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Role of the humoral immune response in the pathophysiology of COVID-19

Melyssa Yaugel Novoa

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Role of the humoral immune response in COVID-19

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“What we know is a drop, what we don't know is an ocean.”

Issac Newton

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Abbreviations

ACE2	Angiotensin-converting enzyme 2
ADCC	Antibody-dependent cellular cytotoxicity
ADCD	Antibody-dependent complement deposition
ADE	Antibody-dependent enhancement
ADP	Antibody-dependent phagocytosis
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavages
DC	Dendritic cells
ICU	Intensive care unit
IFN	Interferon
IFNAR	Interferon-alpha/beta receptor
NET	Neutrophil extracellular traps
NK	Natural killer cells
NSP	Non-structural proteins
ORF	Open reading frame
PRR	Pattern recognition receptors
PSO	Post-symptoms onset
RA	Rheumatoid arthritis
RBD	Receptor-binding domain
RSV	Respiratory syncytial virus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TLR	Toll-like receptors
TMPRSS2	Transmembrane protease serine protease 2
VOC	Variant of Concern
VOI	Variant of Interest
VUM	Variant Under Monitoring

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English Abstract

My PhD thesis is marked by the early years of the COVID-19 pandemic. Despite accumulated knowledge, there is still much to study regarding SARS-CoV-2 infection and the immune response. My objective is to characterize the humoral immune response against the SARS-CoV-2 virus, identify predictive markers of disease severity, and study the immune response after vaccination. In our study, we demonstrated that severe COVID-19 patients had lower levels of sIFNAR1 and higher levels of sIFNAR2 in the serum, compared to asymptomatic patients. These proteins could serve as predictive markers of disease severity. Additionally, we observed that in the most severely affected patients, the serum IgA response was stronger and more rapid than in less severe or asymptomatic patients, suggesting a potential role in the disease's pathophysiology. Furthermore, our work indicates that heterologous vaccination is more immunogenic and protective against different SARS-CoV-2 variants than homologous vaccination. However, the inability of mRNA vaccines to elicit a mucosal response is a gap that should be addressed with new vaccines targeting mucosal pathways. Nevertheless, initial immunization does not sensitize individuals to antibody-dependent enhancement (ADE) of disease. The data generated in this thesis contribute to elucidating certain aspects of the role of the humoral response in the pathophysiology of COVID-19, as well as the immune response developed after vaccination. This work opens the door to new questions and research topics for a better understanding of the immune response in COVID-19.

French Abstract

Mon travail de thèse s'inscrit dans les premières années de la pandémie de COVID-19. Malgré les connaissances accumulées, il reste beaucoup à étudier sur l'infection du SARS-CoV-2 et la réponse immunitaire. Mon objectif est de caractériser la réponse immunitaire humorale contre le virus SARS-CoV-2, d'identifier des marqueurs prédictifs de la gravité de la maladie et d'étudier la réponse immunitaire après vaccination. Dans notre étude, nous avons démontré que les patients atteints de COVID-19 sévère présentaient des niveaux plus bas de sIFNAR1 et des niveaux plus élevés de sIFNAR2 dans le sérum, comparés aux patients asymptomatiques. Ces protéines pourraient être des indicateurs de la gravité de la maladie. Nous avons également constaté que chez les patients les plus gravement atteints, la réponse IgA sérique était plus forte et précoce que chez les patients moins graves ou asymptomatiques. Ces résultats suggèrent un rôle potentiel dans la physiopathologie de la maladie. De plus, notre travail montre que la vaccination hétérologue est plus immunogénique et protectrice contre différents variants du SARS-CoV-2 qu'une vaccination homologue. Cependant, l'incapacité des vaccins à ARN messager (mRNA) à créer une réponse mucoale est une lacune qu'il faudrait combler avec de nouveaux vaccins, ciblant la voie mucoale pour la vaccination. Néanmoins, une première immunisation ne sensibilise pas à une amplification de la maladie dépendante d'anticorps (ADE). L'ensemble des données produites dans ce travail de thèse contribue à éclaircir certains aspects du rôle de la réponse humorale dans la physiopathologie de la COVID-19, ainsi que la réponse immunitaire développée après vaccination. Ce travail ouvre la voie à de nouvelles questions et pistes de recherche pour mieux comprendre la réponse immunitaire dans la COVID-19.

Introduction

Chapter I: Epidemiology and clinical characteristics of COVID-19

Pandemic and disease burden

In December 2019, a new type of coronavirus called SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) originated in Wuhan, China, leading to an outbreak of a distinctive form of viral pneumonia. Within one month after the first case, all 34 provinces of China reported new cases of the new coronavirus disease (COVID-19) and its international spread accelerated from late February. On 11 March 2020, the WHO officially characterized the global COVID-19 outbreak as a pandemic (1). The severity of the situation led to the implementation of control measures, such as quarantines, border closures, and social distancing, with the aim of slowing down the transmission of the virus and reducing the impact on healthcare systems, posing an unprecedented global health and socioeconomic crisis in modern history. So far (as in August 2023) there have been 768,983,095 confirmed cases of COVID-19 worldwide, including 6,953,743 deaths, reported to WHO (**Fig. 1A and Fig.1B**).

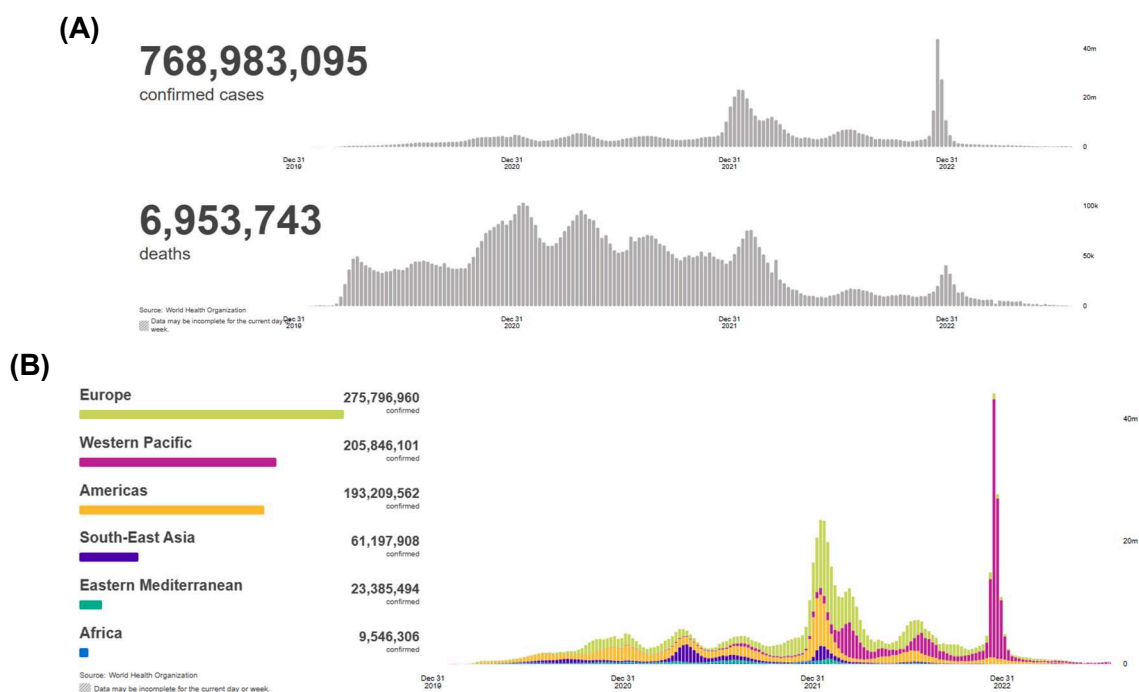


Figure 1. COVID-19 reported cases since the beginning of the pandemic in 2019, worldwide (A) and by region (B), according to the WHO (1).

Different hypotheses have been proposed to understand the origin of this new virus. While the hypothesis of a laboratory escape from Wuhan might be the most mediatized, most of the scientific evidence suggests that SARS-CoV-2 naturally originated from a phylogenetically close bat coronavirus (**Fig. 2**) (2). However, as a significant gap exists between these two viruses, the research to find an intermediate host continues (3). Other closely related coronaviruses infecting pangolins have also been proposed as the progenitor. To fully demonstrate the natural origin of SARS-CoV-2, it will be fundamental to extensively survey the presence of coronaviruses in wild animals and study their evolution and ecology (3)(3).

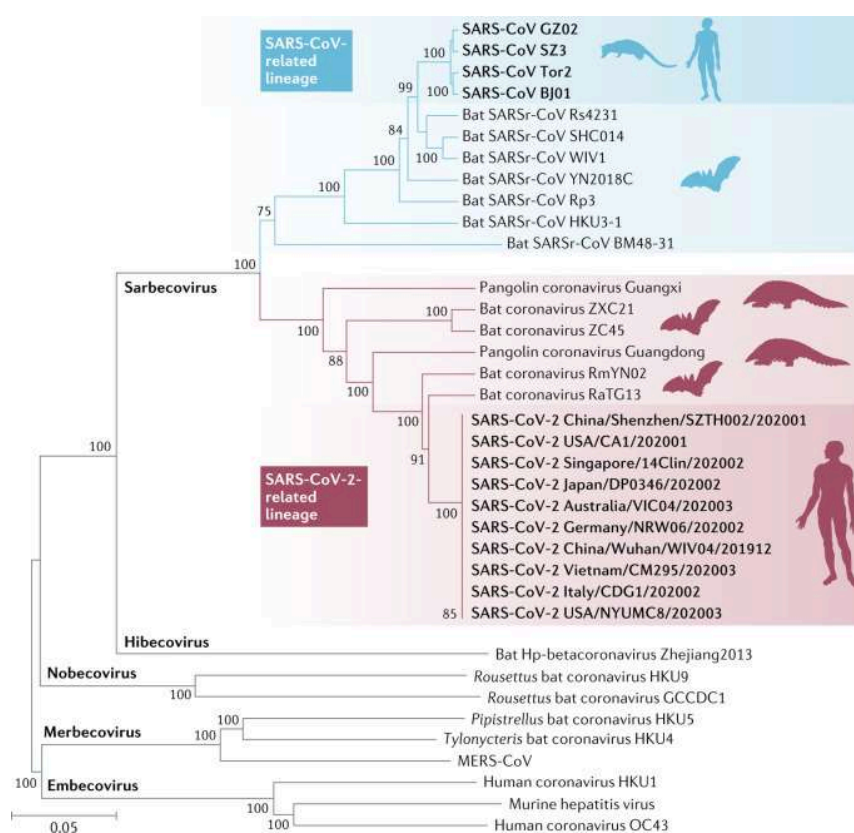


Figure 2. Phylogenetic tree of SARS-CoV-2 virus and related coronaviruses (2).

The construction was performed by the neighbour joining method with use of the program MEGA6 with bootstrap values being calculated from 1,000 trees. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) clusters with closely related viruses in bats and pangolins and together with SARS-CoV and bat SARS-related coronaviruses (SARSr-CoVs) forms the sarbecoviruses. The sequences were downloaded from the GISAID database and GenBank. MERS-CoV, Middle East respiratory syndrome coronavirus.

SARS-CoV-2

Structure

SARS-CoV-2 virus belongs to the subgenus Sarbecovirus of the genus Betacoronavirus, and it shares 79% genome sequence identity with SARS-CoV and 50% with MERS-CoV (4). It is an enveloped, positive-sense, single-stranded RNA virus (4). The genome of SARS-CoV-2 consists of about 30,000 bases and contains a 5' cap structure and a 3' poly-A tail. It has a 5' open reading frame (ORF) and a 3' ORF that comprises 2/3 and 1/3 of the complete genome, respectively. The 5' ORF (ORF1a/b) is translated into pp1a and pp1ab proteins in the host cell's rough endoplasmic reticulum (rER). Proteases cleave these proteins and produce 16 non-structural proteins (NSPs), ranging from NSP1 to NSP16. The 3' ORF of SARS-CoV-2 has both structural and accessory proteins. There are four structural proteins, i.e., Spike (S), Envelop (E), Nucleocapsid (N), and Membrane (M) proteins (**Fig. 3**). The N protein is multifunctional; its main function is to assemble the genomic RNA of the virus into a ribonucleoprotein complex and regulate viral replication. The M protein is responsible for the assembly of viral particles. The E protein regulates replication, pathogenicity, and virus dissemination. In addition, there are nine accessory proteins, i.e., ORF3a, ORF3b, ORF6, ORF7a, ORF7b, ORF8, ORF9b, ORF9c, and ORF10. The accessory proteins do not play roles in virus replication but have important roles in host immune evasion (5).

The SARS-CoV-2 S protein has a full size of 1,273 amino acids, longer than that of SARS-CoV (1,255 amino acids), but it shares 76.7–77.0% with SARS-CoVs from civets and humans, 75–97.7% with bat coronaviruses in the same subgenus and 90.7–92.6% with pangolin coronaviruses (6). The S protein is composed of two subunits: S1 and S2. The S1 subunit is further divided into two functional domains, an N-terminal domain, and a C-terminal domain. Structural and biochemical analyses identified a 211 amino acid region (amino acids 319–529) at the S1 C-terminal domain of SARS-CoV-2 as the receptor-binding domain (RBD), which has a key role in virus entry because it directly interacts with the viral receptor ACE2 (angiotensin-converting enzyme 2) being therefore, the target of most neutralizing antibodies (2). SARS-CoV-2 S protein has the specific feature of insertion of four amino acid residues (PRRA) at the junction of subunits S1 and S2 that generates a polybasic cleavage site (RRAR) specific by furin and other proteases facilitating the interaction of RBD-ACE2 receptor

(7). Moreover, the receptor binding motif (RBM) within the RBD domain mediates contact with the ACE2 receptor and this region differs from SARS-CoV in 5 amino acids critical for receptor binding, resulting in a more compact conformation of the hACE2-binding ridge and better contact with the N-terminal helix of hACE2 than in SARS-CoV. These structural changes have been associated with the higher transmissibility of SARS-CoV-2 compared to SARS-CoV (2).

