs) mutation frequencies in the Spike across SARS-CoV-2 lineages. Heatmaps showing mutation frequencies, indels and substitutions, in immunodominant $CD8^+$ (**A-B**) and $CD4^+$ (**C-D**) TCEs across different SARS-CoV-2 variants. Earliest genome sequences include Wuhan-1 and those with collection dates before March 1st, 2020, Pre-VOC lineages include genomes predating Alpha variant.

Table 3
In-silico prediction of CD8+ T-cell epitope loss in BA.2.86 SARS-CoV-2 spike protein.

										HLA									
Epitope num.	Epitope num. in Grifoni et al.	Mutation	Wu-1	BA.2.86	Other	Length	Antigen	Domain	Start	End	Wu-1	BA.2.86	other	Wu-1	BA.2.86	JN.1	Wu-1	BA.2.86	JN.1	Predicted epitope loss in BA.2.86
2	146	S50L	STQDLFLPFF	**L**TQDLFLPFF		10	S	NTD	50	59	A*01:01			A*26:01 predicted			B*58:01 predicted			partial
											1.184 WB	1.694 WB	-	1.466 WB	4.119	-	1.021 WB	1.085 WB	-	
10	183	L216F del211N (JN.1)	TPINLVRDL	TPINLVRD**F**	TPILVRDLP	9	S	NTD	208	216	B*07:02			B*08:01 predicted			A*26:01 predicted			yes
											0.336 SB	0.717 WB	4,775	1.531 WB	3,286	22.000	6.402	1.895 WB	26.652	
11	185	L216F	LPQGFSAL	**F**PQGFSAL		8	S	NTD	216	223	B*07:02			B*08:01 predicted			B*39:01 predicted			no
											0.225 SB	0.239 SB	-	1.189 WB	0.544 WB	-	3.495	1.388 SB	-	
12	192	H245N	LLALHRSYL	LLAL**N**RSYL		9	S	NTD	241	249	A*02:01 predicted			B*07:02 predicted			B*08:01 predicted			yes
											1.942 WB	2.252	-	1.439 WB	2.521	-	0.291 SB	1.052 WB	-	
20	232	G446S	NLDSKVGGNY	NLDSKH**S**GNY		10	S	RBM	440	449	A*01:01 predicted									no
											0.156 SB	0.130 SB	-	-	-	-	-	-	-	
21	237	N450D L452W L455S (JN.1)	NYNYLYRLF	NY**D**Y**W**YRLF	NYNYLYR**S**F	9	S	RBM	448	456	A*24:02									no
											0.054 SB	0.084 SB	0.093 SB	-	-	-	-	-	-	
28	281	H681R	SPRRARSVA	S**R**RRARSVA		9	S	CS	680	688	B*07:02			B*08:01 predicted						yes
											0.058 SB	6.679		1.685 WB	7.258	-	-	-	-	

Wuhan-Hu-1 (Wu-1). N-terminal domain (NTD), receptor-binding motif (RBM), Cleavage site (CS), Weak binder (WB), Strong binder (SB).
Partial loss when change is > 0.5%. Predicted loss when going from SB to WB or SB/WB to > 2% rank. Known restricting MHC are colored in green and predicted restricted MHCs in blue.

attenuated severity of Omicron variants. Although reinfection rates were higher during the Omicron epidemic than in previous epidemic periods, the symptoms and infectivity have been observed to be milder than those of prior infections, and disease severity is associated with more robust adaptive immune responses.[45–47] Supporting this, studies in mice have demonstrated that Omicron subvariants are inherently less immunogenic than the ancestral virus, resulting in lower humoral and T-cell responses after intranasal challenge.[48,49] The higher magnitude and greater cross-recognition to BA.2.86 observed in individuals exposed to both ancestral/Delta and Omicron variants are likely due to their broader repertoire of TCEs, resulting from cumulative immune priming through different exposures.

Remarkably, we observed that first-infected participants, displayed stronger T-cell responses three years later to both Wuhan and BA.2.86, as well as higher BA.2.86 recognition, compared to participants whose first antigen encounter was through vaccination (first-vaccinated). Since in the first-infected group all infections were mild-to-moderate, we used anti-S binding antibody responses as a proxy for the magnitude of infection to assess its association with T-cell responses after three years. T-cell responses were positively correlated with antibody levels after infection. In contrast, in the first-vaccinated group, T-cell responses did not correlate with antibody levels after primary vaccination. In addition, when comparing to the ancestral Wuhan-Hu-1 (vaccine) strain or early pre-VOC viral lineages, we found no significant enrichment of mutations suggesting that this was not the cause of the differential T-cell responses between the vaccine strain and early infection variants. Thus, our results suggest that instead of a mutation-driven immune priming process, exposure to the whole virus (offering a wider repertoire of antigens) and a stronger immune response after the first encounter might shape a more robust and sustained T-cell immune response. Supporting our hypothesis, previous studies have reported that initial COVID-19 severity imprints the long-term maintenance of SARS-CoV-2 adaptive immunity, with severe cases exhibiting more sustained virus-specific antibodies and memory T-cell responses compared to mild/moderate counterparts.[50] In parallel with our results, a previous study observed differences in transcriptional profiles and epigenetic landscape of S-specific $CD4^+$ T cells between infected and vaccine-primed individuals two years after the encounter, with the infection-primed group showing enrichment for transcripts related to cytotoxicity and IFN-stimulated genes.[51] Additionally, other studies have reported higher T-cell responses over time in first-infected individuals,[52,53] as well as higher frequencies of atypical memory B cell subsets and T_H1 polarization of S-specific follicular helper T cells.[54] These findings warrant further investigation.

Finally, although a significant proportion of LDMs affect TCEs, the emergence of LDMs is not expected to be driven by selective forces exerted by T-cell immunity. LDM emergence and fixation may be driven by multiple evolutionary processes, including genetic drift (chance), or overlapping functional properties, such as ACE2 binding and cleavage for those TCEs falling within the receptor-binding motif or cleavage site of S. Congruent with this, most mutations (substitutions/indels) affecting TCEs occur within the NTD and RBD of S1. In contrast, few mutations affecting TCEs fall within S2, largely reflecting a high degree of protein conservation across coronaviruses, which suggests less tolerance to changes given the high functional constraint.

Our study is limited by a small sample size and a predominantly female cohort, restricting generalizability to broader populations such as older or immunocompromised individuals. Also, we have measured the magnitude of T-cell responses through FluoroSpot which cannot differentiate between $CD4^+$ and $CD8^+$ T-cell responses. However, it provides evidence of BA.2.86 cross-recognition by pre-existing T-cell responses. We observed that exposure history significantly determines the extent of this cross-recognition. Moreover, our findings from the in-silico analysis suggest that BA.2.86 mutations do not exert selective pressure to evade T-cell responses, reinforcing that T-cell-mediated immunity remains largely preserved despite extensive mutations. This underscores the importance of T-cell immunity in counteracting the immune escape of evolving SARS-CoV-2 variants from neutralizing antibodies and suggests it is a crucial target for next-generation COVID-19 vaccines. Additionally, considering exposure history could enhance control strategies.

Table 4
In-silico prediction of CD4+ T-cell epitope loss in BA.2.86 SARS-CoV-2 spike protein.

											HLA									
Epitope num.	Epitope Num. in Grifoni et al.	Mutation	Wu-1	BA.2.86	Other	Length	Antigen	Domain	Start	End	Wu-1	BA.2.86	other	Wu-1	BA.2.86	other	Wu-1	BA.2.86	other	Predicted epitope loss in BA.2.86
1	7	S50L	SVLHSTQDLFLPFFS	SVLH**L**TQDLFLPFFS		15	S	NTD	46	60	**DQB1*02:02**			DQB1*05:01			**DRB1*07:01**			
											21,56	62,47	-	17,69	22,64	-	16,24	21,14	-	
9	31	del211N (JN.1)	KHTPINLVRDLPQGF		KHTPILVRDLPQGF	15	S	NTD	206	220	**DRB1*03:01**									
											7,21	-	7,1							
10	33	del211N (JN.1)	NLVRDLPQGFSALEP		LVRDLPQGFSALEP	15	S	NTD	211	225	**DRB1*03:01**									
											12	-	73,89							
11	34	L216F	LPQGFSALEPLVDLP	**F**PQGFSALEPLVDLP		15	S	NTD	216	230	**DQB1*02:02**			**DQB1*03:03**			**DRB1*05:03**			
											6,33	6,04	-	32,56	32,7	-	19,75	19,32	-	
12	36	H245N	IGINITRFQTLLALH	IGINITRFQTLLAL**N**		15	S	NTD	231	245	**DQB1*06:03**			**DRB1*07:01**			**DRB1*11:02**			
											32,3	31,89	-	21,38	20,73	-	7,31	7,24	-	
14	38	H245N	TRFQTLLALHRSYLT	TRFQTLLAL**N**RSYLT		15	S	NTD	236	250	**DQB1*05:01**			**DRB1*05:03**			**DRB1*06:02**			
											59,59	48,18	-	45,84	49,3	-	16,49	24,5	-	
15	39	H245N	LLALHRSYLTPGDSS	LLAL**N**RSYLTPGDSS		15	S	NTD	241	255	**DRB1*01:01**			**DRB1*07:01**			**DRB1*11:02**			
											57,14	63,77	-	46,18	67,77	-	34,27	52,34	-	
16	45	I332V	QPTESIVRFPNITNL	QPTESIVRFPN**V**TNL		15	S	RBD	321	335	**DRB1*08:03**			**DRB1*15:01**						no
											9,64	10,69	-	0.68 SB	0.8 SB	-				
17	46	I332V, D339H	IVRFPNITNLCPFGE	IVRFPN**V**TNLCPF**H**E		15	S	RBD	326	340	**DRB1*15:01**									
											84,87	83,83	-							
18	47	D339H	CPFGEVFNATRFASV	CPF**H**EVFNATRFASV		15	S	RBD	336	350	**DQB1*03:01**			**DQB1*06:02**			**DQB1*06:03**			
											46,88	49,31		38,46	40,76		55,22	58,29		
20	49	K356T	RFASVYAWNRKRISN	RFASVYAWNR**T**RISN		15	S	RBD	346	360	**DRB1*07:01**			**DRB1*11:02**			**DRB1*13:03**			yes
											33,64	21,26	-	0.43 SB	1.42 WB	-	6,54	13,27	-	
21	61	V445H	GCVIAWNSNNLDSKV	GCVIAWNSNNLDSK**H**		15	S	RBD	431	445	**DQB1*02:02**			**DQB1*06:03**			**DRB1*07:01**			
											24,72	22,5	-	24,93	22,38	-	11,88	11,45	-	
22	63	N450D, L452W, N460K, L455S (JN.1)	GGNYNYLYRLFRKSN	GGNY**D**Y**W**YRLFRKS**K**	GGNYNYLYR**S**FRKSN	15	S	RBD	446	460	**DRB1*11:02**			**DRB1*13:03**						yes
											4.84 WB	3.27 WB	5,72	4.10 WB	17.49	4.00 WB				

Wuhan-Hu-1 (Wu-1). N-terminal domain (NTD), receptor-binding domain (RBD). Weak binder (WB), Strong binder (SB).
Predicted loss when going from SB to WB or SB/WB to > 5% rank. Known restricting MHC are colored in green and predicted restricted MHCs in blue.

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CRediT authorship contribution statement

GM, CD, LS and FB designed the study. CD and GM supervised the immune assays and data analysis. RR, MC, CT performed the T-cell assay and RA coordinated the lab activities. EP, BT, JB and LMMA designed, performed the neutralization antibody assays and normalized data to obtain ID50. RR, AY, MEZ, CT performed the analysis and interpretation of results. CMP performed database management and analyses. ARM, JVA, and ARC recruited and followed up participants, sample and data collection. RR and MEZ wrote the initial draft and CD, GM, LS, FB reviewed the manuscript. All authors contributed, read and approved the final manuscript.

Declaration of Competing Interest

Unrelated to the present work JB received Institutional grants/agreements from/with MSD, HIPRA, GRIFOLS and NESAPOR; personal payments from HIPRA and NESAPOR; and was former CEO and founder of AlbaJuna Therapeutics, S.L. All other authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2024.106402.

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Supplementary data

Initial antigen encounter determines robust T-cell immunity against SARS-CoV-2 BA.2.86 variant three years later

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Tables S1 – S3

Figures S1 – S12

Table S1. Sociodemographic and clinical characteristics by probable variant of infection

		Ancestral/ Delta N=8	Omicron N=23	Ancestral/Delta + Omicron N=18	Non-inf. N=3	p-value
Sex: n (%)						
	Female	7 (87.5 %)	19 (82.6 %)	16 (88.9 %)	2 (66.7 %)	0.865
Age (years): mean (s.d.)		51.13 (11.79)	47.91 (11.31)	48.72 (10.81)	56.33 (5.86)	0.696
Infection pre-1st dose		4 (50 %)	0 (0 %)	17 (94.4 %)	0 (0 %)	**< 0.001**
Breakthrough infections (BTIs): n (%)						**0.001**
0		4 (50 %)	0 (0 %)	0 (0 %)	3 (100 %)	
1	1 symptomatic	3 (37.5 %)	17 (73.9 %)	7 (38.9 %)	0 (0 %)	
	1 asymptomatic	0 (0 %)	1 (4.35 %)	5 (27.8 %)	0 (0 %)	
2	2 symptomatic	0 (0 %)	1 (4.35 %)	1 (5.56 %)	0 (0 %)	
	1 symptomatic + 1 asymptomatic[a]	1 (12.5 %)	4 (17.4 %)	3 (16.7 %)	0 (0 %)	
3	2 asymptomatic + 1 symptomatic[a]	0 (0 %)	0 (0 %)	2 (11.1 %)	0 (0 %)	
Total infections[b]: n (%)						**< 0.001**
	0	0 (0 %)	0 (0 %)	0 (0 %)	3 (100 %)	
	1	7 (87.5 %)	18 (78.3 %)	0 (0 %)	0 (0 %)	
	2	1 (12.5 %)	5 (21.7 %)	13 (72.2 %)	0 (0 %)	
	≥3	0 (0 %)	0 (0 %)	5 (27.8 %)	0 (0 %)	
Vaccine doses: n (%)						0.225
	3	7 (87.5 %)	20 (87 %)	12 (66.7 %)	0 (0 %)	
	4	1 (12.5 %)	3 (13 %)	6 (33.3 %)	3 (100 %)	
Vaccine regimens: n (%)						0.483
3	Pfizer + Pfizer + Moderna	2 (25 %)	11 (47.8 %)	7 (38.9 %)	0 (0 %)	
	Pfizer + Pfizer + Pfizer	5 (62.5 %)	9 (39.1 %)	4 (22.2 %)	0 (0 %)	
	Moderna + Moderna + Moderna	0 (0 %)	0 (0 %)	1 (5.56 %)	0 (0 %)	
4	Pfizer + Pfizer + Pfizer + Pfizer	1 (12.5 %)	2 (8.70 %)	4 (22.2 %)	2 (66.7 %)	
	Pfizer + Pfizer + Moderna + Pfizer	0 (0 %)	1 (4.35 %)	2 (11.1 %)	1 (33.3 %)	
Total exposures[c]: n (%)						**0.001**
	4	6 (75 %)	15 (65.2 %)	0 (0 %)	3 (100 %)	
	5	2 (25 %)	8 (34.8 %)	8 (44.4 %)	0 (0 %)	
	≥6	0 (0 %)	0 (0 %)	10 (55.6 %)	0 (0 %)	
Time since last symptomatic infection (days): median [IQR]		907 [666;1152]	412 [322;389]	389 [240;1130]	.	**0.001**
Time since last vaccine dose (days): median [IQR]		454 [384;509]	517 [504;521]	504 [214;521]	167 [106;178]	0.366
Time since last exposure[d] (days): median [IQR]		454 [384;509]	343 [314;470]	229 [189;358]	167 [106;178]	**0.026**
Seropositive:		8 (100 %)	23 (100 %)	18 (100 %)	3 (100 %)	.
Any comorbidity[e]:		5 (62.5 %)	9 (39.1 %)	10 (55.6 %)	1 (33.3 %)	0.439
Smoking:						0.210
	Active smoker	0 (0 %)	6 (26.1 %)	1 (5.56 %)	1 (33.3 %)	
	Previous smoker	2 (25 %)	6 (26.1 %)	3 (16.7 %)	0 (0 %)	
	Non-smoker	6 (75 %)	11 (47.8 %)	14 (77.8 %)	2 (66.7 %)	
LongCOVID		0 (0 %)	0 (0 %)	0 (0 %)	0 (0 %)	.

[a]Whatever the order

[b]Include symptomatic and asymptomatic breakthrough infections and vaccine doses

[c]Include symptomatic infections and vaccine doses

[d]Include Asthma (3), Cardiac (2), Digestive (2), Autoimmune (2), Mellitus diabetes (1), Dyslipidaemia (3), Arterial hypertension (6), Hypothyroidism (4), Obesity (7), and Allergies (6)

Non-infection (Non-inf.) group is not included in comparisons.

Table S2. Immunodominant CD8$^+$ T-cell epitopes in SARS-CoV-2 spike protein

Epitope number	Epitope number in Grifoni et al.	Epitope Sequence	Domain	BA.2.86	JN.1	Other	Length	Antigen	Start	End
1	138	YTNSFTRGVY	NTD				10	S	28	37
2	146	STQDLFLPFF	NTD	**L**TQDLFLPFF			10	S	50	59
3	154	RFDNPVLPF	NTD				9	S	78	86
4	159	GVYFASTEK	NTD				9	S	89	97
5	160	TEKSNIIRGW	NTD				10	S	95	104
6	162	TLDSKTQSL	NTD				9	S	109	117
7	172	SSANNCTFEY	NTD				10	S	161	170
8	179	FVFKNIDGY	NTD				9	S	192	200
9	182	IYSKHTPINL	NTD			IYSKHTPILV	10	S	203	212
10	183	TPINLVRDL	NTD	TPINLVRD**F**		TPILVRDLP	9	S	208	216
11	185	LPQGFSAL	NTD	**F**PQGFSAL			8	S	216	223
12	192	LLALHRSYL	NTD	LLAL**N**RSYL			9	S	241	249
13	193	WTAGAAAYY	NTD	WTAGAA**D**YY			9	S	258	266
14	200	YLQPRTFLL	NTD				9	S	269	277
15	220	RISNCVADY	RBD				9	S	357	365
16	221	CVADYSVLY	RBD				9	S	361	369
17	225	KCYGVSPTK	RBD				9	S	378	386
18	230	KIADYNYKL	RBD				9	S	417	425
19	231	KLPDDFTGCV	RBD				10	S	424	433
20	232	NLDSKVGGNY	RBM	NLDSK**HS**GNY			10	S	440	449
21	237	NYNYLYRLF	RBM	NY**D**Y**W**YRLF	NYNYLYR**S**F		9	S	448	456
22	247	YFPLQSYGF	RBM				9	S	489	497
23	249	FQPTNGVGY	RBM				9	S	497	505
24	253	QPYRVVVL	RBM				8	S	506	513
25	258	GPKKSTNLV	RBM				9	S	526	534
26	264	EILDITPCSF	S1				10	S	583	592
27	276	IGAEHVNNSY	S1				10	S	651	660
28	281	SPRRARSVA	CS	S**R**RRARSVA			9	S	680	688
29	284	SVASQSIIAY	CS				10	S	686	695
30	285	VASQSIIAY	CS				9	S	687	695
31	286	SIIAYTMSL	CS				9	S	691	699
32	295	FTISVTTEIL	S2				10	S	718	727
33	296	TEILPVSMTK	S2				10	S	724	733
34	300	TECSNLLLQY	S2				10	S	747	756
35	301	LLQYGSFCT	S2				9	S	753	761
36	313	LLFNKVTLA	S2				9	S	821	829
37	320	LTDEMIAQY	S2				9	S	865	873
38	323	MIAQYTSAL	S2				9	S	869	877
39	324	GTITSGWTF	S2				9	S	880	888
40	330	TQNVLYENQK	S2				10	S	912	921
41	332	NQKLIANQF	S2				9	S	919	927
42	339	VLNDILSRL	S2				9	S	976	984
43	341	RLDKVEAEV	S2				9	S	983	991
44	349	RLQSLQTYV	S2				9	S	1000	1008
45	355	YHLMSFPQSA	S2				10	S	1047	1056
46	356	HLMSFPQSA	S2				9	S	1048	1056
47	362	APHGVVFLHV	S2				10	S	1056	1065
48	365	VVFLHVTYV	S2				9	S	1060	1068
49	376	GTHWFVTQR	S2				9	S	1099	1107
50	386	RLNEVAKNL	S2				9	S	1185	1193
51	387	NLNESLIDL	S2				9	S	1192	1200
52	395	QYIKWPWYI	S2				9	S	1208	1216
53	396	KWPWYIWLGF	S2				10	S	1211	1220
54	397	FIAGLIAIV	S2				9	S	1220	1228
55	400	SEPVLKGVKL	S2				10	S	1261	1270