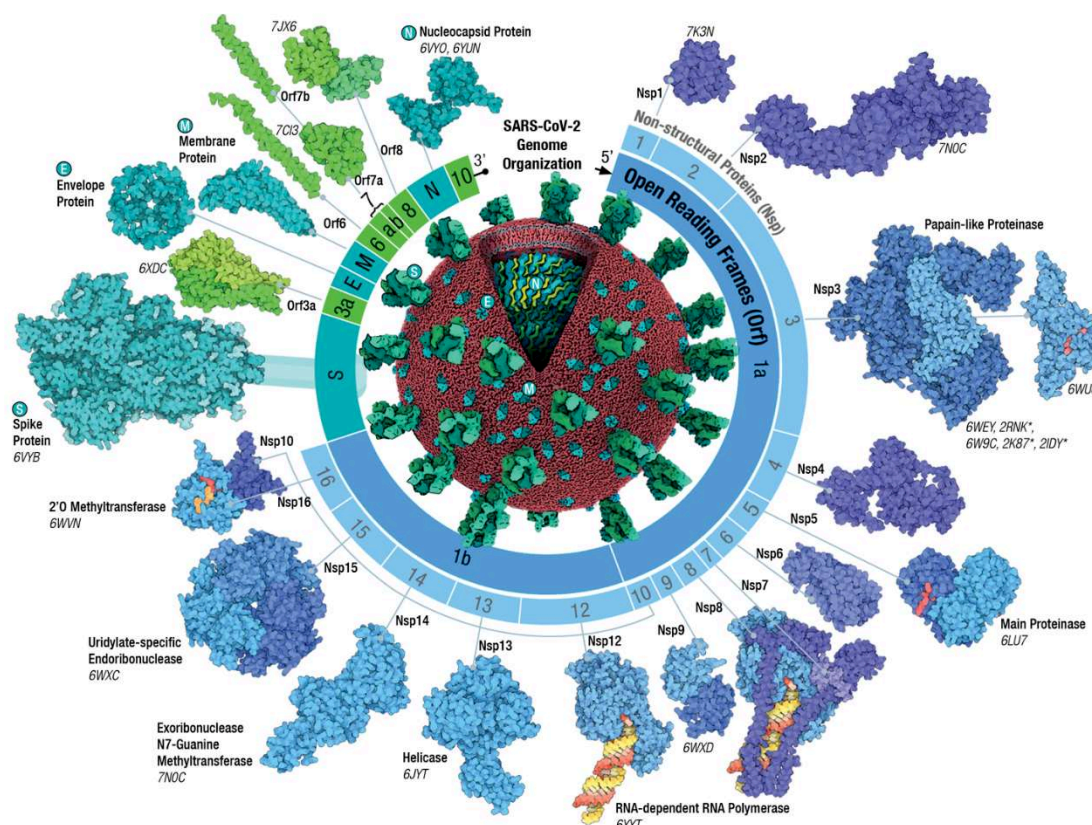


Figure 3. SARS-CoV-2 genome structure and proteins (8).

Structural proteins are coloured in cyan blue, non-structural proteins in dark blue and accessory proteins in green.

SARS-CoV-2 viral replication

Viral entry proteins need to adopt a stable conformation while also undergoing a conformational transition that generates enough energy to overcome the natural repulsion between the virus and cellular membranes. As a result, the S protein undergoes a transition into a metastable state, which tends to transition into a lower-energy state before membrane fusion occurs. The first step is the interaction between the S protein and the ACE2 receptor through the RBD domain. Other host proteins,

such as neuropilin-1, heparin sulfate proteoglycans, C-type lectins, or furin, can also act as viral entry co-factors (9–12). Like SARS-CoV and other coronaviruses, this transition of the S protein in SARS-CoV-2 involves two proteolytic cleavage steps following engagement with ACE2. The first cleavage occurs at the S1-S2 boundary, while the second cleavage occurs at the S2' site within the S2 subunit. In SARS-CoV, both cleavage sites are targeted by proteases in the host cell. However, in the case of SARS-CoV-2, the furin protease cleaves the S1-S2 boundary in the virus-producing cell, while the cleavage of the S2' site occurs in the target cell after binding to ACE2 receptor and still requires the transmembrane protease serine protease 2 (TMPRSS2) in the target cell (13,14) Depending on the entry route of SARS-CoV-2, the cleavage of the S2' site is carried out by different proteases. In one scenario, if the target cell lacks sufficient TMPRSS2, or if the virus-ACE2 complex does not encounter TMPRSS2, the complex is internalized via clathrin-mediated endocytosis into endolysosomes. In this case, cathepsins, which require an acidic environment, cleave the S2' site. In another scenario, in the presence of TMPRSS2, the S2' cleavage occurs at the cell surface. In both entry pathways, cleavage of the S2' site exposes the fusion peptide (FP), and the dissociation of S1 from S2 leads to significant conformational changes in the S2 subunit, particularly in heptad repeat 1 (HR1). This propels the fusion peptide forward into the target membrane, initiating membrane fusion. Fusion between the viral and cellular membranes forms a fusion pore through which viral RNA is released into the host cell cytoplasm for uncoating and replication (13) **(Fig. 4)**. TMPRSS2 is highly expressed in several tissues and body sites and is co-expressed with ACE2 in nasal epithelial cells, lungs, and bronchial branches, which explains some of the tissue tropism of SARS-CoV-2 (6,15).

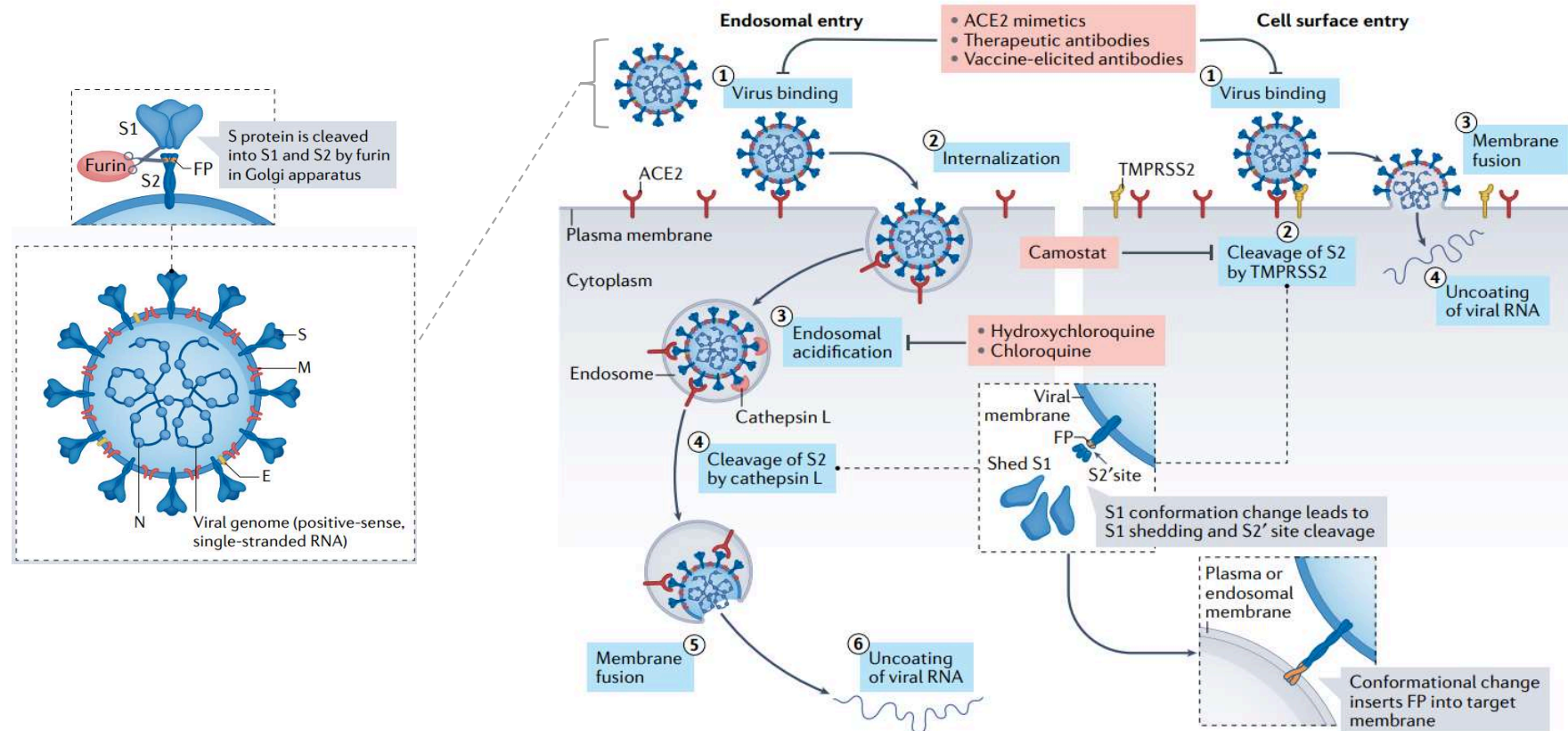


Figure 4. Two distinct SARS-CoV-2 entry pathways (13).

Two spike (S) protein cleavage events are typically necessary for the coronavirus entry process: one at the junction of the S1 and S2 subunits and the other at the S2' site, internal to the S2 subunit. In the case of SARS-CoV-2, the polybasic sequence at the S1–S2 boundary is cleaved during virus maturation in an infected cell, but the S2' site is cleaved at the target cell following ACE2 binding. Virus binding to ACE2 (step 1) induces conformational changes in the S1 subunit and exposes the S2' cleavage site in the S2 subunit. Depending on the entry route taken by SARS-CoV-2, the S2' site is cleaved by different proteases. Left: If the target cell expresses insufficient TMPRSS2 or if a virus–ACE2 complex does not encounter TMPRSS2, the virus–ACE2 complex is internalized via clathrin-mediated endocytosis (step 2) into the endolysosomes, where S2' cleavage is performed by cathepsins, which require an acidic environment for their activity (steps 3 and 4). Right: In the presence of TMPRSS2, S2' cleavage occurs at the cell surface (step 2). In both entry pathways, cleavage of the S2' site exposes the fusion peptide (FP) and dissociation of S1 from S2 induces dramatic conformational changes in the S2 subunit, especially in heptad repeat 1, propelling the fusion peptide forward into the target membrane, initiating membrane fusion (step 5 on the left and step 3 on the right). Fusion between viral and cellular membranes forms a fusion pore through which viral RNA is released into the host cell cytoplasm for uncoating and replication (step 6 on the left and step 4 on the right). Several agents disrupt interaction between the S protein and ACE2: ACE2 mimetics, therapeutic monoclonal antibodies targeting the neutralizing epitopes on the S protein and antibodies elicited by vaccination block virus binding to ACE2 and thus inhibit both entry pathways. By contrast, strategies targeting post-receptor-binding steps differ between the two pathways. Being a serine protease inhibitor, camostat mesylate restricts the TMPRSS2-mediated entry pathway. Hydroxychloroquine and chloroquine block endosomal acidification, which is necessary for cathepsin activity, and thus restrict the cathepsin-mediated entry pathway.

Variants

SARS-CoV-2 is classified in different ways, and it is often discussed in the context of lineages, sublineages, and variants (16). A lineage is a group of closely related viruses with a common ancestor and their descendants are sublineages, while a variant is defined as a viral genome that may contain one or more mutations (16). A lineage or group of lineages with similar genetic changes may be designated by the World Health Organization (WHO) as a Variant of Interest (VOI), Variant of Concern (VOC), or Variant Under Monitoring (VUM) due to shared attributes and characteristics that may require public health action (16). To date there have been five SARS-CoV-2 variants declared as VOCs by the WHO based on the altered transmissibility and immune escape capabilities. Every VOC demonstrated superior transmissibility compared to the previous variants, leading to their dominance in specific regions or globally. The Alpha variant (PANGO lineage B.1.1.7) prevailed regionally, while the Beta variant (B.1.351) and the Gamma variant (P.1) dominated in southern Africa and South America, respectively. In contrast, the Delta variant (B.1.617.2/AY sublineages) and the various sublineages of the Omicron variant (B.1.1.529/BA sublineages, including BA.1, BA.2, and BA.5) gained global prominence (17). As Omicron infections are 98% of current COVID-19 cases, since March 15th, 2023, the WHO variant tracking system considers the classification of Omicron sublineages independently as VUM, VOI or VOCs (18). Currently, VOIs are XBB.1.5 and XBB.1.16; and VUMs are BA.2.75, CH.1.1, XBB.1.9.1, XBB.1.9.2 and XBB.2.3 (18). **Fig. 5** depicts the phylogeny of SARS-CoV-2 variants described since the beginning of the pandemic (19).

It is known that RNA viruses have a higher mutation rate compared to DNA viruses. However, SARS-CoV-2 has lower rates of recombination, insertions and deletions, and point mutations than other RNA viruses due to the proofreading activity of the replication-associated exoribonuclease (ExoN) (20). The first notable change in SARS-CoV-2 evolution was a single spike substitution (D614G) that appeared early in the pandemic and gained ~20% growth advantage becoming dominant in Europe (PANGO lineage B.1). From October 2020 other variants emerged with distinct grades of transmissibility and antigenicity (21). These variants are distinguished by higher

numbers of non-synonymous mutations in the S protein, particularly the Omicron variant (15) (**Fig. 6**).

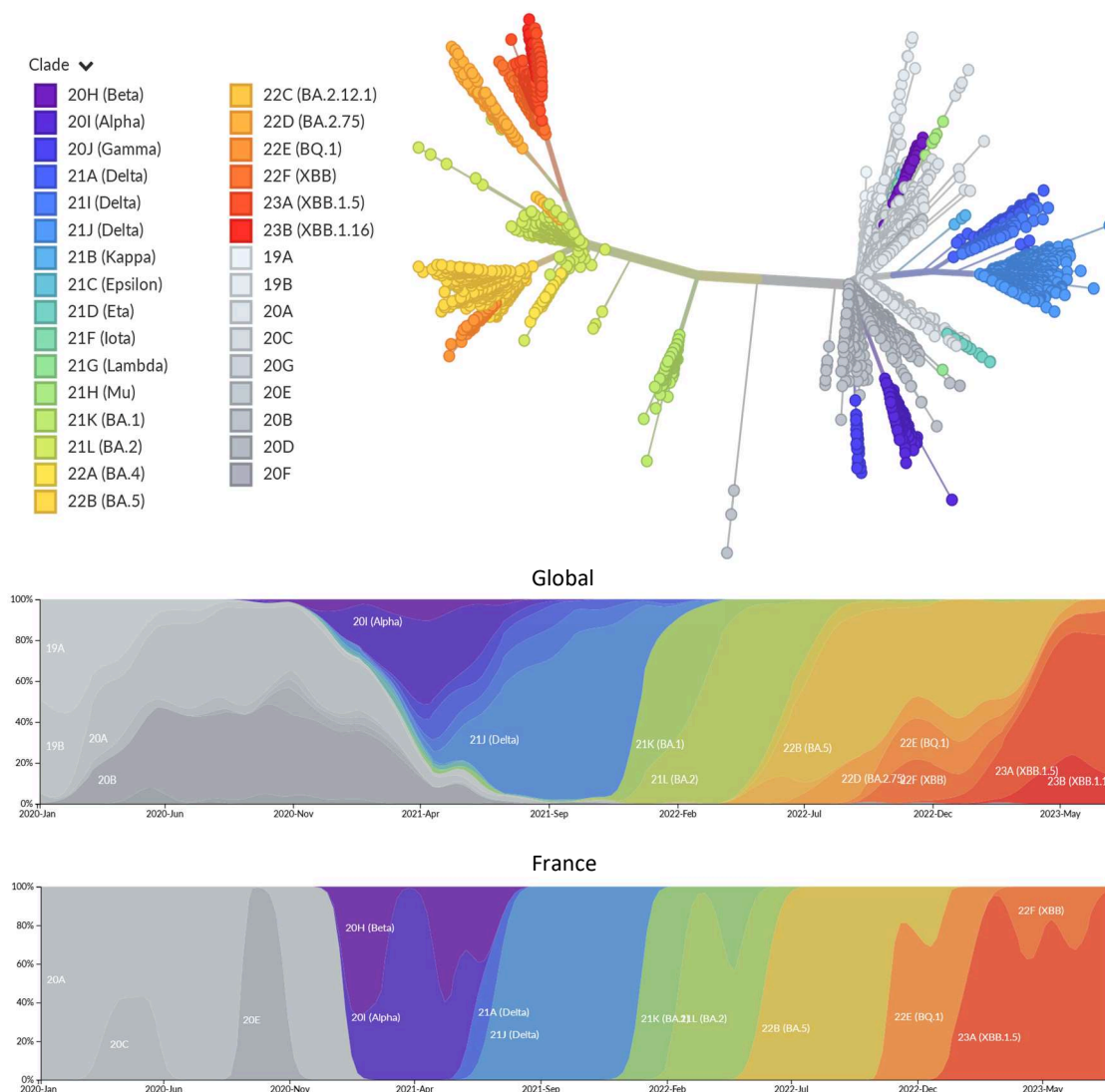


Figure 5. SARS-CoV-2 phylogeny evolution since the beginning of the COVID-19 pandemic (19).