Table S3. Immunodominant CD4⁺ T-cell epitopes in SARS-CoV-2 spike protein

Epitope number	Epitope number in Grifoni et al.	Epitope Sequence	Domain	BA.2.86	JN.1	Other	Length	Antigen	Start	End
1	7	SVLHSTQDLFLPFFS	NTD	SVLH**L**TQDLFLPFFS			15	S	46	60
2	17	NNATNVVIKVCEFQF	NTD	NNATNV**F**IKVCEFQF			15	S	121	135
3	19	CEFQFCNDPFLGVYY	NTD				15	S	131	145
4	22	SSANNCTFEYVSQPF	NTD				15	S	161	175
5	23	CTFEYVSQPFLMDLE	NTD				15	S	166	180
6	25	LMDLEGKQGNFKNLR	NTD				15	S	176	190
7	27	EFVFKNIDGYFKIYS	NTD				15	S	191	205
8	28	NIDGYFKIYSKHTPI	NTD				15	S	196	210
9	31	KHTPINLVRDLPQGF	NTD			KHTPILVRDLPQGF	15	S	206	220
10	33	NLVRDLPQGFSALEP	NTD			LVRDLPQGFSALEP	15	S	211	225
11	34	LPQGFSALEPLVDLP	NTD	**F**PQGFSALEPLVDLP			15	S	216	230
12	36	IGINITRFQTLLALH	NTD	IGINITRFQTLLAL**N**			15	S	231	245
13	37	ITRFQTLLALHRSYL	NTD				15	S	235	249
14	38	TRFQTLLALHRSYLT	NTD				15	S	236	250
15	39	LLALHRSYLTPGDSS	NTD	LLAL**N**RSYLTPGDSS			15	S	241	255
16	45	QPTESIVRFPNITNL	RBD	QPTESIVRFPN**V**TNL			15	S	321	335
17	46	IVRFPNITNLCPFGE	RBD	IVRFPN**V**TNLCPFGE			15	S	326	340
18	47	CPFGEVFNATRFASV	RBD				15	S	336	350
19	48	VFNATRFASVYAWNR	RBD				15	S	341	355
20	49	RFASVYAWNRKRISN	RBD	RFASVYAWNR**T**RISN			15	S	346	360
21	61	GCVIAWNSNNLDSKV	RBD	GCVIAWNSNNLDSK**H**			15	S	431	445
22	63	GGNYNYLYRLFRKSN	RBD	GGNY**D**YLYRLFRKSN	GGNYNYLYR**S**FRKSN		15	S	446	460
23	70	QPYRVVVLSFELLHA	RBD				15	S	506	520
24	77	SIIAYTMSLGAENSV	CS				15	S	691	705
25	78	AENSVAYSNNSIAIP	S2				15	S	701	715
26	80	SIAIPTNFTISVTTE	S2				15	S	711	725
27	81	TNFTISVTTEILPVS	S2				15	S	716	730
28	83	STECSNLLLQYGSFC	S2				15	S	746	760
29	84	NLLLQYGSFCTQLNR	S2				15	S	751	765
30	86	TQLNRALTGIAVEQD	S2				15	S	761	775
31	90	NFSQILPDPSKPSKR	S2				15	S	801	815
32	92	KPSKRSFIEDLLFNK	S2				15	S	811	825
33	93	SFIEDLLFNKVTLAD	S2				15	S	816	830
34	96	AGFIKQYGDCLGDIA	S2				15	S	831	845
35	101	FNGLTVLPPLLTDEM	S2				15	S	855	869
36	103	TDEMIAQYTSALLAG	S2				15	S	866	880
37	108	IPFAMQMAYRFNGIG	S2				15	S	896	910
38	109	QMAYRFNGIGVTQNV	S2				15	S	901	915
39	116	VQIDRLITGRLQSLQ	S2				15	S	991	1005
40	127	ELDKYFKNHTSPDVD	S2				15	S	1151	1165
41	129	GINASVVNIQKEIDR	S2				15	S	1171	1185

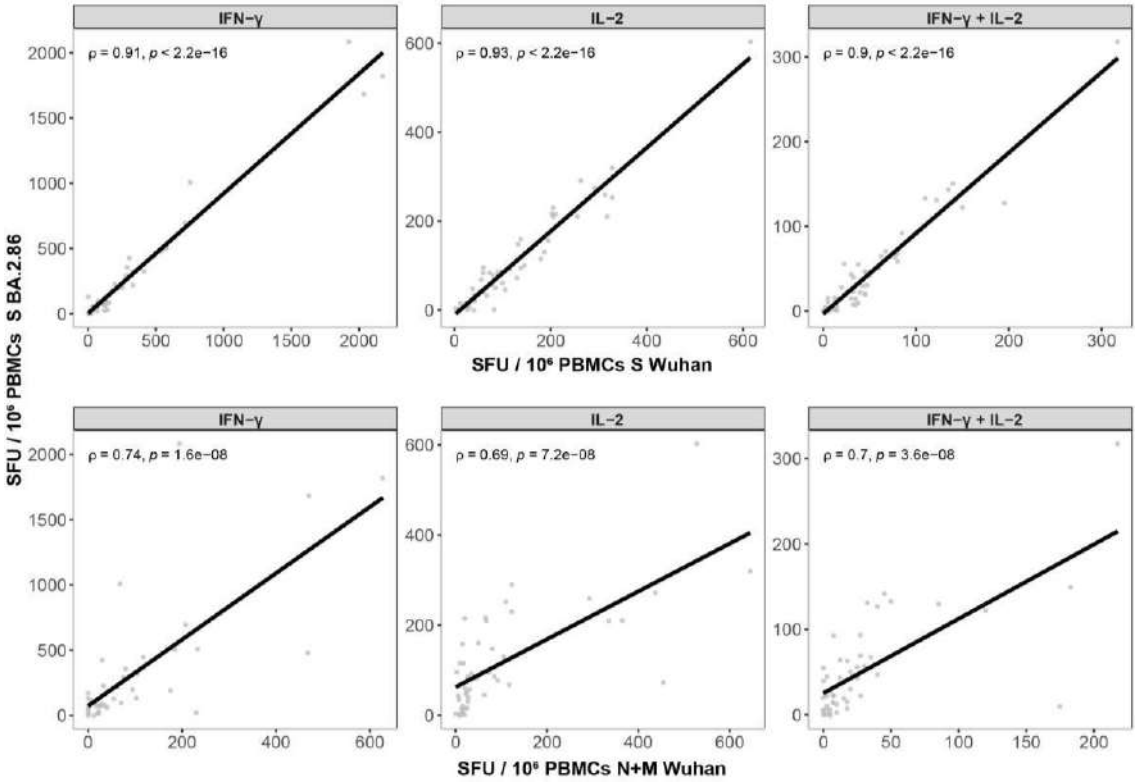

Figure S1. Correlations of T-cell responses between BA.2.86 and Wuhan. Spearman's correlation coefficient ρ (Rho) between the magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) to spike (S) from BA.2.86 variant and to S, or nucleocapsid and membrane (N+M) from ancestral. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