As SARS-CoV-2 receptor ACE2 is expressed in different mammal species and in different parts of the human body, it can rapidly spread. ACE2 binding and the furin cleavage site between S1 and S2 are the principal targets of the VOCs' new mutations contributing to the efficient transmission in humans. For example, Alpha and Delta variants furin cleavage site (FCS) optimization contributed to the 65% and 55% higher relative transmissibility of these variants compared to their precedent ones. In contrast

to these variants, Omicron success is linked to an altered entry phenotype with a preference for the endosomal entry pathway (22) coupled with significant immune escape enabling efficient infection of vaccinated or previously infected individuals. These features of Omicron map to different regions of spike, with S2 determining endosomal entry and the RBD controlling proteolysis and syncytia formation (22).

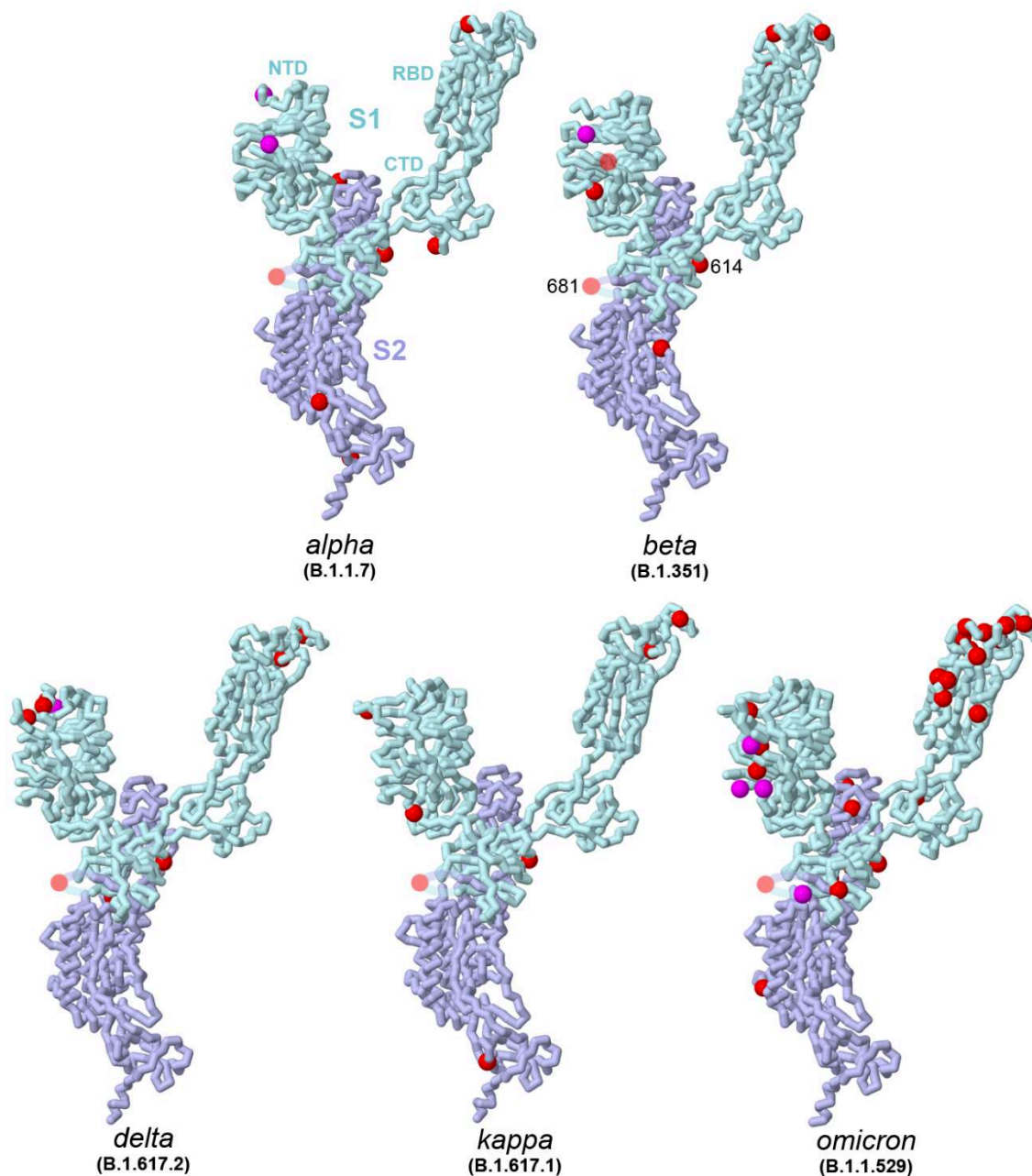


Figure 6. Important variants of SARS-CoV-2 spike. Mutations are showed in red and deletions in magenta (23).

Important variants of SARS-CoV-2 spike with mutations in red and deletions in magenta. The active spike is cleaved into two functional pieces, S1 and S2, shown in turquoise and blue. S1 is composed of several functional domains: the N-terminal domain (NTD), the receptor-binding domain (RBD), and two C-terminal domains (CTD).

COVID-19 symptoms and clinical characteristics

Signs and symptoms of COVID-19 can vary. The most common symptoms include fever, cough, fatigue, anorexia, shortness of breath, and muscle pain. Other non-specific symptoms like sore throat, nasal congestion, headache, diarrhea, nausea, vomiting, loss of smell (anosmia), and loss of taste (ageusia) may also occur. Neurological manifestations such as dizziness, agitation, weakness, seizures, and stroke-like symptoms have been reported. Older individuals and immunosuppressed patients may exhibit atypical symptoms like fatigue, reduced alertness, mobility, loss of appetite, and confusion, without fever. Children may not exhibit fever or cough as frequently as adults. Moreover, as the virus evolves, the clinical symptoms also change according to the SARS-CoV-2 variant of infection. Symptoms that were not that common with the Wuhan variant, now are present in most of the new infections with Omicron variants (**Fig. 7**) (24). Nevertheless, clinical definitions of disease severity are still the same according to the World Health Organization (WHO) (**Table 1**) (25).

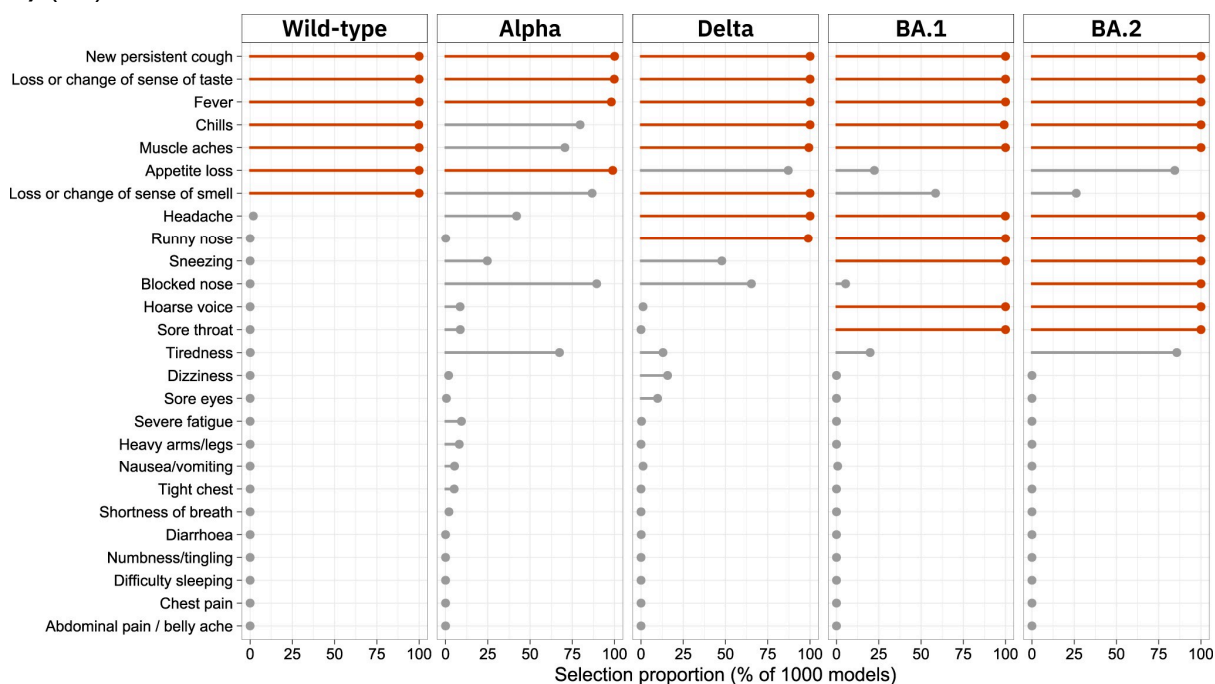


Figure 7. Clinical symptoms variation according to different SARS-CoV-2 variants (26).

Table 1. Clinical definitions of disease severity in COVID-19 (25).

Clinical severity	Principal symptom	Definition
Mild disease		Symptomatic patients meeting the case definition for COVID-19 without evidence of viral pneumonia or hypoxia.
Moderate disease	Pneumonia	Adolescent or adult with clinical signs of pneumonia (fever, cough, dyspnoea, fast breathing) but no signs of severe pneumonia, including SpO ₂ ≥ 90% on room air. Child with cough or difficulty breathing + fast breathing and/or chest indrawing and no signs of severe pneumonia. Fast breathing: < 2 months: ≥ 60 breaths/min; 2–11 months: ≥ 50; 1–5 years: ≥ 40. The diagnosis can be made on clinical grounds; chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications.
Severe disease	Severe pneumonia	Adolescent or adult with clinical signs of pneumonia (fever, cough, dyspnoea) plus one of the following: respiratory rate > 30 breaths/min, severe respiratory distress, or SpO ₂ < 90% on room air. Child: with clinical signs of pneumonia (cough or difficulty breathing + fast breathing or chest wall indrawing) + at least one of the following: <ul style="list-style-type: none"> • SpO₂ < 90% • Very severe chest indrawing, grunting, central cyanosis, or presence of any other general danger sign (inability to breastfeed or drink, lethargy or unconsciousness or convulsions). The diagnosis can be made on clinical grounds; chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications
Critical disease	Acute respiratory distress syndrome (ARDS)	Onset: within 1 week of a known clinical insult (i.e. pneumonia) or new or worsening respiratory symptoms. Chest imaging: radiograph, CT scan or lung ultrasound: bilateral opacities, not fully explained by volume overload, lobar or lung collapse, or nodules. Origin of pulmonary infiltrates: respiratory failure not fully explained by cardiac failure or fluid overload. Need objective assessment (e.g. echocardiography) to exclude hydrostatic cause of infiltrates/oedema if no risk factors present. <p>Oxygenation impairment in adults:</p> <p>⇒ Air blood gases (ABG) available</p> <ul style="list-style-type: none"> • Mild ARDS: 200 mmHg < PaO₂/FiO₂ ≤ 300 mmHg (with PEEP or CPAP ≥ 5 cmH₂O) • Moderate ARDS: 100 mmHg < PaO₂/FiO₂ ≤ 200 mmHg (with PEEP ≥ 5 cmH₂O) • Severe ARDS: PaO₂/FiO₂ ≤ 100 mmHg (with PEEP ≥ 5 cmH₂O). <p>⇒ ABG not available (Kigali modification)</p> <ul style="list-style-type: none"> • SpO₂/FiO₂ < 315 suggests ARDS (including non-ventilated patients)