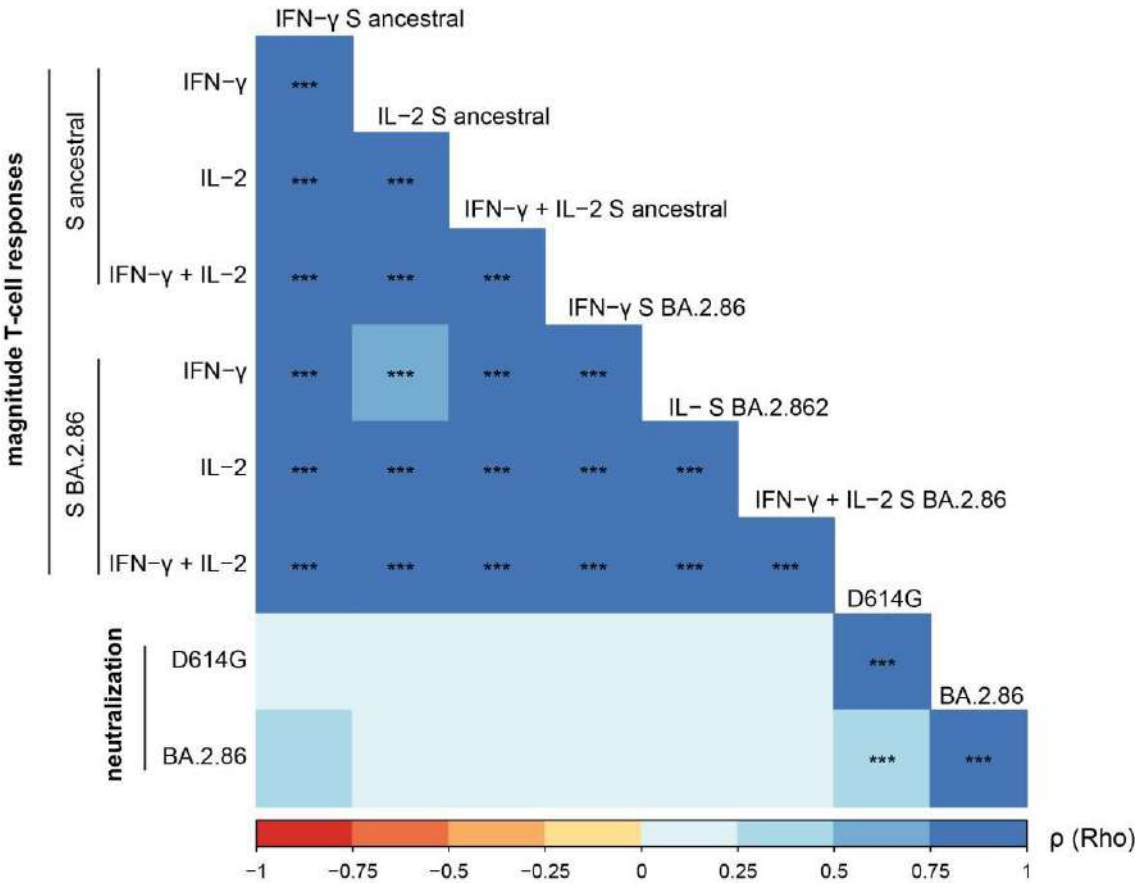

Figure S2. Correlations of T-cell responses and plasma neutralizing activity. Heatmap illustrating the Spearman's correlation coefficient ρ (Rho) between the magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and plasma neutralizing activity as ID50. p-values: * ≤ 0.05, ** ≤ 0.01 and *** ≤ 0.001. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), peripheral mononuclear cells (PBMCs), spike (S), receptor binding domain (RBD), spot-forming units (SFU).

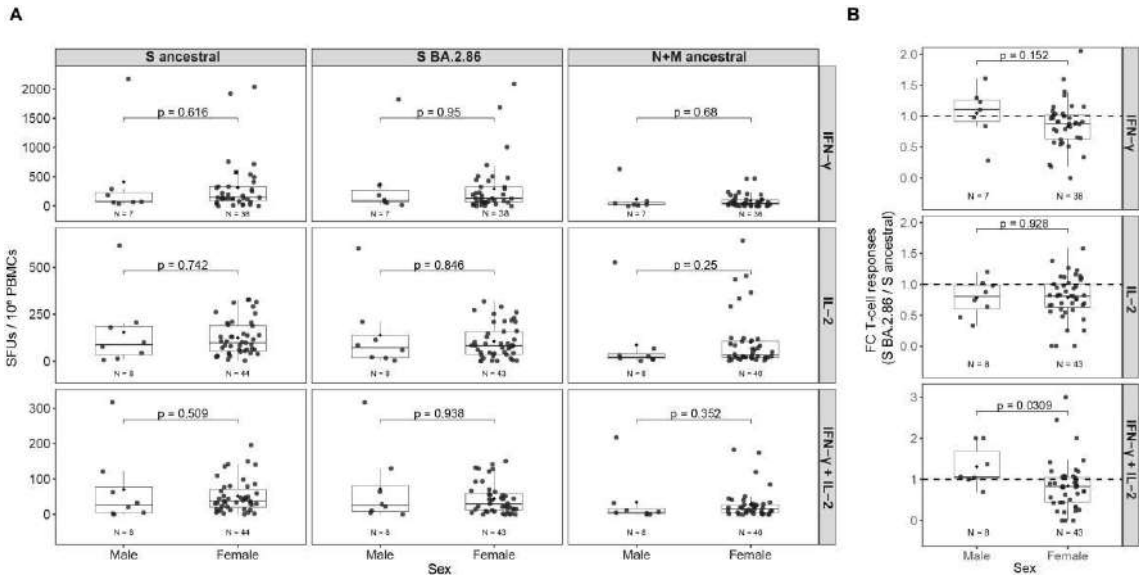

Figure S3. T-cell responses by sex. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**B**) BA.2.86 cross-recognition as fold change in T-cell responses to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral) by sex. T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

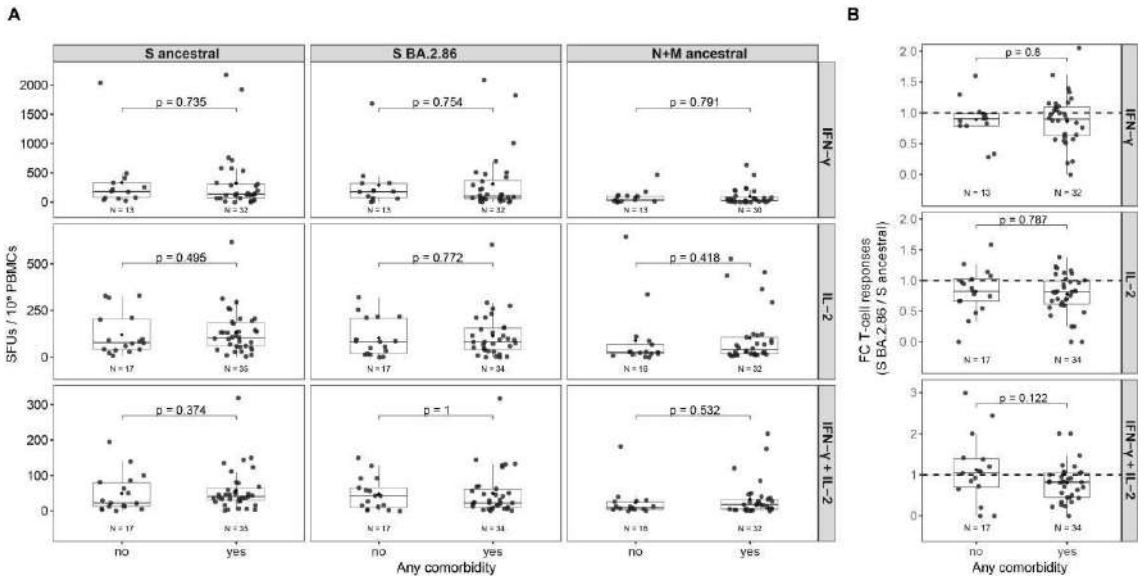

Figure S4. T-cell responses by any comorbidity. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**B**) BA.2.86 cross-recognition as FC in T-cell responses to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral) by any comorbidity. T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Any comorbidity includes Asthma (3), Cardiac (2), Digestive (2), Autoimmune (2), Mellitus diabetes (1), Dyslipidaemia (3), Arterial hypertension (6), Hypothyroidism (4), Obesity (7), and Allergies (6). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

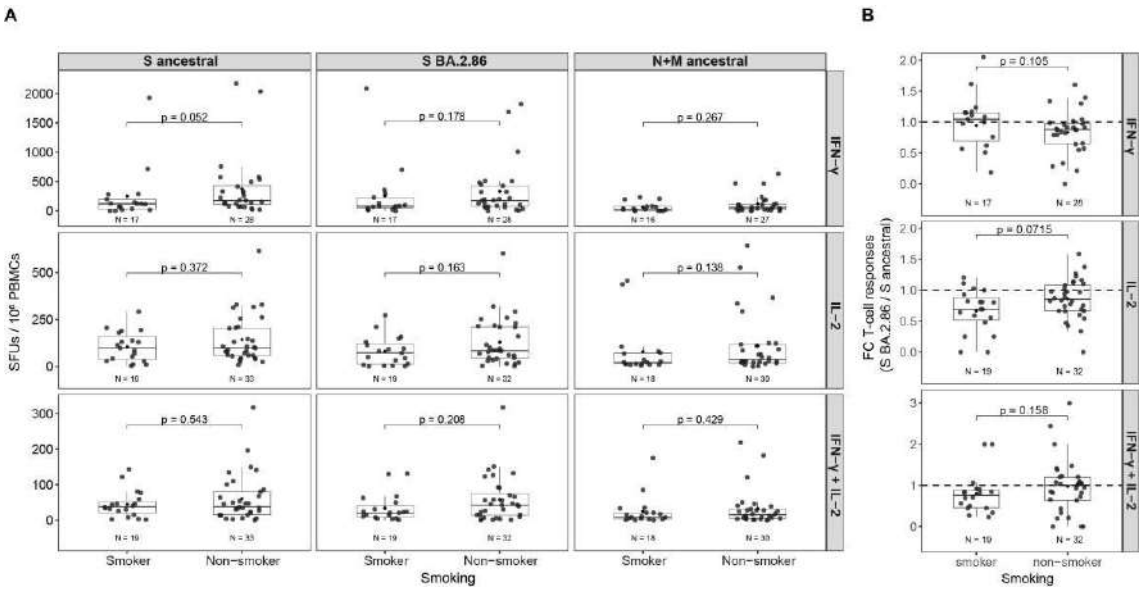

Figure S5. T-cell responses by smoking. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**B**) BA.2.86 cross-recognition as FC in T-cell responses to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral) by smoking. T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

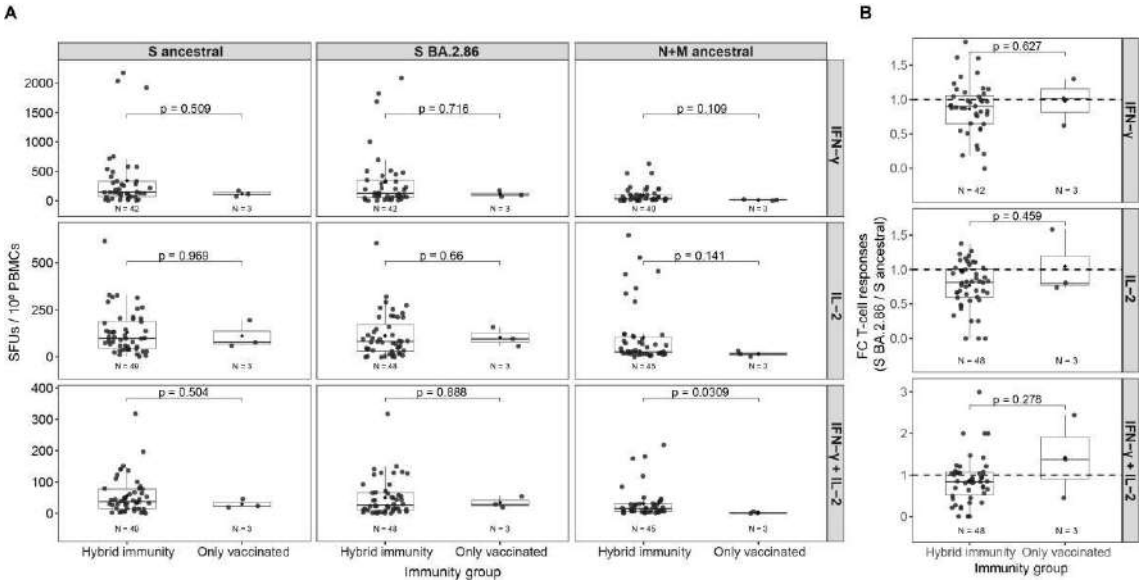

Figure S6. T-cell responses by immunity groups. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**B**) BA.2.86 cross-recognition as FC in T-cell responses to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral) by immunity groups. T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

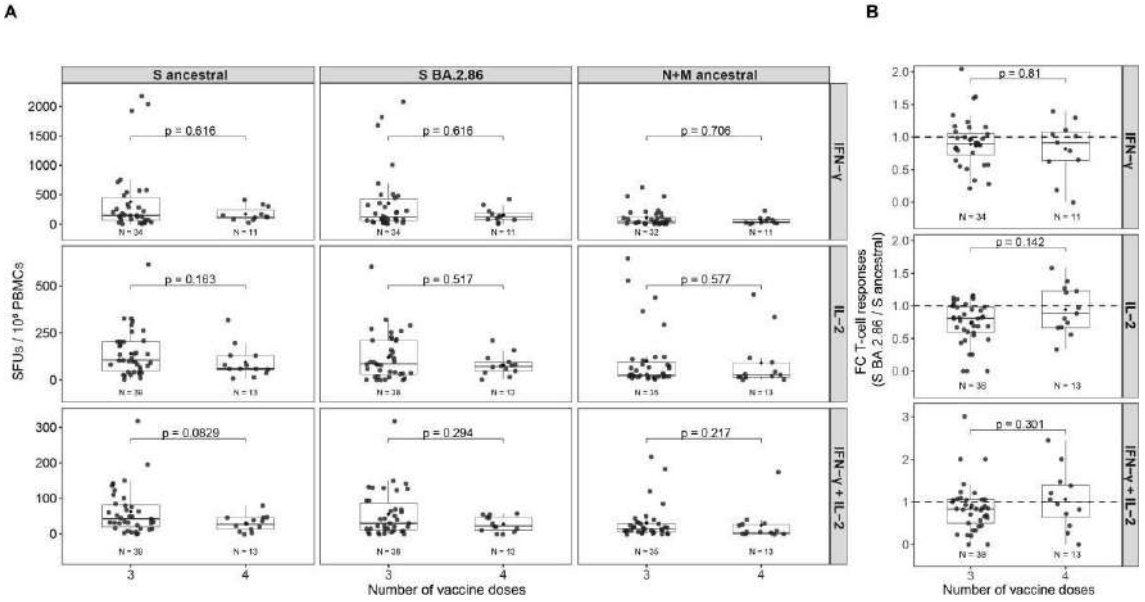

Figure S7. T-cell responses by number of vaccine doses. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**B**) BA.2.86 cross-recognition as FC in T-cell responses to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral) by number of vaccine doses. T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

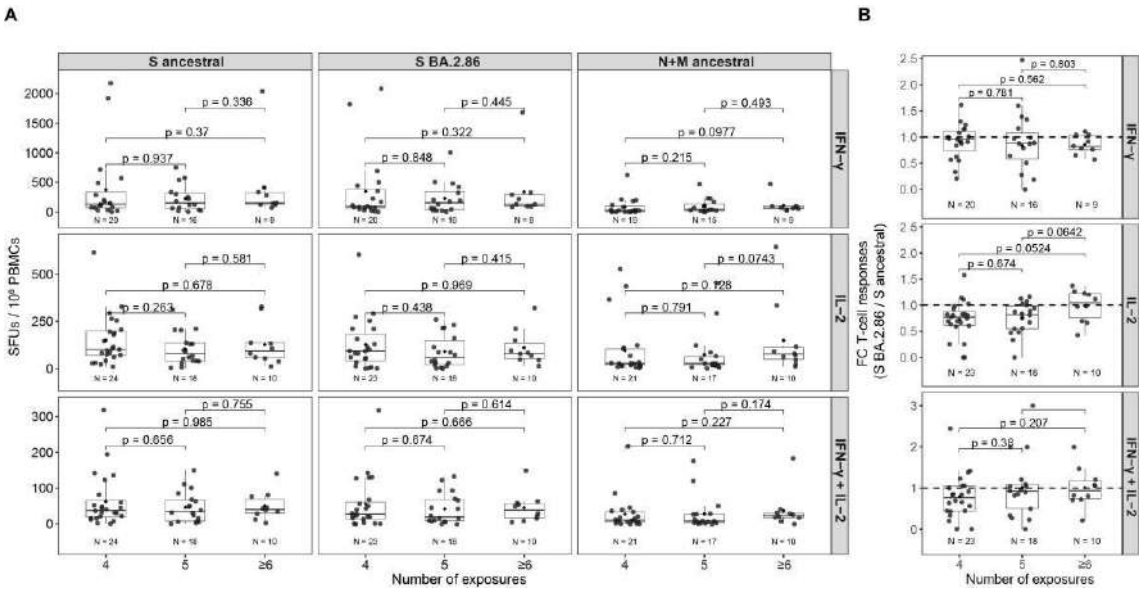

Figure S8. T-cell responses by number of total exposures. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and (**B**) BA.2.86 cross-recognition as FC in T-cell responses to BA.2.86 with respect to ancestral strain (BA.2.86 / ancestral) by number of total exposures (vaccine doses and infections). T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

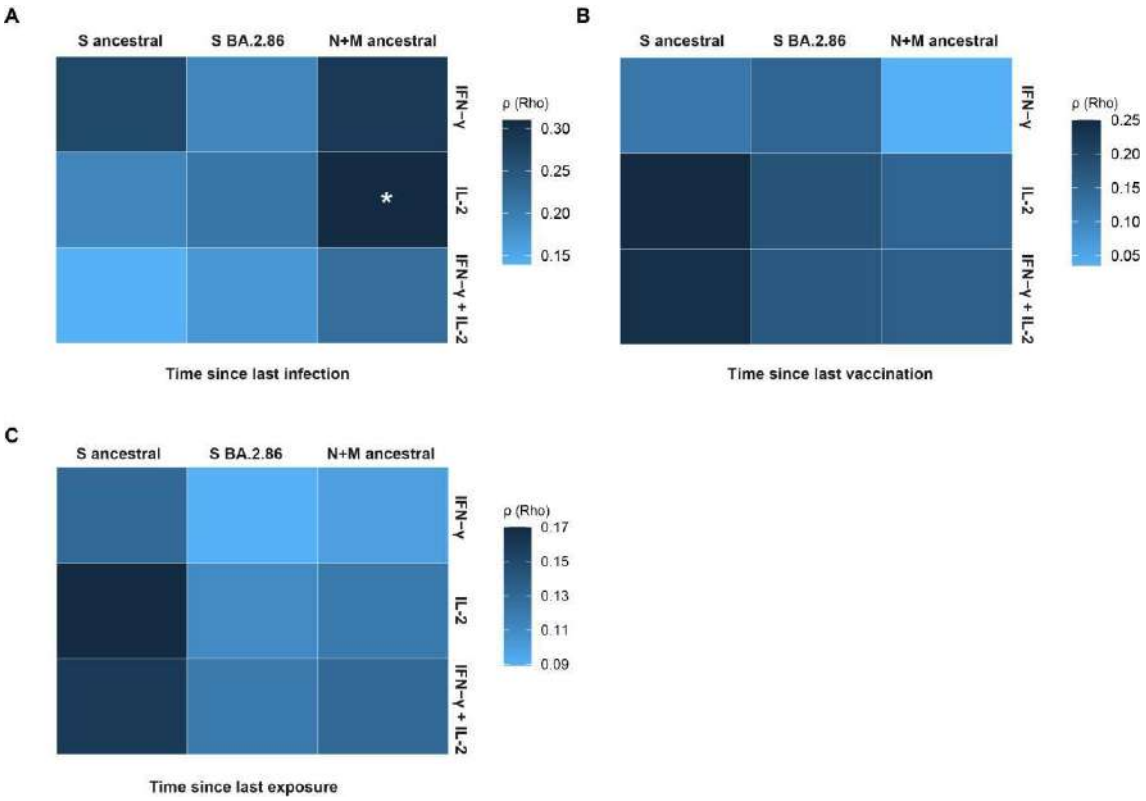

Figure S9. Correlations of T-cell responses and time since last infection (A), vaccination (B) or exposure (C). Heatmaps illustrating the Spearman's correlation coefficient ρ (Rho) between the magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) and time since last exposures. p-values: * ≤ 0.05. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), receptor binding domain (RBD), spot-forming units (SFU).