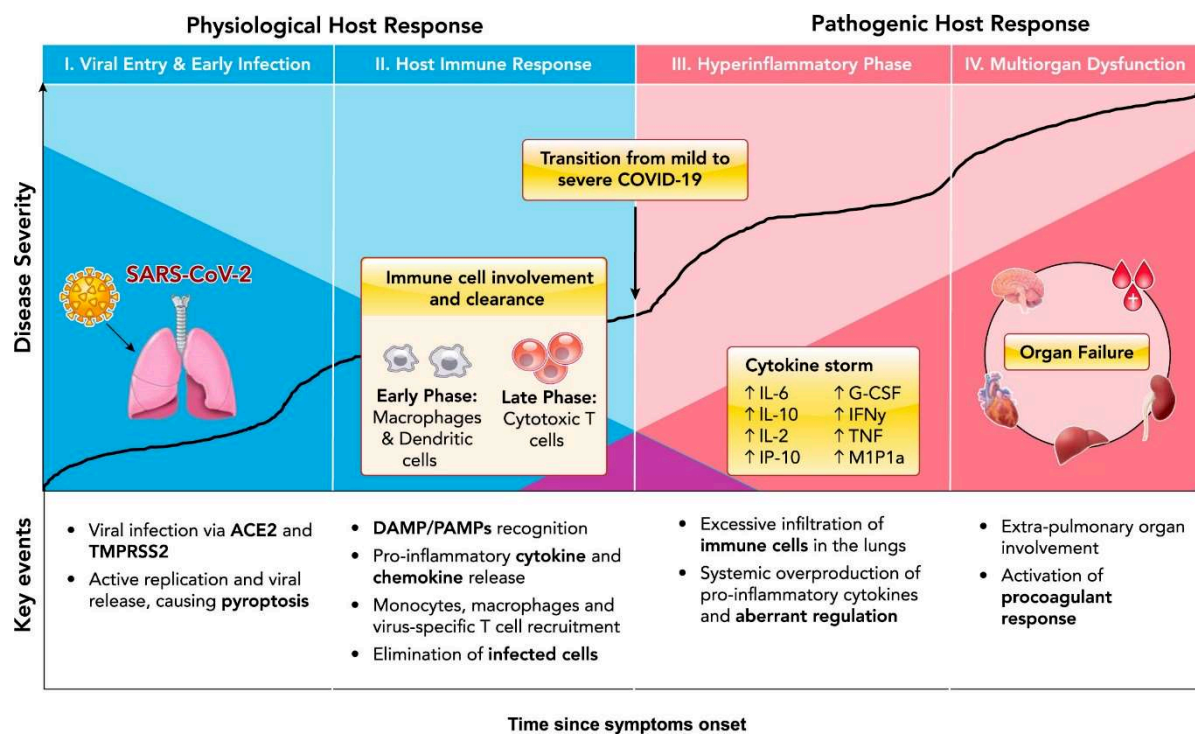
		<p>Oxygen impairment in children: note OI and OSI.^a Use OI when available. If PaO₂ not available, wean FiO₂ to maintain SpO₂ ≤ 97% to calculate OSI or SpO₂/FiO₂ ratio:</p> <ul style="list-style-type: none"> • Bilevel (NIV or CPAP) ≥ 5 cmH₂O via full face mask: PaO₂/FiO₂ ≤ 300 mmHg or SpO₂/ FiO₂ ≤ 264 • Mild ARDS (invasively ventilated): 4 ≤ OI < 8 or 5 ≤ OSI < 7.5 • Moderate ARDS (invasively ventilated): 8 ≤ OI < 16 or 7.5 ≤ OSI < 12.3 • Severe ARDS (invasively ventilated): OI ≥ 16 or OSI ≥ 12.3.
	Sepsis [110][111]	<p>Adults: acute life-threatening organ dysfunction caused by a dysregulated host response to suspect or proven infection. Signs of organ dysfunction include: altered mental status (delirium), difficult or fast breathing, low oxygen saturation, reduced urinary output, fast heart rate, weak pulse, cold extremities or low blood pressure, skin mottling, laboratory evidence of coagulopathy, thrombocytopenia, acidosis, high lactate or hyperbilirubinaemia.</p> <p>Children: suspected or proven infection and ≥ 2 age-based systemic inflammatory response syndrome (SIRS) criteria,^b of which one must be abnormal temperature or white blood cell count. b SIRS criteria: abnormal temperature (> 38.5 °C or < 36 °C); tachycardia for age or bradycardia for age if < 1 year; tachypnoea for age or need for mechanical ventilation; abnormal white blood cell count for age or > 10% bands.</p>
	Septic shock [110][111]	<p>Adults: persistent hypotension despite volume resuscitation, requiring vasopressor to maintain MAP ≥ 65 mmHg and serum lactate level > 2 mmol/L. Children: any hypotension (SBP < 5th centile or 2SD below normal for age) or two or three of the following: altered mental status; bradycardia or tachycardia (HR < 90 beats/min [bpm] or < 160 bpm in infants and heart rate < 70 bpm or > 150 bpm in children); prolonged capillary refill (> 2 sec) or weak pulse; fast breathing; mottled or cool skin or petechial or purpuric rash; high lactate; reduced urine output; hyperthermia or hypothermia.</p>
	Acute thrombosis	<p>Acute venous thromboembolism (i.e. pulmonary embolism), acute coronary syndrome, acute stroke.</p>
	MIS-C	<p>Preliminary case definition: children and adolescents 0–19 years of age with fever ≥ 3 days AND two of the following: rash or bilateral non purulent conjunctivitis or muco-cutaneous inflammation signs (oral, hands or feet); hypotension or shock; features of myocardial dysfunction, pericarditis, valvulitis, or coronary abnormalities (including ECHO findings or elevated troponin/NT-proBNP); evidence of coagulopathy (PT, PTT, elevated D-dimers); acute gastrointestinal problems (diarrhoea, vomiting or abdominal pain); AND elevated markers of inflammation such as ESR, C-reactive protein, or procalcitonin AND no other obvious microbial cause of shock syndrome AND evidence of COVID-19 (RT-PCR, antigen test or serology positive), or likely contact with patients with COVID-19.</p>

Pathophysiology of COVID-19

The pathogenesis of the SARS-CoV-2 induced pneumonia occurs in two phases: (1) the viral phase, which is characterized by the extension of the viral replication and virus-mediated tissue damage (27); and (2) the immune phase, characterized by the recruitment of immune cells causing local and systemic inflammation that can persist even after viral clearance (27). The damages that occurred during the first phase determine the second one, and it is not well established the exact contribution of viral loads in the disease course (28). However, pulmonary complications are associated with excessive vascular permeability, microthrombi deposition, and with systemic symptoms like olfactory, cardiac, hepatobiliary, and renal dysfunction, as well as gastrointestinal (GI) symptoms. These manifestations are multifactorial, including direct viral injury to the cells, tissues and vasculature, excessive inflammatory cytokines release, auto-antibodies-dependent tissue damage and gut dysbiosis. Moreover, autopsies from COVID-19 deceased patients have shown that it is more the over-activation of the immune system or vascular damage what kills the patients rather than an excessive viral-induced tissue damage (27). On binding to epithelial cells in the respiratory tract, SARS-CoV-2 starts replicating and migrating down to the airways and enters alveolar epithelial cells in the lungs. The rapid replication of SARS-CoV-2 in the lungs may trigger a strong immune response (2). Histopathological changes in patients with COVID-19 occur mainly in the lungs. Histopathology analyses showed bilateral diffused alveolar damage, hyaline membrane formation, desquamation of pneumocytes and fibrin deposits in lungs of patients with severe COVID-19. Exudative inflammation was also shown in some cases. Immunohistochemistry assays detected SARS-CoV-2 antigen in the upper airway, bronchiolar epithelium, and submucosal gland epithelium, as well as in type I and type II pneumocytes, alveolar macrophages and hyaline membranes in the lungs (2). **Fig. 8** shows the evolution of severity in COVID-19 according to the pathophysiology of the disease.

Time is crucial for controlling viral infections starting with the innate immune response. Type I interferons (IFN-I) are a very important key to limiting viral replication and promote viral immunity (29). In severe COVID-19 patients it has been identified an imbalance in the IFN response, explained either by host genetic mutations altering the function of Toll-like receptors (TLRs) or other signaling molecules associated to the

IFN pathways; and, by auto-antibodies against IFN that inhibit their function (30–33). Another important aspect is that several SARS-CoV-2 accessory and non-structural proteins like ORF9b, ORF6, ORF7a, Nsp6, Nsp8 and Nsp13 interfere with the correct IFN signaling pathways for example by downregulating the phosphorylation-mediated



activation of different molecules involved in those pathways (**Table 2**) (5,34).

Figure 8. Pathophysiological phases of COVID-19 severity (35).

Cytokines are important immune mediators delivered in a first time by the infected cells and tissue damaged cells that results in the recruitment of innate immune cells (monocytes, macrophages, neutrophils, natural killer (NKs), and dendritic cells (DCs)) that they will also continue to secrete proinflammatory cytokines that allow the adaptive immune response to be called (T and B cells). There is an uncontrolled release of proinflammatory cytokines in severe patients called “cytokine storm” that exacerbate the infiltration of immune cells and therefore an increase in local and systemic inflammation (27). In addition, DCs and T cell reduction numbers in severe COVID-19 patients do not allow the correct clearance of infected cells, nor the type 2 immune response present in these patients. Moreover, resident macrophages that are important in tissue homeostasis and repair are found to be depleted in severe patients, maybe due to direct impact of viral infection or inflammation-induced cell death (27).

Table 2. SARS-CoV-2 proteins interfering with IFN induction and signalling (5).

IFN production inhibition					IFN signalling inhibition				
Protein	Mechanism	Experimental approach	Cellular model	Ref.	Protein	Mechanism	Experimental approach	Cellular model	Ref.
NSP1	Multiple targets, may be upstream and downstream of IRF3	Luciferase assay	HEK293T cells	(36)	NSP1	Suppress STAT1 phosphorylation	Western blotting	HEK293T cells	(36)
NSP6	Suppress IRF3 phosphorylation	Western blotting	HEK293T cells	(36)	NSP6	Suppress STAT1 and STAT2 phosphorylation	Western blotting	HEK293T cells	(36)
NSP8	Suppress the phosphorylation of IRF3, TBK1	Western blotting	HEK293T cells	(37)	NSP13	Suppress STAT1 and STAT2 phosphorylation	Western blotting	HEK293T cells	(36)
NSP12	Inhibit nuclear translocation of IRF3	Immunofluorescence assays	HEK293T cells	(38)	ORF3a	Suppress STAT1 phosphorylation	Western blotting	HEK293T cells	(36)
NSP13	Physical binds with TBK1, Suppress the phosphorylation of IRF3, TBK1, and NF- κ B, Suppress nuclear translocation of NF- κ B	Luciferase assay, Western blotting, Immunofluorescence assays	HEK293T, HeLa cells	(36)	ORF6	Inhibit STAT1 nuclear translocation	Immunofluorescence assays	HEK293T cells	(36,39)
NSP14	Inhibit nuclear translocation of IRF3	Luciferase assay, Immunofluorescence assays	293 FT cells	(40)	ORF7a	Suppress STAT2 phosphorylation	Western blotting	HEK293T cells	(36)
NSP15	Inhibit nuclear translocation of IRF3	Luciferase assay, Immunofluorescence assays	293 FT cells	(40)	ORF7b	Suppress STAT1 and STAT2 phosphorylation	Western blotting	HEK293T cells	(36)

ORF6	Inhibit nuclear translocation of IRF3	Luciferase assay, Western blotting, Immunofluorescence assays	HEK293T	(36)		ORF8	Interact with MHC-I and mediate its down regulation	Western blotting	HEK293T cells	(41)
ORF9b	Interact with MDA5, MAVS, TRIF, TBK1, STING, and RIG-1, suppress the phosphorylation of TBK1 and IRF3, suppress nuclear translocation of IRF3	Luciferase assay, Western blotting, Immunofluorescence assays	HEK293T, HeLa cells	(42)		ORF10	Degrade MAVS	Western blotting	HEK293T cells	(43)
M	Suppress the phosphorylation of IRF3, TBK1, IKK α/β , p65	Luciferase assay, qRT-PCR, Western blotting	HEK293T	(44)						

Auto-antibodies targeting nuclear antigens, phospholipids, T and B cell antigens as well as chemokines and cytokines have been reported for severe COVID-19 patients, and they might contribute to the immune cell depletion described before, as well as vascular damage (clots formation, endothelial damage, venous and arterial embolisms) (30,45). All these steps contributing to the pathogenesis of the SARS-CoV-2 infection are described locally, but if we think about the expression of the ACE2 receptor in the human body (**Fig. 9**), it is easy to imagine that after the respiratory mucosa gets infected, all the other ACE2⁺ tissues in the body are having the same altered inflammatory phenotype. Therefore, the last phase of the pathophysiology of COVID-19 is multiorgan failure. This phase is characterized by systemic inflammation and functional complications in different organs. For example, significantly elevated levels of classical markers of cardiac injury and failure like cardiac troponin (46), acute kidney injury (47), diarrhea and abdominal pain (48), elevated levels of liver enzymes and liver injury (49), pancreatic dysfunction (50), hypoxia as well as metabolic acidosis (51).

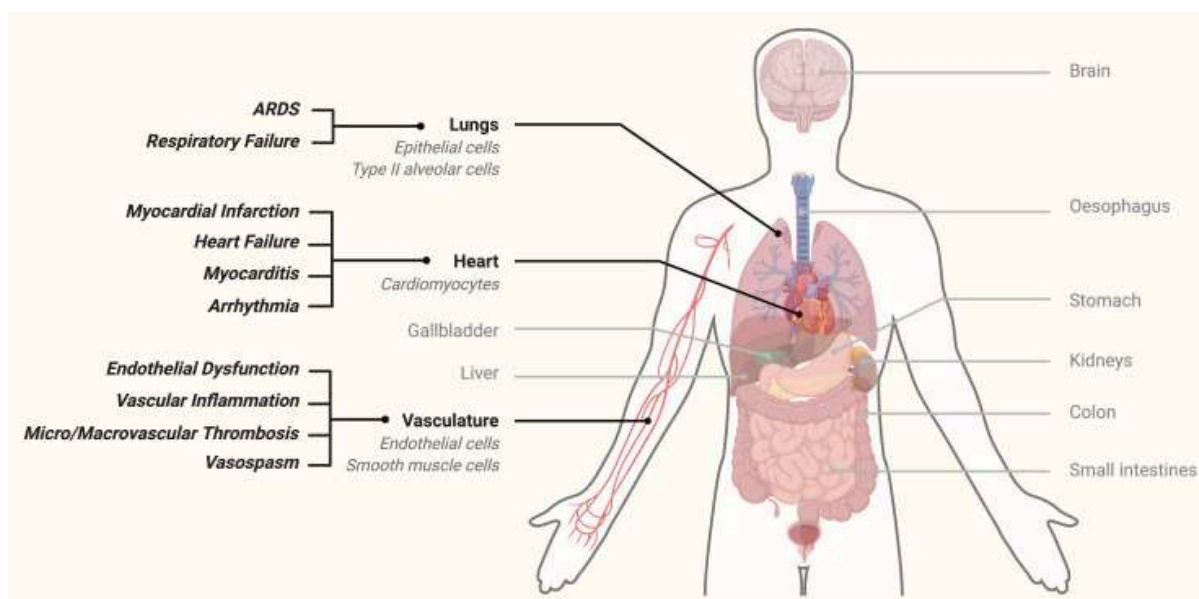


Figure 9. ACE2 expression in the human body and the symptoms associated with SARS-CoV-2 infection in ACE2⁺ tissues (15).

Physiopathology of long COVID-19

COVID-19 patients can suffer from a post-acute sequela (known as long COVID) which is a multisystemic condition defined as the continuation or development of new symptoms 3 months after the initial SARS-CoV-2 infection, with these symptoms

lasting for at least 2 months with no other explanation (52). **Fig. 10** resumes the impact of long COVID on numerous organs. The incidence is estimated at 10–30% of non-hospitalized cases, 50–70% of hospitalized cases, and 10–12% of vaccinated cases (53). It is associated with all ages, however, a higher incidence has been observed in ages between 36-50 years and in patients with mild acute illness, as this population represents most overall COVID-19 cases (53).

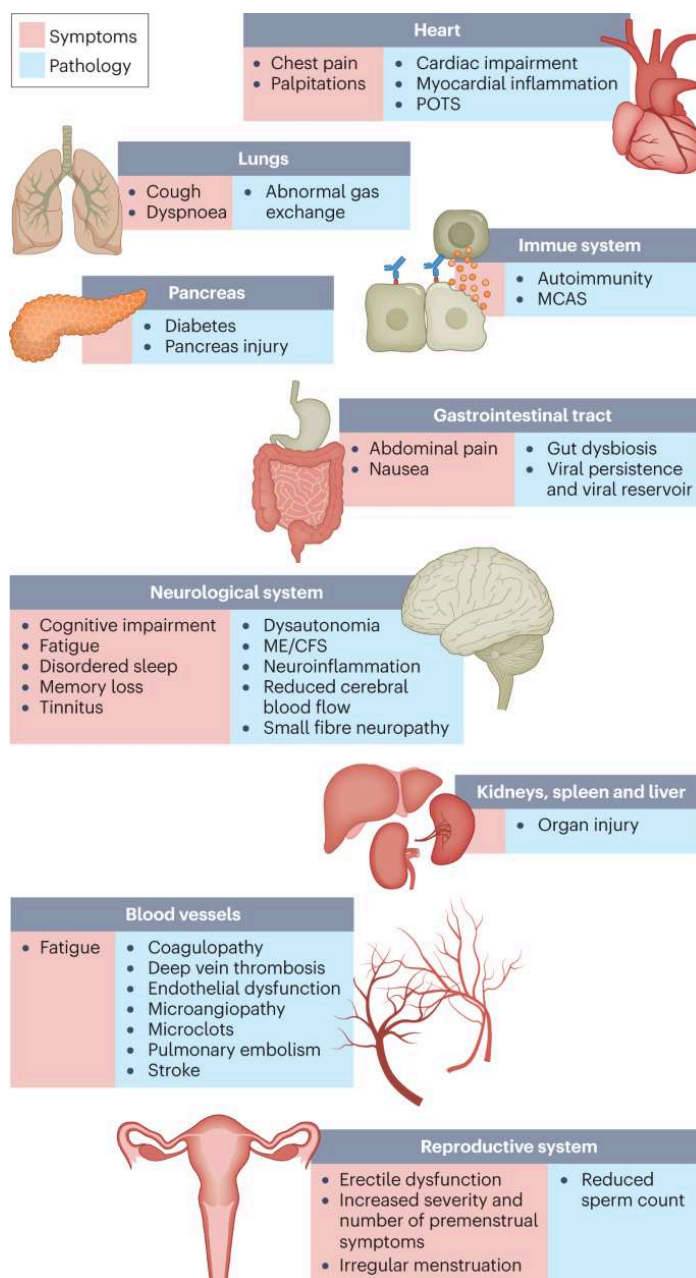


Figure 10. Symptoms and pathologies associated with long COVID (53).