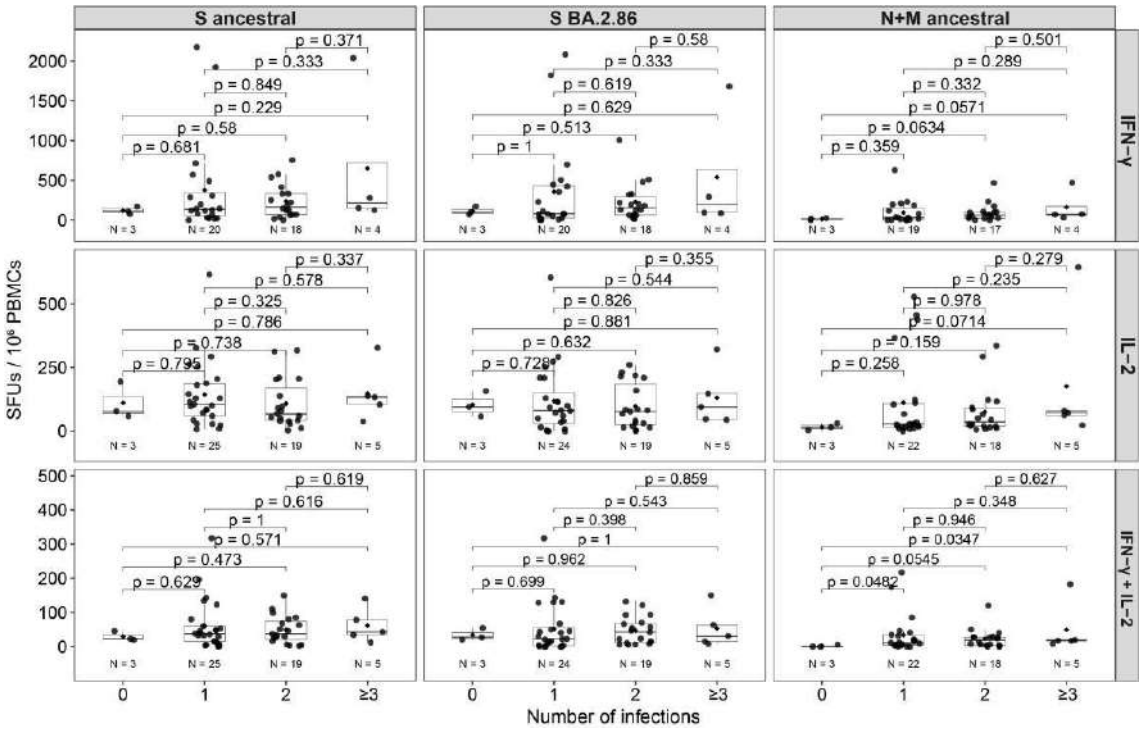

Figure S10. T-cell responses by number of infections. (**A**) Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional). T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Interferon-gamma (IFN-γ), interleukin-2 (IL-2), membrane (M), nucleocapsid (N), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

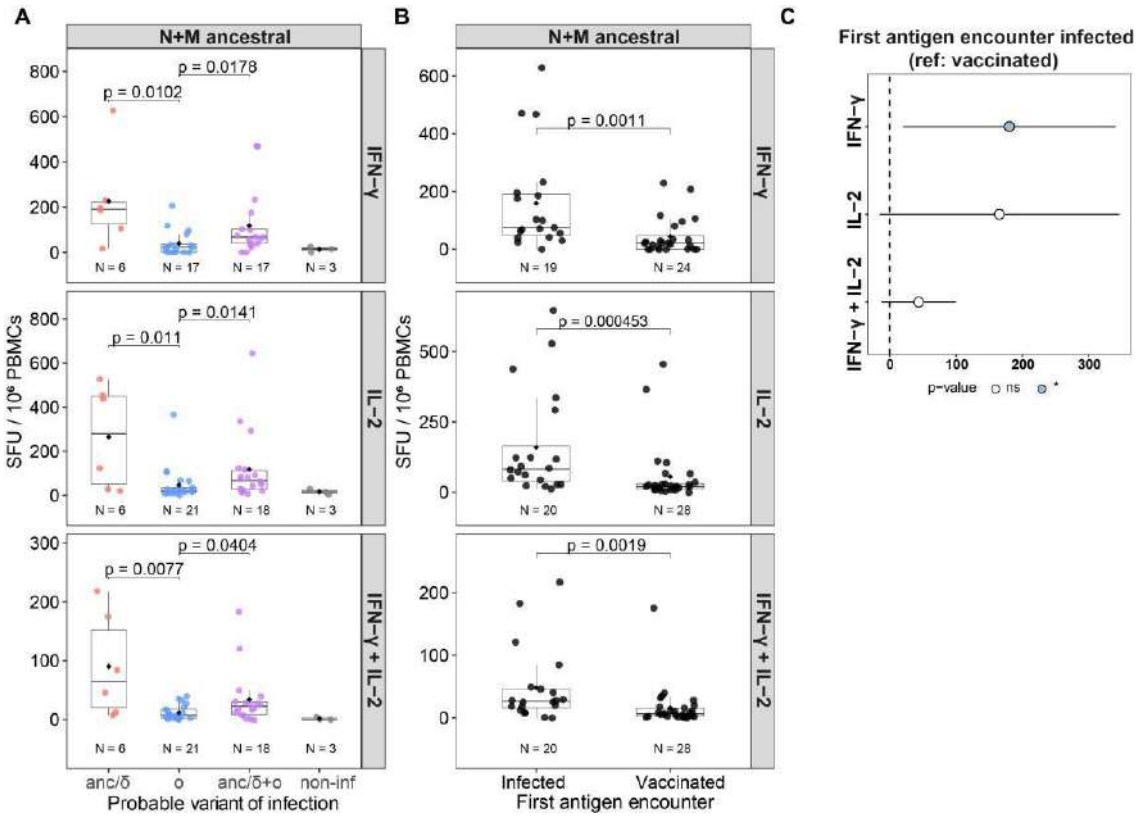

Figure S11. Factors influencing T-cell responses to ancestral nucleocapsid (N) and membrane (M) antigens. Magnitude of T-cell responses as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) by probable variant of infection (**A**) and first antigen encounter (**B**). T-cell responses were compared by Wilcoxon rank-sum test. Boxplots represent median (bold line), the mean (black diamond), 1st and 3rd quartiles (box), and largest and smallest values within 1.5 times the interquartile range (whiskers). Ancestral/Delta (anc/δ), Omicron (o), Ancestral/Delta + Omicron (anc/δ+o), and non-infected (non-inf). (**C**) Forest plot showing the association of being infected before vaccination with magnitude of T-cell responses. The represented values and CI show the SFU / 10^6 PBMCs increase in individuals infected before vaccination compared to individuals not infected prior vaccination. Multivariable linear regression models were fitted to calculate the estimates (dots) and 95 % confidence intervals (lines) and adjusted for the number of vaccine doses, total infections and the probable variant of infection. p-values: * ≤ 0.05. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), peripheral mononuclear cells (PBMCs), spike (S), spot-forming units (SFU).

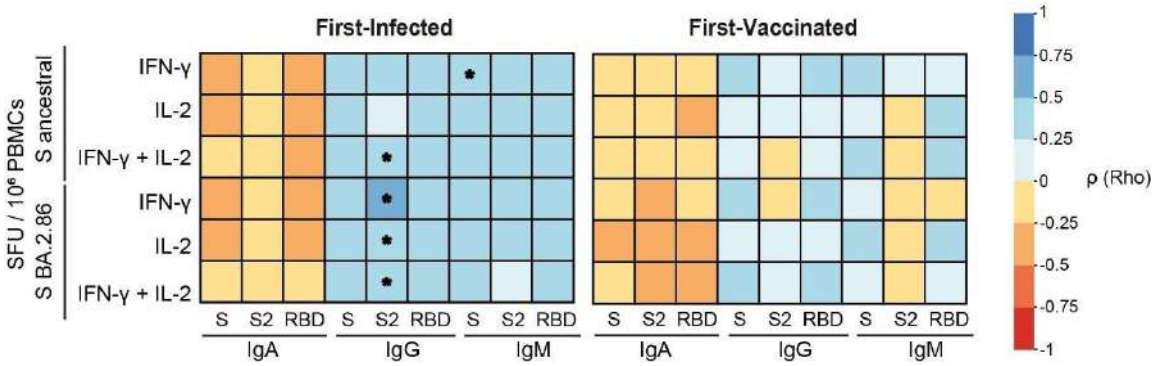

Figure S12. Correlations of T-cell responses with plasma antibody levels by first antigen encounter groups at the same timepoint. Heatmaps illustrating the Spearman's correlation coefficient ρ (Rho) between the as SFU / 10^6 PBMCs of T-cells secreting IFN-γ, IL-2 or IFN-γ + IL-2 (polyfunctional) with the antibody responses (median fluorescence intensity (MFI) of IgA, IgG, and IgM) to S, S2 and RBD from ancestral strain. p-values: * ≤ 0.05. Interferon-gamma (IFN-γ), interleukin-2 (IL-2), peripheral mononuclear cells (PBMCs), spike (S), receptor binding domain (RBD), spot-forming units (SFU).

Discussion

Decoding immune responses to SARS-CoV-2 through longitudinal studies

The establishment of the CovidCatCentral cohort early in the pandemic in Spain (March-April 2020), combined with longitudinal sample collection during twelve cross-sectional visits up to December 2024, enabled a comprehensive evaluation of long-term antibody and T-cell responses following SARS-CoV-2 infection and COVID-19 vaccination in a real-scenario with different exposures. This cohort consisted of two groups of primary HCWs: individuals infected (mostly) during the first wave and subsequently vaccinated (pre-exposed or first-infected) and naïve individuals who were vaccinated (naïve or first-vaccinated), providing the opportunity to investigate the impact of first antigen encounter on adaptive immune responses over time. In addition, the KinetiCoVac cohort, though smaller in size, included healthy adults closely monitored up to 55 visits between February 2021 and March 2023. This cohort offered unprecedented insight into the fine details of antibody kinetics post-COVID-19 vaccination and long-term T-cell responses. Leveraging our expertise in antibody detection using Luminex technology, we swiftly and effectively adapted this highly sensitive and specific high throughput assay to SARS-CoV-2 [675]. Furthermore, our Luminex assay demonstrated strong concordance with commercial ELISA [676] and showed a robust correlation with surrogate neutralizing activity [677]. While antibody detection is relatively straightforward, assessing antigen-specific T-cell responses is more challenging owing to low precursor frequencies and the variability in MHC haplotypes in the population [343]. Thus, we optimized IFN-γ/IL-2 FluoroSpot and AIM assays that provide highly sensitive functional profiling of antigen-specific T cells independent of HLA type. Importantly, I had the privilege of contributing to both cohorts, participating in a wide range of activities, from processing samples and performing laboratory assays to analyzing and writing up the results. This hands-on involvement provided invaluable experience and a deeper understanding of the methodologies and data that underpin the findings presented in this thesis.