The impacts of long COVID on numerous organs with a wide variety of pathology are shown. The presentation of pathologies is often overlapping, which can exacerbate management challenges. MCAS, mast cell activation syndrome; ME/CFS, myalgic encephalomyelitis/chronic fatigue syndrome; POTS, postural orthostatic tachycardia syndrome.

Nowadays there are no validated effective treatments and although there are some diagnostic tools for some effects of long COVID (like MRI scans to detect cardiovascular impairment) most of the diagnostic tools are in development (53). As for COVID-19, long COVID prevalence and symptomatology can vary depending on vaccination status and SARS-CoV-2 variants infection. For example, it has been shown that the prevalence of long COVID was higher after breakthrough infection with Omicron BA.2 in triple-vaccinated individuals than with Omicron BA.1 (54). In addition, long COVID risk is increased after the second or third reinfection, even in vaccinated individuals. Several hypotheses are being studied to decipher the pathogenesis of long COVID (**Fig 11**) (53).

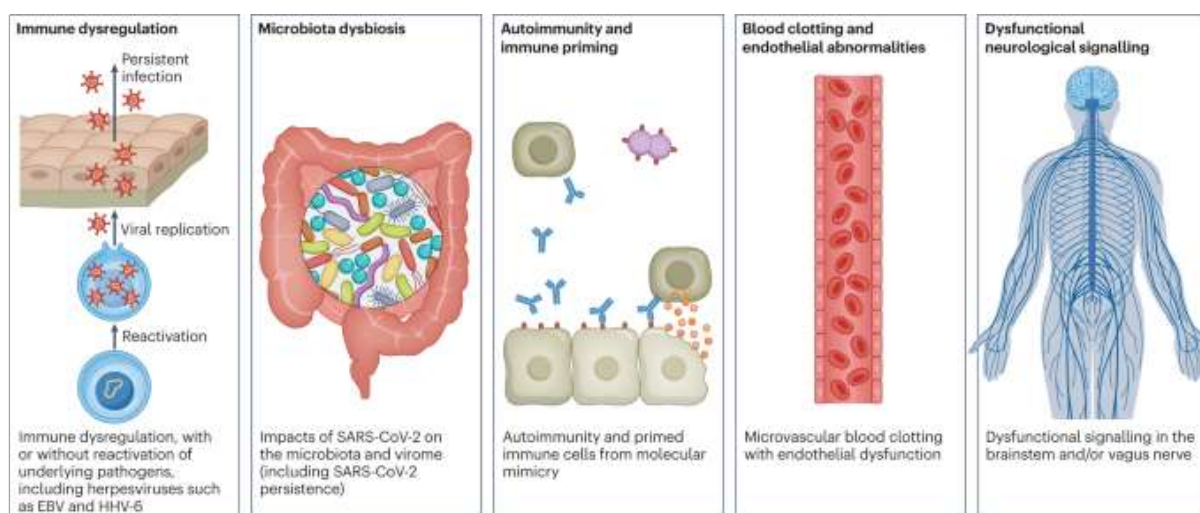


Figure 11. Different hypotheses that might explain long COVID pathophysiology (53).

These hypothesis include: (1) immune dysregulation (higher levels of exhausted T cells (55), reduced CD4⁺ and CD8⁺ effector memory cell numbers (56), highly activated innate immune cells (57), elevated expression of type I and III interferons (57), high levels of inflammatory cytokines like IL-1b, IL-6, and TNF (58) that can also facilitates the reactivation of underlying pathogens such as EBV and herpes viruses (59); (2) microbiota dysbiosis that can last for 14 months (60) (less presence of butyrate-producing bacteria (61), higher levels of fungal translocation to the blood (62), and SARS-CoV-2 viral persistence in the gut (63)); (3) autoimmunity (high levels of autoantibodies including autoantibodies that target connective tissue, vascular endothelium, platelets, cytokines, complement components or cell surface proteins (45,59)); (4) blood clotting and endothelial abnormalities (higher presence of

microclots contributing to thrombosis (64), long-lasting reduction in vascular density and changes in the size and fitness of blood cells potentially affecting the oxygen delivery (65)); (5) dysfunctional neurological signaling (activation of the kynurenine pathways which is associated with cognitive impairment (66), neuroinflammation (67), myelin loss, microglial reactivity (68)).

Role of microbiota

One of the tissues with high levels of ACE2 and TMPRSS2 expression is the GI tract, and GI symptoms are present in nearly 50% of the COVID-19 patients, sometimes preceding respiratory symptoms, and associated with a worse prognosis (69,70). Microbiota dysbiosis is one of the elements in the investigations about the pathological effects of SARS-CoV-2 infection in the GI tract. As in chronically inflammatory diseases, like intestinal bowel disease (IBD) or Chron's disease, an "abnormal" intestinal microbiota might increase the imbalance of the immune response in the intestine (71,72). One possible hypothesis is that damage caused by SARS-CoV-2 infection in the intestinal epithelium leads to leaks in the gut and the possibility that opportunistic pathogens or endotoxins pass to the basal layer exacerbating the systemic inflammatory response. Dysbiosis could also negatively influence the correct mucosal immune response in the upper respiratory airways through the gut-lung axis enhancing the no-control of the infection and creating more inflammation (**Fig 12**) (69). Plasma markers of gut permeability including lipopolysaccharide-binding protein (LBP), fecal calprotectin, fatty acid-binding protein 2 (FABP2), and peptidoglycans are higher in severe COVID-19 patients (73,74). The fecal microbiome of patients with COVID-19 showed decreased bacterial diversity (75–78) and reduced abundance of short-chain fatty acid (SCFA)-producing bacteria from the *Lachnospiraceae*, *Ruminococcaceae*, and *Eubacteriaceae* families as well as increased opportunistic pathogens from *Enterobacteriaceae* families compared with the fecal microbiome of healthy individuals (69). Specifically, the abundance of *Faecalibacterium*, *Eubacterium*, *Coprococcus*, *Ruminococcus*, *Lachnospira* and *Roseburia* are decreased, whereas that of *Enterococcus*, *Rothia* and *Lactobacillus* are increased (**Fig 13**) (77,79–81). Several studies have associated butyrate-producing genera

Faecalibacterium and *Roseburia* negatively correlated with disease severity (69,79,82).

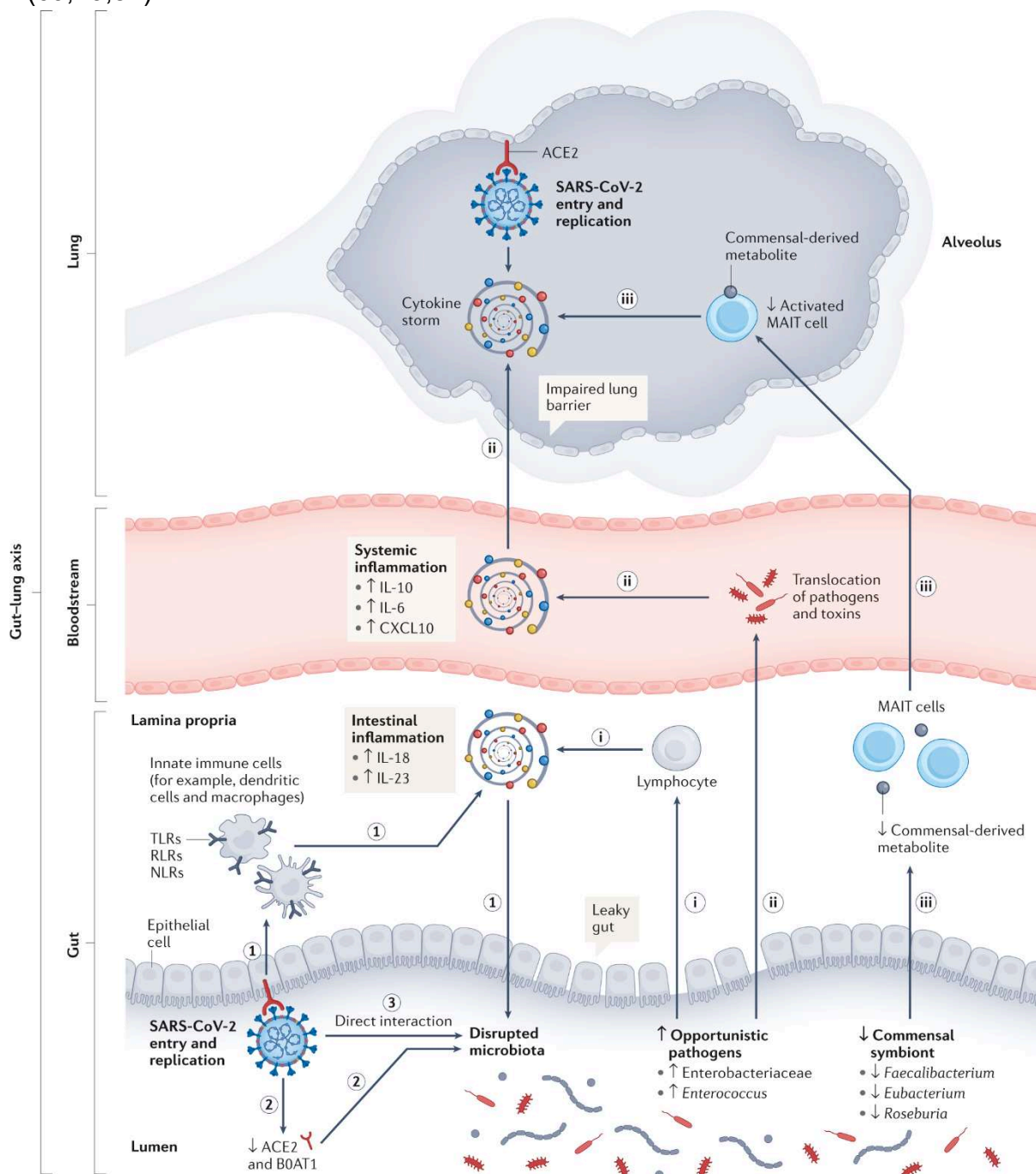


Figure 12. Pathophysiology of COVID-19 linked to the intestinal microbiota (69).

Dysbiosis has been also associated to the risk of developing long COVID (69) (**Fig 10**). An altered microbiota can remain for at least 30 days after infection clearance and those changes, including for example the depletion of immunomodulatory bacterial

species like *F. prausnitzii*, are like in chronic inflammatory conditions (69). *Bacteroides vulgatus* and *Ruminococcus gnavus* are increased in long COVID patients and the former have been associated to post-infectious irritable bowel syndrome (PI-IBS), suggesting that this pathogen is potentially linked to the pathogenesis of both long COVID and PI-IBS (69). The relative abundance of members of *Bifidobacterium* and *Ruminococcus* remain depleted in patients with COVID-19 compared with controls at 6-month follow-up, while *Atopobium parvulum*, *Actinomyces johnsonii* and *Actinomyces sp. S6 Spd3* are enriched in those patients (83). However, bacterial species, including *B. longum* and *Blautia wexlerae*, at admission were negatively associated with the development of long COVID at 6 months, implying a potential protective role of these species in the convalescent phase (83). In conclusion, a person's gut microbiome configuration at the time of infection might affect their susceptibility to long-term complications of COVID-19 (69).

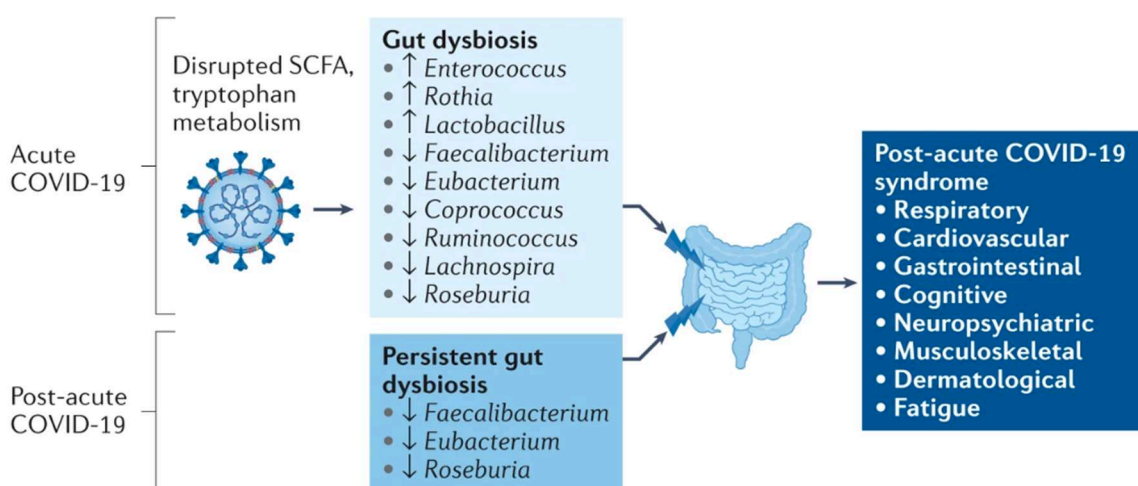


Figure 13. Principal modifications in the gut microbiota composition in acute COVID-19 and long COVID (69).

Chapter II: Immune response to SARS-CoV-2 infection

Innate response and predicting factors of disease severity

Innate immunity is the first line of defense against infections. Innate immune responses limit viral entry, translation, replication and assembly, help identify and remove infected cells, and coordinate and accelerate the development of adaptive (84). The first alarm signal after viral encounter is through the pattern recognition receptors (PRRs). These receptors are localized in the cell surface, endosomes and cell cytosol and respond to pathogen-associated molecular patterns (PAMPs) to trigger inflammatory response and cell death that limit viral replication (85). The winners in the expression of this PRRs are the innate immune cells, including macrophages, monocytes, DCs, neutrophils and innate lymphoid cells (ILCs) like NKs. The five principal PRRs families include TLRs, retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and the absent in melanoma 2 (AIM2)-like receptors (ALRs) (85).