Acquisition and maintenance of adaptive immune responses to SARS-CoV-2

Our longitudinal study revealed that most individuals maintained seropositivity for anti-S IgG and, to a lesser extent, for anti-S IgA for over 20 months following SARS-CoV-2 infection during the first wave in the absence of vaccination and reinfections (Chapter 1). Furthermore, S-specific antibody and T-cell responses persisted for over two years

after COVID-19 vaccination and up to three years for those infected before vaccination (Chapter 2 and 3). However, most individuals experienced BTIs, particularly by Omicron subvariants, contributing to the maintenance of immune responses. These findings align with other studies reporting the persistence of SARS-CoV-2 humoral and T-cell responses for up to two years following infection alone [512,515,678] and for more than two years post-COVID-19 vaccination [679–681]. Assessing the long-term durability of immunity from infection alone was challenging due to the widespread rollout of COVID-19 vaccines, which resulted in a small number of unvaccinated individuals available for analysis. However, by including the measure of responses to non-vaccine antigens such as the N protein and conducting intensive follow-up, particularly in Chapter 3, we could capture undiagnosed BTIs and comprehensively evaluate adaptive immune responses to different exposures.

Antibody kinetics demonstrated a prototypical antiviral response, consistent with findings from other studies [526,682–684]. Notably, our studies in Chapters 1-3 provided more precise resolution due to numerous visits during the follow-up and the simultaneous evaluation of multiple isotypes and antigens, including several VOCs. Following exposure, a rapid seroconversion of IgM, IgG, and IgA isotypes was observed, targeting all SARS-CoV-2 antigens in the case of infection and the S protein in the case of mRNA vaccination. Antibody levels peaked shortly thereafter and subsequently declined within 3-8 months after exposure, varying by isotype, viral antigen-specificity, and type of immunization. IgM levels dropped sharply to seronegativity and did not increase following re-exposure. In contrast, IgG and IgA exhibited a biphasic decline: IgG decayed gradually over time, while IgA showed an initial sharp decline that slowed and stabilized over time. Surges following re-exposures restored and even enhanced IgG and IgA responses. This pattern aligns with the expected kinetics of primary and secondary immune responses, including a rapid activation of MBCs and impaired production of IgM due to pre-existing immunity. These kinetics reflect the process of B-cell development and its contraction phase, characterized by an early phase of high antibody production by short-lived plasmablasts, followed by a later phase dominated by antibodies produced by bone marrow LLPCs. These findings are consistent with observations of S-specific long-lasting MBCs during the same period in the CovidCatCentral cohort [685], and

with reports of S-specific MBCs and LLPCs following SARS-CoV-2 infection and vaccination [409,523,527,574]. The prominence and longevity of IgG in peripheral circulation compared to IgA support its central role in systemic immunity and may be explained by differences in antibody half-life and compartmentalization. Indeed, it has been described that most of the neutralizing activity in sera is attributable to IgG [683]. Additionally, all S antigens demonstrated similar persistence over time, but anti-N antibodies declined more rapidly than those directed to S antigen after SARS-CoV-2 infection, as reported by others [170,369,509,686,687]. This could be attributed to the fact that the N protein is not expressed on the surface of the virions and is, therefore, less exposed.

In results from Chapter 3, SARS-CoV-2 infection in previously vaccinated individuals induced greater and more persistent antibody responses than vaccination alone (primary or booster), highlighting the benefits of hybrid immunity. The enhanced and more durable anti-S IgG and IgA responses observed with three compared to two mRNA doses provide evidence supporting the efficacy of booster regimens. Our anti-S IgG half-life (in months) estimates were of 8.5 after SARS-CoV-2 infection in previously vaccinated individuals and 4.5 after booster dose. However, some studies have reported longer half-lives: up to 17 months after infection [688], 23 months after primary vaccination and 3 years after booster dose [689]. These discrepancies likely arise from different study designs, population immunity scenarios, and analytical methods. Moreover, our group further investigated binding antibody levels as a correlate of protection, and higher anti-RBD IgG and IgA levels were associated with protection over three 6-month follow-up periods sequentially dominated by BA.1, BA.2, BA.5, BQ.1, and XBB Omicron subvariants, although the strength of this association declined over time [69,690], also reported by others [515,668,691]. However, immune responses are more complex, and it is important to consider other factors such as T- and B-cell responses, Fc-effector functions of antibodies, and the use of “co-correlates” of protection where multiple immune mechanisms may coordinately and complementarily contribute to protect against infection, disease or severe disease, given that each component play a distinct role in each category and the requirements of immunity for effective protection vary depending on the specific protection [263]. The mechanisms underlying why some

pathogens or vaccines induce long-lasting immune responses while others elicit only short-lived remain incompletely understood. For example, smallpox infection or vaccination generates humoral and T-cell responses that can persist for decades. Smallpox-specific MBCs have been shown to remain stable for over 50 years, constituting ~0.1% of total circulating IgG^+ B cells, and memory T-cell responses have been detected up to 75 years [343,692]. In contrast, humoral immune responses to Influenza A by vaccines are generally shorter-lived (6-12 months) compared to natural infection, in which neutralizing responses to the 1918 H1N1 Influenza A virus have been detected 90 years after the pandemic [693,694]. Neutralizing antibody responses to seasonal HCoVs are also short-lived but T-cell responses have been detected for up to 17 years [443]. Several factors are thought to influence the longevity and robustness of immune responses, including antigen persistence, antigen complexity, vaccine formulation, host factors, and pathogen evasion strategies [324,352,360,695–697]. Understanding these mechanisms is critical for optimizing vaccine design and improving long-term protection against infectious diseases.

Determinants of adaptive immune responses to SARS-CoV-2

We observed in Chapters 1 to 3 the high heterogeneity of SARS-CoV-2 adaptive immune responses. Results from Chapters 1 and 2 elucidated determinants influencing antibody responses to SARS-CoV-2 infection and COVID-19 vaccination, offering valuable insights into the interplay of host factors and immune responses, as supported by other studies [498,501,644,698–701]. The factors with the greatest positive influence on antibody responses in the short- (5-9 months) and long-term (12 months) after SARS-CoV-2 infection during the first wave were symptomatic disease (fever, anosmia/hypogeusia) and hospitalization, likely reflecting the impact of more severe infection (higher viral load with longer antigen exposure) and heightened immune activation. In contrast, smoking and obesity negatively influenced short-term antibody levels. Notably, occupation, specifically being a physician or nurse compared to other primary HCWs (social worker, customer service, technician, driver, maintenance worker, IT worker, X-ray technician, others) negatively influenced long-term SARS-CoV-2 IgG and IgA levels. These results highlight lifestyle, clinical, and occupational factors as modulators. Regarding determinants of COVID-19 vaccine responses, having had a prior SARS-CoV-2 infection

was associated with stronger long-term (11 months) responses. In those pre-exposed individuals, post-vaccination responses were associated to hospitalization and symptomatic disease (anosmia/ageusia, dyspnea, fever and shivers) during the first COVID-19 episode, and longer intervals since disease onset and vaccination. In addition, earlier morning vaccination hours were associated with higher IgG responses in pre-exposed participants, suggesting that immune responses may be influenced by the body's circadian rhythms. Some studies have reported advantages of morning SARS-CoV-2 and influenza vaccination [702–705], while others have found no significant differences between timings [706,707]. Lower responses were observed in smokers, physicians and nurses, and those without adverse events after vaccination, suggesting suboptimal immune priming in these groups.

In addition, the number of SARS-CoV-2 infections and the specific infecting variants shaped adaptive immune responses, as reported [594,708–710]. Interestingly, Omicron infections in vaccinated individuals generated lower T-cell responses compared to infections with pre-Omicron (ancestral/delta) or both pre-Omicron and Omicron variants. Likewise, Omicron infections in pre-exposed individuals generated lower antibody levels over time. This might be explained by the accelerated antigen clearance due to pre-existing immunity [711], immune imprinting, and the attenuated severity of Omicron variants. Unlike ancestral or earlier variants, Omicron infections generally cause milder disease, primarily affecting the upper respiratory tract rather than the lower respiratory tract [712]. Supporting this, studies in mice have demonstrated that Omicron subvariants are inherently less immunogenic than the ancestral virus, resulting in lower humoral and T-cell responses after intranasal challenge [713,714]. These insights emphasize the need to further investigate determinants influencing immune responses and address modifiable factors such as smoking and vaccination timing to tailor vaccination strategies for high-risk groups.

First antigen encounter with SARS-CoV-2 shapes immune responses over time

Our findings indicate that hybrid immunity offers an advantage over infection or vaccination alone, particularly for humoral immunity, characterized by higher and more sustained antibody levels with increased variants cross-recognition. However, no significant differences were observed in long-term T-cell responses. These observations

on superior antibody responses and no differences in T-cell responses are consistent with other studies [423,433,435,499,538,592,594,715,716] and are likely due to broader antigenic exposure and sustained immune activation during infection. In addition, it provides mucosal immunity, which is not effectively elicited by intramuscular mRNA vaccines [377,441,471,474,652,658].

Hybrid immunity presents a highly complex scenario involving antigenic exposures from infection and vaccination, occurring in varying orders, with distinct variants, and over different timeframes. Therefore, initial exposure to the antigen, whether through infection or vaccination, may influence subsequent immune responses to later encounters, as seen in Chapter 4. Interestingly, we found that individuals whose first antigen encounter was infection (first-infected), followed by vaccination, exhibited higher levels of ancestral anti-S and anti-RBD IgG and IgA from the beginning of the pandemic until the emergence of the Omicron variant. The appearance of Omicron marked a turning point in antibody kinetics: from this period onward, individuals whose first antigen encounter was vaccination (first-vaccinated), followed by infection, demonstrated higher anti-S and anti-RBD IgG and IgA, not only against the ancestral strain but also against BA.1, BA.2, BA.4/5, BQ.1.1, XBB Omicron subvariants. In contrast, T-cell responses showed the opposite pattern: first-infected individuals developed more robust and cross-reactive T-cell responses three years later. In addition, first-vaccination was associated with an increased risk of BTIs during the period of Omicron emergence, and this trend was reversed in the later periods, when first-vaccination exhibited a decreased risk of infection, indicating changes depending on the context of immunity and variant evolution. These findings might reflect the phenomenon of immune imprinting in antibody responses, where repeated exposure to the ancestral S through infection and subsequent vaccination in first-infected individuals led to back-boosting of antibodies against ancestral SARS-CoV-2 when Omicron re-exposures. Conversely, when exposed to Omicron subvariants, first-vaccinated individuals appeared to generate more tailored responses to these variants. Notably, the observed differences between first antigen exposures in antibody responses diminished with increasing antigen exposures, as reported in the literature that repeated heterotypic variant exposures mitigate immune imprinting effects [638,639,691,717]. Our findings also align with the observed S-

specific MBCs kinetics and the enrichment of S-specific atypical MBCs in first-infected individuals during the same period in the CovidCatCentral cohort [685], a cell phenotype driven by chronic or repeated antigenic exposure and inflammation [718]. Whether this enrichment is detrimental or beneficial for an efficient SARS-CoV-2 antibody response remains uncertain [719]. These findings suggest the need for adapted vaccination strategies depending on prior SARS-CoV-2 exposures and including heterotypic variants.