Following SARS-CoV-2 infection, TLRs 1, 2, 4 and 6 (in the cell surface) and TLR3 (in endosomes) can be activated by viral proteins, transducing the signal through Myd88 and/or TRIF. On one hand, Myd88 will activate NF- κ B and ERK1/2 than will further activate the transcription of pro-inflammatory cytokines like IL-6, IL-1 β and type I IFNs, and other innate immune sensors like and NLRP3 further activating the inflammasome and pyroptosis which will conduct to cell death and potential viral clearance (84). On the other hand, signaling through TRIF also activates type I IFN production and several TLR4- and TLR3-dependent transcription factors, some of which have direct antiviral activity (84). Inborn errors in *TLR7* gene have been linked to severe disease in young individuals suggesting that TLR7 might have a protective role during SARS-CoV-2 infection via the activation of IFN response (84,86).

Viral RNA can also activate PRRs in the cytosol of the infected cells. This is the case for RLRs like RIG-I and MDA5 which provide key regulation of IFN pathways (84). The production and release of IFNs stimulate downstream signaling through IFN receptors (IFNAR1/IFNAR2 for type I IFNs; IFNLR1/IL10R β for type III IFNs) that allows the production of hundreds of interferons stimulated genes (ISGs) with different antivirals

functions. However, the fine-tuning of a type I IFN response is critical because both over and underactivation of IFN signaling can be deleterious to the host (84). Severe forms of coronavirus disease 2019 (COVID-19) involve a dysregulation of the immune response that results in insufficient or delayed type I interferon response (87,88) (**Fig. 14**). In the pathophysiology part, we mentioned that in severe COVID-19 patients, there are circulating type I IFN autoantibodies (30,32), reducing their IFN response and these autoantibodies increase with age, which correlates with the more severe disease the older the patient is. Moreover, it is not only the IFN production which is altered in COVID-19. IFNAR expression and signaling can also modulate the IFN response. Some studies at the genome, transcriptomic and single-cell levels have identified IFNAR2 as a risk factor for severe COVID-19 (89,90). There is an upregulation of the expression of IFNAR2 in asymptomatic patients and moreover, in individuals who recovered from severe COVID-19 there are reduced levels of type I-IFN producing pDCs and an increased expression of IFNAR2 on several myeloid cell subsets at steady state (91). In addition, SARS-CoV-2 infection increases the ubiquitination of IFNAR1, thereby decreasing the cell-surface expression of this protein in vitro (92).

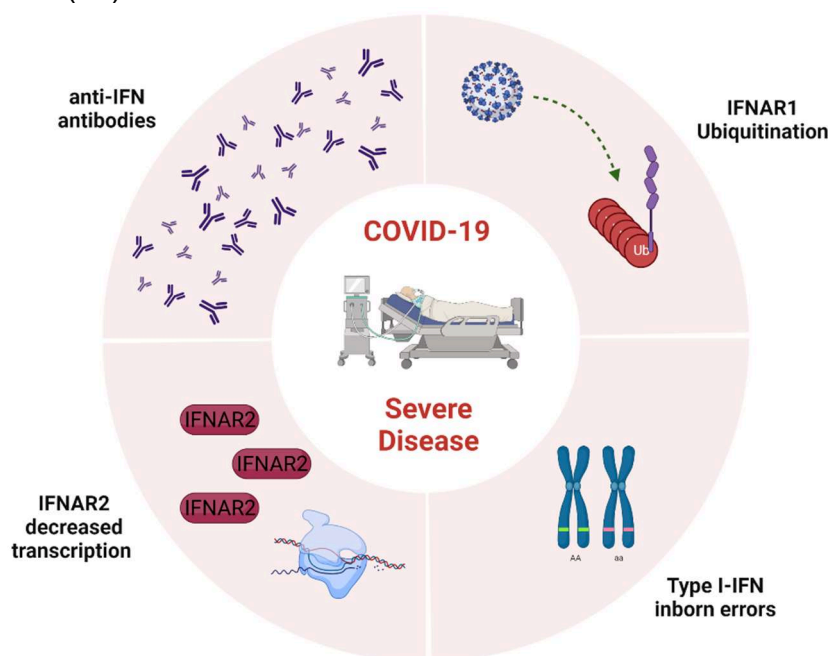


Figure 14. IFN-related anomalies associated to severe disease in COVID-19.

Adaptive immune response against SARS-CoV-2

The immune response to pathogens is characterized by the activation of innate and adaptive responses, and their humoral and cellular components. Unlike the innate immune system, which provides a general, non-specific response, adaptive immunity offers a highly specific and tailored defense. This sophisticated system is characterized by its ability to recognize, remember, and target specific pathogens, enabling the body to mount a precise and powerful response.

Cellular immune response

T cell responses are important components of adaptive immunity and at the beginning of the pandemic there were concerns about the induction of this part of the immunity during SARS-CoV-2 infection because of the potentially weak immunogenicity of coronaviruses (93). Indeed, SARS-CoV-2 is immunogenic and virus-specific CD4⁺ T cell responses predominantly of Th1 and T follicular helper (Tfh) types are associated to uncomplicated COVID-19 disease evolution (94,95). In contrast to other virus like flaviviruses where T cell response recognize prevalently non-structural proteins (96), in COVID-19 there is a strong T cell response against the S protein probably due to its large size and its high level of expression (94). There is also a T cell response against the N, M, E and non-structural proteins (93) but the more immunodominant epitopes for CD4⁺ and CD8⁺ T cell response are found in the S, N and M proteins (93). However, Human Leukocyte Antigen (HLA) restriction studies have defined thousands of SARS-CoV-2 T cell epitopes which is an indicative of the large breadth of the cellular response against the virus. Murine and non-human primate models have shown the protective role that T cell response can have in COVID-19 (93). For instance, depletion of CD8⁺ T cells in convalescent rhesus macaques reduced protection against SARS-CoV-2 rechallenge (97). In patients, an early CD4⁺ T cell response is associated with a better disease outcome as well as a good balance of regulatory and cytotoxic SARS-CoV-2-reactive CD4⁺ T cells in blood (95,98). Nevertheless, a delayed CD8⁺ T cell response is associated to a more severe prognosis, probably due to inability of T cells to control viral replication in the lungs sufficiently fast (93,95,99). CD4⁺ and CD8⁺ T cells are detected in the upper respiratory tract following SARS-CoV-2 infection, However, in lungs of covid severe patients there are less CD8⁺ T cells than in asymptomatic, indicating a lack of

successful T cell response in severe COVID. Moreover, severe or fatal COVID-19 can be characterized by profound disruption of germinal centers (GCs) and loss of Bcl-6-expressing GC-Tfh cells (100,101). CD4⁺ and CD8⁺ T cells and memory B cells generated in response to infection are present in the lung, bone marrow, spleen, and multiple lymph nodes for at least six months after infection (102), regardless of disease severity (103); however while most individuals present a durable CD4⁺ T cell response, approximately 30% did not have CD8⁺ T cells suggesting an impairment of memory CD8⁺ T cells in some individuals (104).

In the face of the pandemic, multiple vaccines were rapidly developed and approved worldwide, with 13,492,159,872 doses administered to date. As of June 2023, the vaccines that have been authorized to be used are described in **Table 2**. These vaccines have demonstrated efficacy against different variants, including the Omicron variant. However, certain factors such as gender, age, comorbid conditions, and immunosuppression can influence the immune response after vaccination, leading to lower antibody concentrations and neutralization capacities in some individuals (105–108). **Table 2** listed the current SARS-CoV-2 vaccines authorized up to date.

After vaccination, with all the technologically different vaccines (adenoviral vectors, inactivated viral vaccine, mRNA, recombinant protein) a T cell response is elicited (109–111). Nevertheless, vaccination with a mRNA vaccine has shown to elicit a more robust CD4⁺ and CD8⁺ T cell response, higher levels of circulating Tfh and good functioning of GCs in comparison to inactivated viral vaccines which only elicits CD4⁺ T cell response (111,112). A heterologous prime-boost vaccination improves the T cell response (93). SARS-CoV-2-specific T cell response decreased 2-fold or less after six months of vaccination and in contrast to antibody response, hybrid immunity resulted in a lesser boost to the T cell response (111,113) and a broader set of epitopes targeted (114), suggesting a superior advantage for this type of immunity.

In the context of variants, T cell responses are largely preserved. This aligns with the extensive experimental validation of thousands of SARS-CoV-2 class I and class II T cell epitopes, making it extremely challenging for the virus to mutate multiple epitopes simultaneously and escape T cell recognition at the population level while maintaining viral fitness (93).

Table 3. SARS-CoV-2 licensed vaccines (115–117).

Vaccine	Platform	Strain	Population	Licensed
Cominarty (Pfizer/BioNTech)	mRNA	Original strain (PV) (B)	> 6 months	EMA, WHO
		Original strain + Omicron BA.1 variant (B)	> 12 years	EMA, FDA
		Original strain + Omicron BA.4-5 variants (B)	> 5 years	EMA, FDA
Spikevax (Moderna)	mRNA	Original strain (PV) (B)	> 6 months	EMA, WHO
		Original strain + Omicron BA.1 variant (B)	> 6 years	EMA, FDA
		Original strain + Omicron BA.4-5 variants (B)	> 6 years	EMA, FDA
Vaxzevria (AstraZeneca)	Adenoviral vector	Original strain (PV) (B)	> 18 years	EMA, WHO
Jcovden (Janssen)	Adenoviral vector	Original strain (PV) (B)	> 18 years	EMA, WHO, FDA
Nuvaxovid (Novavax)	Protein	Original strain (PV) (B)	> 12 years	EMA, WHO, FDA
COVID-19 Vaccine Valneva (Valneva)	Inactivated	Original strain (PV) (B)	18-50 years	EMA
VidPrevtyn Beta (Sanofi Pasteur)	Protein	Beta variant (B)	> 18 years	EMA
Bimervax (HIPRA Human Health S.L.U.)	Protein	Alpha + Beta variants (B)	> 16 years	EMA
Sinopharm (Beijing Institute of Biological Products)	Inactivated	Original strain (PV) (B)	> 18 years	WHO
Sinovac-CoronaVac	Inactivated	Original strain (PV) (B)	> 18 years	WHO
Bharat Biotech BBV152 COVAXIN	Inactivated	Original strain (PV) (B)	> 18 years	WHO
Covovax (NVX-CoV2373)	Protein	Original strain (PV) (B)	> 12 years	WHO
SII/COVISHIELD	Adenoviral vector	Original strain (PV) (B)	> 18 years	WHO

(PV): Primary vaccination; (B): Boost

Humoral immune response

Antibodies play a crucial role in protection against viral diseases via various mechanisms involving both their Fab and corresponding Fc portions. The Fab-mediated mechanisms include neutralization, in which the entry of the virus into the host cell is sterically blocked. Fc mechanisms include complement activation, antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent phagocytosis (ADP). However, antibody effector functions can also exacerbate inflammation and generate more damage, as in the antibody-dependent enhancement (ADE) observed in dengue disease (118).

The systemic and mucosal immune response against SARS-CoV-2 as well as after vaccination is well detailed in **my review in the journal Mucosal Immunology in October 2022** (119) and annexed to this manuscript. However, here I will summarise what we know so far about the systemic and mucosal humoral immune response to COVID-19.

Systemic humoral response in COVID-19

The proportions of total IgA, IgM and IgG antibodies and of total IgG subclasses are not modified following SARS-CoV-2 infection⁶. However, anti-SARS-CoV-2 antibody production varies with disease severity and depends on patient characteristics, such as sex and age (**Fig. 15**). Severe patients have higher levels of anti-SARS-CoV-2 antibodies (120–130) as well as men compared to women (8,18,19) and in elderly compared to youngers (128,131,132). Serum IgG, IgA and IgM antibodies are elicited principally against the spike (S1, S2 and RBD domains) and nucleocapsid proteins (123,125,127,129,131,133–139) (**Fig. 16**). In addition, the affinity of the antibodies against the SARS-CoV-2 prefusion spike detected in the serum and nasal washes has been shown to be significantly higher in asymptomatic adults than in symptomatic COVID-19 patients (140), suggesting that the antibody response in asymptomatic patients is more effective at controlling the infection than that in symptomatic patients.

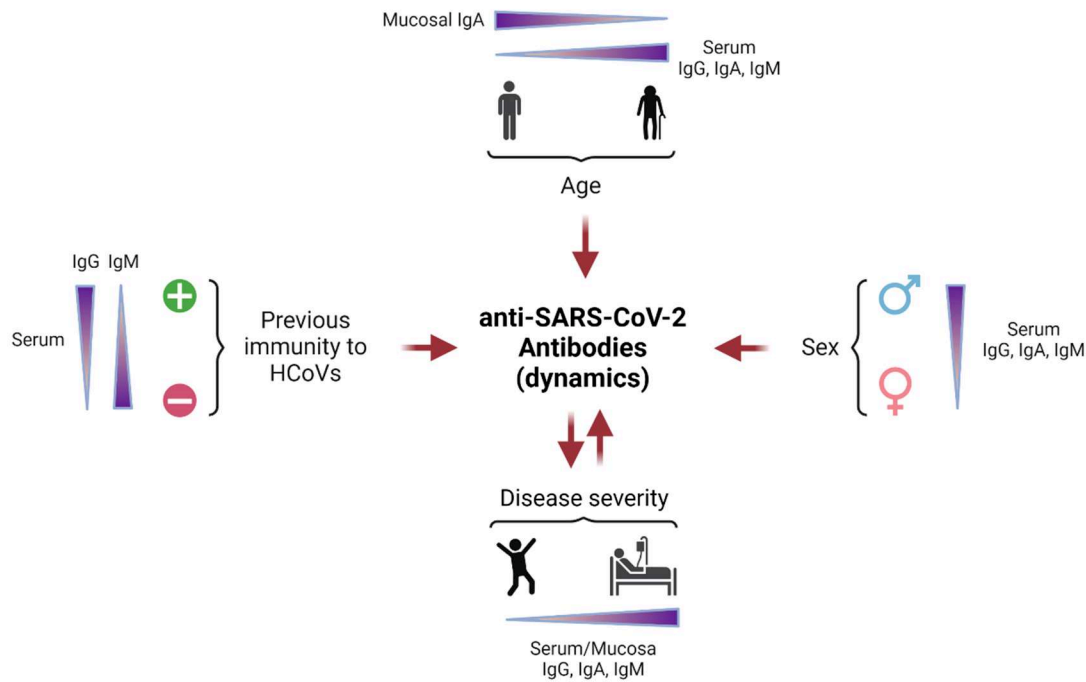


Figure 15. Factors affecting the dynamics of anti-SARS-CoV2 antibodies.

Patients' characteristics can modulate anti-SARS-CoV2 antibody response. Specific IgG, IgA and IgM in serum is elevated in old patients while mucosal IgA is diminished, contrary to young patients who have lower anti-SARS-CoV2 antibody titers in serum and higher levels of mucosal IgA. Males have higher titers of specific IgG, IgA and IgM in serum than females. Patients that had been previously infected with another human coronavirus (HCoV) (represented with the "+" symbol) develop more anti-SARS-CoV2 IgG than IgM in serum; and the contrary happens in patients with no previous immunity to HCoV (represented with the "-" symbol). Disease severity can also modulate patient's specific response as the more severe, the higher the titers of anti-SARS-CoV2 IgG, IgA and IgM in serum. At the same time the quality and quantity of these antibodies can modify disease severity.

In terms of kinetics, specific IgA is the predominant isotype in the first week post-symptoms onset (PSO); there is then a peak of anti-SARS-CoV-2 IgM between 10 and 15 days PSO and an IgG peak around day 20 PSO (124,126,136,138,139,141,142) (**Fig. 16**). IgM levels decrease significantly one-month PSO (139), but specific IgA and IgG levels in the blood remain stable more than 6 weeks PSO (142,143) and IgG can be detected for up to 1 year PSO (124,125,135,136,144–146). However, asymptomatic antibodies lose their anti-SARS-CoV-2 antibodies faster than severe disease patients, suggesting that the immune system is stronger stimulated in the late ones. The presence of anti-SARS-CoV-2 IgA in serum is associated with gastrointestinal symptoms in COVID-19, whereas no such association has been found for IgG (138). SARS-CoV-2 can replicate in human enterocytes (147) and may activate the local production of anti-SARS-CoV-2 IgA, contributing to an increase in specific IgA levels in the blood. Some

studies have found that antibodies from COVID-19 patients can cross-react with the spike proteins of seasonal human coronaviruses (HCoVs). The impact of prior immunity to HCoVs on SARS-CoV-2 infection is still under debate with some studies suggesting that higher levels of specific IgG and IgA against HCoVs are associated with milder COVID-19 symptoms, and others indicating the contrary (4,148–154).

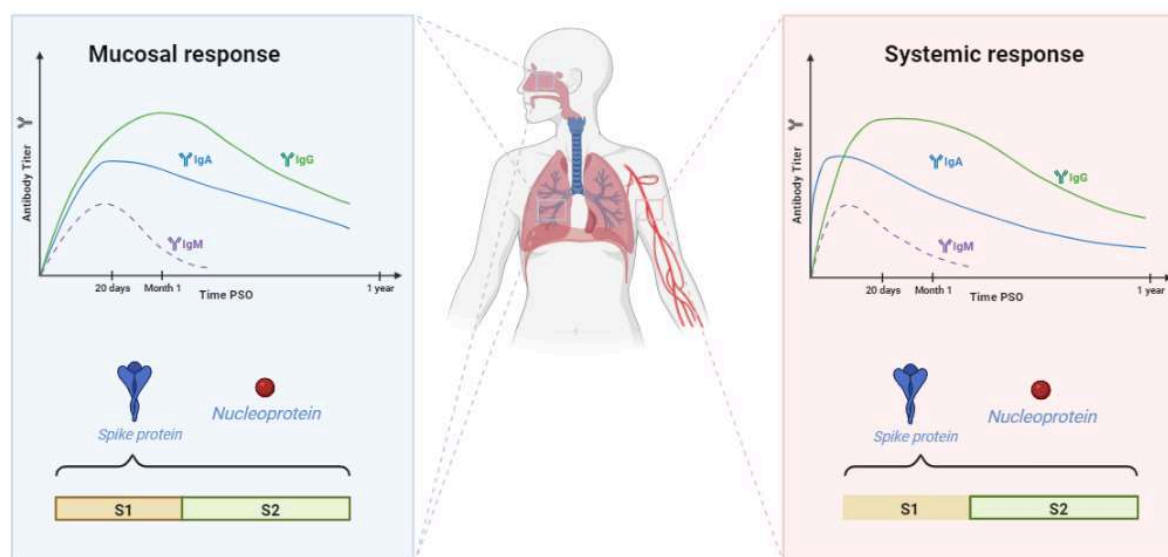


Figure 16. Mucosal and systemic humoral immune response to SARS-CoV2.

Both mucosal and systemic humoral immune response against SARS-CoV2 virus are characterised by a transient IgM response detectable until 1 month post-symptom onset (PSO). Mucosal response is dominated by IgG and IgA, while systemic response is firstly dominated by IgA. Specific IgA and IgG antibodies are detected in mucosal tissues even at 9-month PSO and until one year PSO in serum. Both responses are directed against the Spike and Nucleoprotein. Even though both responses target epitopes in S1 and S2 subunits from the Spike protein, the mucosal response is more diverse (represented by the marked line around the rectangles S1 and S2) than the systemic response which has the epitopes principally against S2 subunit (marked with the line only in the S2 rectangle).

Mucosal humoral immunity in COVID-19

The mucosal immune response to SARS-CoV-2, particularly in the nasal and respiratory tract, plays an important role in preventing the entry and spread of the virus. Specifically, anti-spike IgM and anti-RBD IgA, are associated with lower viral loads in the nasopharynx, indicating that it may help limit disease severity by initiating early viral clearance (155). Additionally, a robust nasal antibody response, particularly anti-RBD IgA, has been correlated with the resolution of systemic symptoms such as fatigue, fever, headache, and joint or muscle pain (155).

Specific antibodies against SARS-CoV-2 have been detected in various mucosal tissues, including saliva, nasopharynx, bronchoalveolar lavages (BAL), and trachea (33,137,141,145,155–161). These antibodies target different regions of the spike

protein, including the NTD, RBD, FP, HR1, and HR2 regions, which are distributed throughout the S1 and S2 subunits (140). Notably, both monomeric and dimeric IgA antibodies have been found in the BAL of COVID-19 patients, suggesting different origins for mucosal antibodies (137). Dimeric IgA likely arises locally, while monomeric IgA can reach the airways through a process called transudation, which is more likely to occur in damaged lung tissue observed in severe COVID-19 cases (162,163).

The levels and dynamics of antibodies in mucosal tissues differ from those in serum, for example, the IgG/IgA ratio shows a gradient down the respiratory tract, with higher levels of IgA in saliva and higher levels of IgG in BAL (137). IgG antibodies against SARS-CoV-2 peak later and persist longer in both serum and mucosal tissues while IgM and IgA antibodies appear earlier but decline more rapidly (156). Anti-RBD IgA levels are found to be higher in saliva compared to serum at later stages of infection (137). However, the duration and levels of specific antibodies in mucosal tissues can vary depending on disease severity, with higher antibody levels observed in moderate/severe disease compared to asymptomatic/mild cases (145).

While the mucosal immune response is an essential component in the defense against SARS-CoV-2, it may not provide sufficient protection against certain variants like Omicron as reinfections are common. The specific role of antibodies during the infectious process and their ability to protect against severe disease are still subjects of ongoing research and investigation.

Antibody protective and pathological functions in COVID-19

Fc-effector functions, such as ADP, have been associated with protection against other coronaviruses or HIV. Shiakolas et al. showed that six monoclonal antibodies from SARS-CoV patients that cross-reacted with SARS-CoV-2 virus-induced ADP but not neutralization in vitro, and that this Fc-effector function was associated with milder haemorrhagic disease in animal models (164). In vivo studies of SARS-CoV-2 infection have demonstrated that the humoral immune response, including neutralizing antibodies, antibody-dependent cellular cytotoxicity (ADCC), and ADP, contributes to protection (165–168). While antibody levels in the serum gradually decrease over time, neutralizing antibody titers and memory B-cell responses remain detectable and play a role in protection against reinfection (127,136,142,145,169) (**Fig. 17**). The quality of antibody effector functions correlates with their magnitude

(158), their specificity for RBD in both serum and nasal secretions (127,157), and with disease severity (170), and dysfunctions in NK cells may hinder proper ADCC in severe COVID-19 cases (143,171). Antibody effector functions other than neutralization, such as ADCC, antibody-dependent complement deposition (ADCD), and ADP, are associated with better protection. Less is known about the Fc-effector functions of antibodies against SARS-CoV-2 in mucosal tissues, and most studies to date have focused on antibody neutralization capacity. Neutralization has been observed with IgA antibodies in nasal washes and saliva of convalescent individuals, and particularly anti-RBD IgA, have been found to be more neutralizing than IgG antibodies in the lung (137,158,170,172). Breast milk contains anti-spike IgA antibodies that persist for several months postpartum, potentially providing protection to breastfed newborns. Robust neutralization and ADP were detected in nasal washes from convalescent individuals who had experienced mild or severe disease (158,173).

As the number of SARS-CoV-2 variants increases, the protective functions of antibodies have been questioned. Neutralizing antibodies elicited against one variant have reduced efficacy against other variants (140,144,173–175), however, sequential boosters with different variants can strengthen neutralizing capacity (176–178). Some monoclonal antibodies have demonstrated cross-reactivity and neutralization against the Omicron variant, indicating the potential for a broader antibody response to overcome antigenic shifts (166). Fc-effector functions can also be influenced by the presence of new variants, with some variants affecting specific functions like ADCD. Nevertheless, Fc-effector functions are less affected by new variants than neutralization suggesting that Fc-dependent effector functions may make a greater contribution to decreasing the number of severe cases after infection with a variant than neutralization alone (179).

As discussed before in the immunopathology of the COVID-19, the severe disease is characterized by a dysregulated immune response, leading to a cytokine storm that contributes to rapid disease progression and death in some patients. The exact mechanisms underlying the immune pathophysiology of COVID-19 are not fully understood, however some parts of the puzzle are being found (**Fig. 17**). Neutrophils play a crucial role in the immune response and can initiate various defense mechanisms, such as neutrophil extracellular trap formation (NETosis). In vitro

studies have shown that IgA antibodies, particularly serum IgA, can stimulate NETosis and may persist in tissues, leading to local inflammation (180–182). Patients with severe disease exhibit a rapid switch to the IgA2 isotype, which is associated with NETosis (183) and in other disease like rheumatoid arthritis (RA) have shown that differences in glycosylation patterns between IgA1 and IgA2 may contribute to the greater stimulation of NETosis by IgA2. It remains unknown whether IgA1 glycosylation patterns are modified in COVID-19 patients, but these data are potentially important, to shed light on the pathological role of IgA isotypes associated with disease severity.

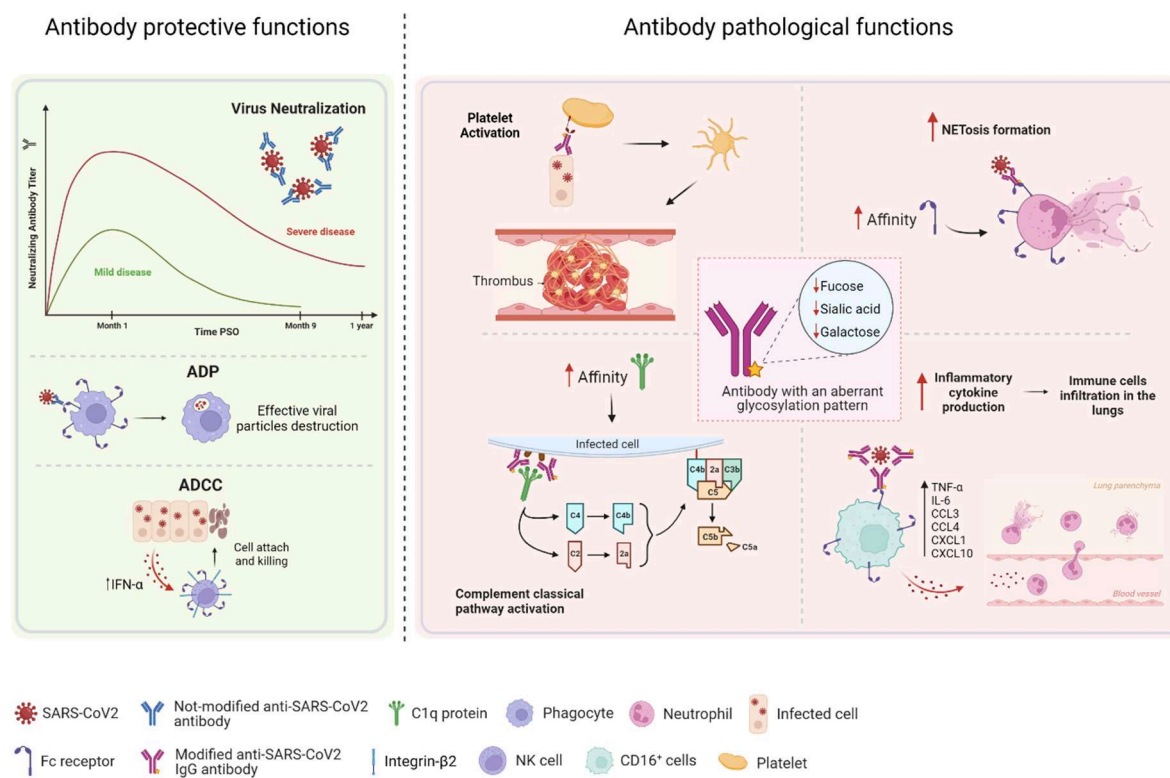


Figure 17. Protective and pathological functions of SARS-CoV2 antibodies.

Anti-SARS-CoV2 antibodies can exert protective functions like neutralization, antibody-dependent phagocytosis (ADP) or antibody-dependent cellular cytotoxicity (ADCC). Neutralizing antibody titers are higher in severe disease patients than in mild disease patients and these antibodies are detected until one year post-symptom onset (PSO); however they decrease faster in mild disease patients. Serum from non-hospitalized patients as well as convalescent plasma from recovered patients elicit more Fc-dependent effector functions such as ADP and ADCC than serum from hospitalized patients. Pathological effects associated to antibodies in COVID-19 disease are related in part to aberrant glycosylation patterns presented in anti-SARS-CoV2 IgG in severe disease patients but not in mild disease patients. These structural modifications can trigger inflammatory process like cytokine production, immune cells infiltration in the lungs or platelet-mediated thrombosis. This new glycosylation pattern increases the antibody affinity to the C1q protein activating then the complement through the classical pathway; and increase the affinity to FcγR producing more Fc-dependent functions like neutrophil extracellular traps formation (NETosis) and ADCC that are associated to more inflammation.

Another immune response-related pathology observed in severe COVID-19 cases is small-vessel vasculitis driven by deposits of IgA-C3 immune complexes, which is known as IgA vasculitis or Henoch-Schönlein purpura (HSP) and has also been reported in children with COVID-19 (184,185). SARS-CoV-2 infection may induce the secretion of inflammatory cytokines like IL-6, affecting the glycosylation process and leading to the synthesis of galactose-deficient IgA1, which forms immune complexes in the blood (186). Complement activation, specifically through C4b and C3bc, was restricted to anti-spike protein IgG antibodies and correlated with disease severity. Patients with severe disease exhibit lower galactosylation levels in all IgG subclasses, and the binding capacity of IgG to receptors and complement components is positively correlated with disease severity (143,187). Immune complexes formed by the spike protein and anti-S IgG antibodies can induce the production of inflammatory molecules and neutrophil infiltration in the lungs. These complexes, when the IgG antibodies have aberrant glycosylation patterns, enhance platelet-mediated thrombosis. It has been observed that levels of anti-spike IgG fucosylation return to normal within a few weeks after SARS-CoV-2 infection, suggesting that higher levels of antibody-dependent inflammation occur primarily during seroconversion (188). However, early-phase spike-specific IgG antibodies in patients with severe disease can induce the production of inflammatory molecules, leading to lung damage and microvascular thrombosis. The question remains whether the observations made during infection can be applied to vaccination.