The fight between adaptive immune responses and SARS-CoV-2 emerging variants

The rapid development of vaccines with high protective efficacy against severe COVID-19 raised hopes of controlling the pandemic. However, two key factors have limited the long-term effectiveness of these vaccines: the waning of antibodies over time and the emergence of immune-resistant viral variants, leading to common BTIs, though often asymptomatic or mild. This prompted us to recognize the importance of evaluating the potential for cross-recognition from pre-existing immunity generated by infections with earlier variants and/or vaccination. Our findings revealed consistent kinetic patterns of antibody responses, varying by isotype, to the RBD of Alpha, Delta, and BA.1, BA.2, BA.4/5, BQ.1.1, and XBB Omicron subvariants. In Chapters 1, 2, and 3, we detected lower cross-recognition to RBDs from Alpha, Beta, Gamma, and Delta variants, though these responses demonstrated similar persistence as to ancestral RBD over time. In contrast, cross-recognition to the RBDs from BA.1, BA.2, BA.4/5, BQ.1.1, and XBB Omicron subvariants were highly limited, characterized by significantly lower antibody levels and shorter duration compared to ancestral or Alpha, Beta, and Delta variants. These results highlight the significant impact of humoral immune escape by Omicron subvariants and align with literature showing that neutralizing activity is robust against the ancestral virus but progressively diminishes to Alpha, Beta, Gamma, Epsilon, Kappa, and Delta variants and is lost to Omicron subvariants [459,515,618–624,715,720,721]. Importantly, while neutralizing activity against the highly mutated BA.2.86 Omicron subvariant was severely compromised, S-specific T-cell responses remained preserved, albeit with a small loss in cross-recognition. This slight loss of S cross-recognition may be attributed to the six immunodominant TCEs we predicted to have reduced peptide presentation due to BA.2.86 mutations. These findings from Chapter 5 are concordant with research reporting robust T-cell cross-recognition of SARS-CoV-2 emerging variants

[382,430,456,515,709,722–725], emphasizing the critical role of preserved T-cell responses against SARS-CoV-2 emerging variants. Beyond their well-documented association with protection against severe disease, accumulating evidence indicates that T-cell responses are pivotal in controlling established infections, halting viral replication at its earliest stages, as well as their function when properly antibody responses are absent [390,483,726].

The selection of viral mutations generally favors virus survival and often displays a high degree of synergism [83,727], which implies the necessity of evaluating mutations in combination rather than independently. Accordingly, we included the entire S antigen from the variants in our T-cell assays to assess their recognition comprehensively. In contrast, we focused on the RBD for antibodies due to poor performance in the Luminex assays when using complete S protein from Omicron subvariants. The decision to produce and use only the RBD from the variants was further justified by its role as the main target of nAb and its higher density of mutations, which have a more pronounced impact on humoral immunity. In addition, our novel bioinformatic analysis in Chapter 5 enabled us to investigate the impact of mutations at the TCE level. We found that most mutations affecting TCEs are concentrated within the NTD and RBD of S1, in line with recent findings [691,728–730]. The preserved T-cell responses suggest that most targeted epitopes are in stable regions and that T-cell immunity is not a major driving force in SARS-CoV-2 viral evolution. This is coherent with the literature, which indicates that the primary drivers of SARS-CoV-2 evolution and fitness are enhanced ACE2 receptor affinity and humoral immune evasion. During the initial two years of the pandemic, viral evolution was mainly driven by increased transmissibility, likely due to the large population of SARS-CoV-2-naïve individuals. However, as more people gained immunity through vaccination or infection, selected pressures shifted toward mutations that facilitate escape from humoral responses [691]. These insights underline the urgent need to develop a pan-coronavirus vaccine capable of effectively boosting both humoral and T-cell responses. Such a vaccine should incorporate antigens beyond the S protein to provide broader and more durable protection against future variants.

Strengths and limitations

Many of the results and questions regarding COVID-19 were generated at specific moments during the evolving course of the pandemic. The growing body of literature is

vast, with numerous findings, although often contradictory, emerging over time. The entire field of COVID-19 research has been a large-scale, real-time experiment. While many researchers have contributed to this effort, it is important to recognize that the quality of studies varies significantly and that some conclusions or statements have been made based on insufficient evidence.

Within this complex research landscape, this PhD thesis has contributed to a deeper understanding of the kinetics and characteristics of adaptive immune responses to SARS-CoV-2 and the first authorized mRNA vaccines. By analyzing the longitudinal behavior of antibodies and T-cell responses elicited by SARS-CoV-2 infection and vaccination in well-characterized cohorts with multidimensional assays, this work provides critical insights into the duration, quality, and determinants influencing these responses, the impact of hybrid immunity, and the cross-reactivity against variants. These findings form a cornerstone for understanding the current complex immunological context and offer a robust foundation for optimizing control strategies and guiding the development of next-generation COVID-19 vaccines.

Despite these contributions, this thesis has several limitations that should be acknowledged. One major limitation is the composition of our cohorts, which consisted predominantly of white, middle-aged female individuals who experienced mainly mild-to-moderate COVID-19, which do not fully represent the overall population, particularly of immunocompromised or elderly who may exhibit distinct immune responses and durability. Another limitation is that our findings are based solely on systemic immune responses, without assessing mucosal immunity, which plays a critical role in SARS-CoV-2 defense. However, other studies performed by our group in children measured antibody responses in both saliva and serum, demonstrating a good correlation between these compartments [648]. Moreover, the lack of viral sequencing prevented the identification of the infecting variant and viral load, which could have provided valuable insights into immune responses to specific variants and the severity of infection. Other minor limitations were that all participants received mRNA vaccines, restricting our ability to compare immune responses across different vaccine types or regimens. The small sample size of T-cell assays constrained the statistical power to analyze their association with protection. Furthermore, the high complexity of the immune landscape

resulted in a small sample size for analysis when stratified by number of exposures, infections, order of hybrid immunity exposures, or the infecting variant. Finally, from a methodological perspective, the inability to directly convert MFI units into standardized units complicates comparisons with other studies. Similarly, the limited availability of cells restricted the scope of conditions and tests that could be performed. These limitations highlight areas of future research, including the need to expand cohort diversity, enhance sample sizes, and integrate additional immune compartments to provide a more comprehensive understanding of SARS-CoV-2 immunity.

The Road Ahead

In the near future, further analysis will be built upon the findings of this thesis. I am currently analyzing the AIM results from longitudinal kinetics of T-cell responses following vaccination conducted in another prospective cohort, which, in contrast, included a random sample of HCWs in a hospital setting. We will also assess the Fc-effector functions by applying the techniques I acquired during an international research stay at Burnet Institute (Australia), which will complement results from isotype subclasses and binding Fc-effector functions with this cohort. All this information on SARS-CoV-2 adaptive immune responses will enable the identification of co-correlates of protection through a systems immunology approach. Furthermore, as part of the END-VOC consortium (Horizon Europe project), our group will analyze samples from diverse cohorts, encompassing various populations, exposures, and other relevant factors.

Future research should prioritize the development of next-generation pan-coronavirus vaccines capable of eliciting both systemic and mucosal immunity, thereby offering durable and broad protection against existing and emerging coronaviruses. Moreover, vaccination strategies must account for factors influencing adaptive responses to optimize their effectiveness, particularly in vulnerable populations. These efforts will enhance pandemic preparedness and contribute to equitable and robust global health strategies.

Conclusions

1. SARS-CoV-2 infection and COVID-19 vaccination elicit persistent IgG and IgA antibody and T-cell responses over three years of follow-up, although most individuals experienced asymptomatic or mild breakthrough infections, which contributed to the maintenance of responses.

2. SARS-CoV-2 immune responses exhibit high heterogeneity. Factors with highest impact are hybrid immunity compared to vaccination or infection alone and receiving booster doses versus primary vaccination, substantially enhancing the magnitude and persistence of antibody responses. Conversely, smoking, obesity, and occupation (being physician or nurse) are negatively associated with long-term antibody responses, albeit the effects are smaller. These findings highlight the benefits of booster doses and the importance of addressing modifiable risk factors to optimize control strategies and improve public health outcomes.

3. The nature and order of SARS-CoV-2 antigenic encounters shape adaptive immune responses over three years, in parallel with viral evolution. In the context of hybrid immunity, infection followed by vaccination is associated with higher antibody levels and protection during the pre-Omicron period, whereas this pattern is reversed after Omicron emergence. Infection followed by vaccination also leads to more robust, long-lasting T-cell responses. These results suggest the need of tailored vaccination strategies that account for prior SARS-CoV-2 exposures and include heterotypic variants.

4. Pre-existing immunity from ancestral SARS-CoV-2 infection and/or COVID-19 vaccination shows reduced antibody cross-recognition to Alpha, Beta, Gamma, and Delta variants, with greater escape for Omicron subvariants, and absent neutralization to BA.2.86. However, T-cell responses to BA.2.86 remain largely preserved, underscoring the need for next-generation pan-coronavirus vaccines to effectively boost humoral and T-cell immunity against existing and future coronaviruses.

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