Humoral immune response to vaccination

Vaccination and natural infection can modulate the immune response to SARS-CoV-2. Individuals who have recovered from COVID-19 and subsequently received vaccination exhibit higher antibody levels and greater neutralization capacity compared to those who have not had prior infection (106,189–191). Cross-protection against new variants is conferred by vaccination in most of the cases, with evidence of a better neutralizing response and higher antibody titers in seropositive individuals (190,192–196), however, breakthrough infections can occur (197). Different hypothesis might explain these new infections even if the patient is immunized (naturally or by vaccination). In one part, the neutralizing antibodies elicited after a prior immunization are less efficient to neutralize new variants that are antigenically different (198,199). In addition, it might be an uncomplete clearance of the virus and

a further viral reactivation (200). However, a broader immune response is elicited after recurrent infections which might protect against subsequent infections due to the more specific response to the conserved epitopes between variants (101,201). Whether there will be a moment where there will not be more breakthrough infections because the immune system will be highly prepared and broadly enough to face new variants, it is still to be discovered. Nevertheless, the other HCoV's have shown that reinfections are cyclic and hard to eliminate (154). To enhance the immune response and protection against new variants, additional doses (boosters) and heterologous or multivalent vaccination strategies have been shown to be effective (176,193–196,202–204). These approaches increase the breadth of neutralization and stimulate the formation of memory B cells that target more conserved regions of the virus (203). Heterologous vaccination schedules, such as combining different vaccines, have been found to generate stronger neutralizing activity compared to homologous vaccination schedules (204). Vaccination induces structurally different antibodies compared to natural infection, resulting in effector functions that contribute to reducing the likelihood of severe disease (205,206). Vaccination elicits antibodies with increased Fc-dependent effector activity, such as engaging in FcγR pathways and complement activation, in addition to their Fab-mediated neutralization activity (173,205,207). These structural modifications enhance the ability of antibodies to reduce infection and therefore might contribute to vaccine-induced protection.

The mucosal immune response is crucial for preventing infection, as SARS-CoV-2 is a respiratory virus. It is known the compartmentalization that presents the immune system, and particularly the mucosal immune system. Intramuscular vaccines are not the best target to elicit a mucosal immune response against respiratory pathogens, however, the positive effect of SARS-CoV-2 vaccination is clear and without doubts. After vaccination, while antibody levels in the mucosa are lower compared to serum, seropositive individuals exhibit a stronger mucosal response, probably due to a recall response (108,160,171,208–210). Nevertheless, the functionality of the mucosal immune response against different variants is not yet well understood but as in serum, cross-neutralization in the mucosa is lower against variants compared to the initial strain (211). Although antibody-dependent enhancement (ADE), where antibodies facilitate infection instead of neutralizing it, has been observed in vitro (11,212–216), the evidence suggests that it is not a significant concern in vivo (217–223). In vivo

studies in nonhuman primates and humans have shown no increase in disease severity or infection rates due to ADE (219). Furthermore, the risk of severe disease is not higher in COVID-19 patients treated with convalescent plasma or in vaccinated individuals (201,221,222,224,225).

Current intramuscular vaccines primarily reinforce systemic immune responses, while the activation of existing mucosal responses in previously infected individuals is relatively weak. Therefore, there is an urgent need to improve SARS-CoV-2 vaccine design to target the mucosal immune response and achieve sterilizing immunity more effectively.

Objectives of my PhD

The role of systemic and mucosal immunity during COVID-19 pathology remains uncertain. My PhD work has been centered on the COVID-19 pandemic. Very early some studies started to describe higher levels of SARS-CoV-2 antibodies as well as a stronger neutralizing response in severe patients, compared to asymptomatic ones. These studies suggested that the immune response was not able to efficiently control the infection and that antibodies might contribute to the development of severe disease. As a respiratory virus that infects in the first instance mucosal tissues, mucosal approaches should be done in addition to systemic ones. Therefore, **we wanted to study the humoral immune response against SARS-CoV-2 in the systemic and mucosal compartments.** We published an updated review article about the topic in the journal *Mucosal Immunology* (119). For our research, we were principally focused on the potential pathological role of antibodies, notably IgA, in COVID-19. For that, we had access to different cohorts of patients from the CHU Nord of Saint-Etienne as early as March 2020 as well as to cohorts of vaccinated individuals.

At the same time, the cytokine storm was described for COVID-19 and the pathophysiology of the disease started to be investigated. Key elements of the innate immune response like the IFN pathways were found to be dysregulated in the more severe forms of the disease avoiding the correct function of the antiviral response elicited against viral infections. However, **the role of the IFN receptors (IFNARs) as disease predictors** was not studied at that moment and we wanted to evaluate this point in our retrospective cohort of severe and mild COVID-19 patients.

The correct functioning of the mucosal immune response is very linked to the microbiota as shown for chronically inflammation disorders and some studies started to show that in severe COVID-19 patients, there is intestinal dysbiosis. As IgA plays a crucial role in the interaction microbiota-immune system and SARS-CoV-2 can replicate in the gut, **we wanted to study the intestinal and lung microbiota in COVID-19 patients.**

In addition, with the unprecedentedly rapid development of new vaccines, principally with the development of mRNA technology, SARS-CoV-2 vaccines were licensed

less than one year after the start of the pandemic. Therefore, **we wanted to study the immune response elicited by the vaccination**, especially comparing different immunization schemes and the protection against emerging viral variants. One question raised by the scientific community and that we wanted to address was the possible ADE effect that the vaccination could have or even the natural infection vis a vis of the new variants that were already creating new waves of infection.

Therefore, the objectives of the present work are:

- 1- To determine potential predictive factors of COVID-19 disease severity.
 - 1.1- Innate immunity markers: soluble IFNARs (sIFNARs) (Article 1: Yaugel-Novoa et al., Lancet Microbe (April 2023)).
 - 1.2- Microbiota dysbiosis and intestinal IgA in COVID-19 patients (Article 2: In preparation).
- 2- To better understand the role of the humoral immune response in the pathophysiology of the disease.
 - 2.1- Systemic and mucosal immune response in COVID-19 (Article 3: Submitted).
- 3- To study the immune response against SARS-CoV-2 elicited after vaccination (Article 4: Pozzetto et al., Nature (October 2021); Article 5: Saade et al., NPJ Vaccines (April 2023); Article 6: Yaugel-Novoa et al., Vaccines (March 2023)).

Results

Result 1: Potential predictive factors of COVID-19 disease severity.

Article 1: Innate immunity markers: soluble IFNARs (sIFNARs).

(Correspondance published in The Lancet Microbe in April 2023)

As previously discussed in the introduction of this manuscript (see Pathophysiology of COVID-19), IFNs are key factors in the antiviral response but in COVID-19 severe disease there is an imbalance in this part of the innate response. Some studies have associated disease severity to the presence of autoantibodies against type I-IFN and/or IFN- ω (30,32), type I-IFN inborn errors (226–228), as well as IFNAR expression (89–91). Moreover, SARS-CoV-2 infection increases the ubiquitination of IFNAR1, thereby decreasing the cell-surface expression of this receptor in vitro (92). IFNAR2 is expressed in three isoforms, one attached to the cell membrane (IFNAR2c), and two others in a soluble form with different length (IFNAR2a and IFNAR2b) (229). The three forms can interact with type I IFN and with IFNAR1 but IFNAR2a and b cannot activate signalling (230). However, since nothing was described about the potential effect of soluble forms of the IFNARs and their association with disease severity in COVID-19, we wanted to evaluate whether they could be predictor factors of disease severity. This study was published in the journal Lancet Microbe in April 2023 (231).

Association of IFNAR1 and IFNAR2 with COVID-19 severity

Type I interferons propagate and amplify the antiviral response at the start of an infection and are crucial for effective antiviral immunity. Type I interferons bind interferon alpha/beta receptors 1 (IFNAR1) and 2 (IFNAR2), which are ubiquitous, but expressed differentially depending on the cell type.¹

Interferon pathways have been identified as involved in COVID-19 and are associated with disease severity.² Genome-wide association studies, transcriptomic studies, and single-cell studies, have identified IFNAR2 as a risk factor for severe COVID-19.³ However, the correlation between serum amounts of soluble forms of IFNAR in individuals with COVID-19 at early stages of infection and COVID-19 disease severity has not been investigated.

In this Correspondence, we compared serum IFNAR1 and IFNAR 2 amounts (quantified by two commercial sandwich ELISA kits: LS-F17211 and ab264610) in 77 individuals. This study included ten individuals who were PCR-negative for SARS-CoV-2 and 67 who were PCR-positive (ten asymptomatic patients, 20 patients with mild disease, 17 hospitalised patients [not intensive care unit], and 16 patients in the intensive care unit). Written informed consent for participation was obtained from all participants and ethics approval was obtained from Comité Protection des Personnes Ile de France V

(NCT04648709). All samples were collected at the time of patient inclusion in the study (days 3–7 after symptom onset).

Although we found no differences in IFNAR1 concentrations between groups separated by disease severity, IFNAR1 amounts seemed to be inversely correlated to COVID-19 severity (appendix p 1). These results are in concordance with findings from other studies that associated autosomal recessive IFNAR1 deficiency with severe COVID-19.⁴ By contrast, IFNAR2 concentrations were significantly higher in patients with severe COVID-19 (appendix p 1). IFNAR2 is expressed in three isoforms, two of which are soluble but cannot activate signalling after interaction with type I interferon. Therefore, the action of type I interferon might depend on the relative abundances of IFNAR2 isoforms, as suggested by Aliaga-Gaspar and colleagues in 2021.⁵ The higher amounts of soluble IFNAR2 in serum observed in our study might underlie an impairment of the immune response in patients with severe COVID-19, which decreases sensitivity to interferon beta and therefore antiviral activity. Our results contrast with the genome-wide association studies or transcriptomic studies. However, these studies did not quantify the soluble IFNAR isoforms in the serum of individuals with COVID-19. Our study contributes to a better understanding of the interferon response damage during SARS-CoV-2 infection. Whether the increased serum concentrations of soluble IFNAR2 in patients with COVID-19 are due to splicing mechanisms, as

is the case in patients with multiple sclerosis,⁵ remains unclear and requires further studies. We believe serum IFNAR1 and IFNAR2 concentrations in patients with COVID-19 can be used as predictors of disease severity and response to interferon treatment outcomes.

We declare no competing interests. We would like to thank the Agence Nationale de Recherches sur le Sida/Maladies infectieuses émergentes, MSD and the Agence Nationale de la Recherche for their financial support.

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See Online for appendix

THE LANCET

Microbe

Supplementary appendix

This appendix formed part of the original submission. We post it as supplied by the authors.

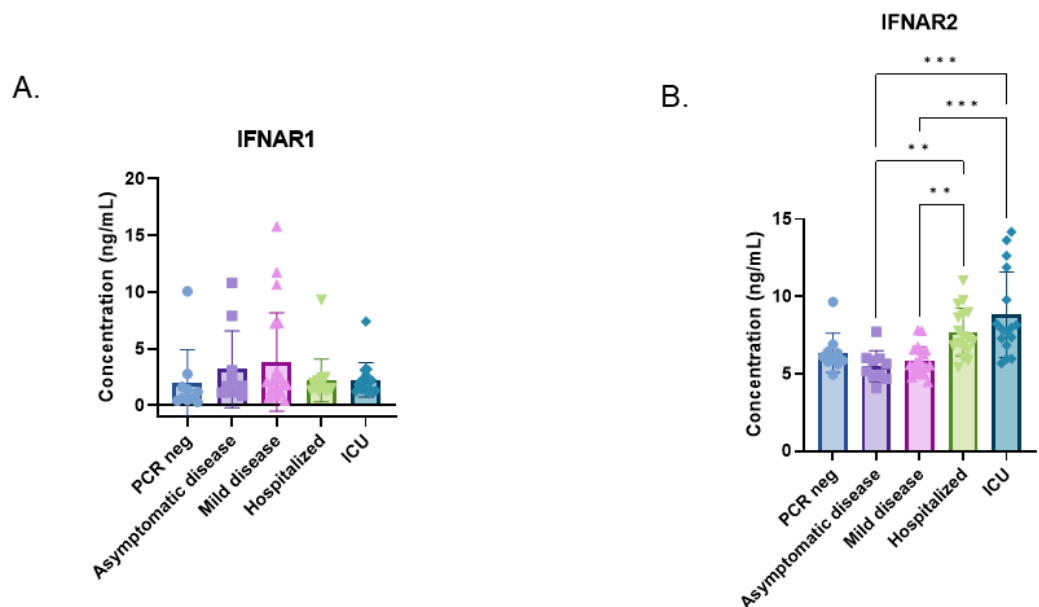
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1 Appendix

2 Fig. 1. sIFNAR2 but not sIFNAR1 levels are elevated in COVID-19 severe patients

3 Soluble levels of A. IFNAR1 and B. IFNAR2 in the serum of COVID-19 patients (77), as
4 quantified by ELISA. Patients were classified into groups on the basis of disease severity:
5 10 individuals PCR-negative for SARS-CoV-2, 10 individuals with asymptomatic disease,
6 20 with mild disease, 17 hospitalized patients (not ICU), and 16 ICU patients. Statistical
7 comparisons were performed with GraphPad Prism 9.5.0 (GraphPad, San Diego, CA, USA).
8 The statistical significance between groups was determined by a Kruskal-Wallis test and
9 Dunn's multiple comparison test. Statistical significance was defined as $p < 0.05$.

Fig. 1



10

11 Materials and methods

12 Cohort

13 Seventy-seven individuals (10 PCR-negative and 67 PCR-positive for SARS-CoV-2) were
14 included in prospective longitudinal cohort study conducted at CHU Nord de Saint-Etienne
15 (Saint-Etienne, France). They were divided in groups based on their disease severity at the

16 time of study inclusion (10 asymptomatic patients, 20 mild disease patients, 17 hospitalized
17 patients (not ICU), and 16 ICU patients). Blood samples were collected at the time of patient
18 inclusion which corresponded to three to seven days after symptom onset. Asymptomatic
19 patients were defined as PCR-positive patients without clinical symptoms at the beginning
20 of the study. Written informed consent for participation was obtained from all subjects;
21 ethics approval was obtained from CPP Ile de France V (NCT04648709).

22

23 **IFNAR1 and IFNAR2 quantification**

24 IFNAR1/2 serum levels were quantified by two commercial sandwich ELISA kits: IFNAR1
25 ELISA kit (LSbio, Ref: LS-F17211) (Range: 0.156 - 10 ng/ml; Intra-Assay: CV<5.6% Inter-
26 Assay: CV<9.6%), and IFNAR2 ELISA kit (abcam, Ref: ab264610) (Range: 46.875 pg/ml
27 - 3000 pg/ml; Intra-Assay: CV=5% Inter-Assay: CV=2.3%). Manufacturer's instructions
28 were followed.

29

30 **Statistical analysis**

31 All statistical calculations and graphs were performed with GraphPad Prism 9.5.0
32 (GraphPad, San Diego, CA, USA). The statistical significance between groups was
33 determined by a Kruskal-Wallis test and Dunn's multiple comparison test. Statistical
34 significance was defined as $p < 0.05$.

35

36

Discussion of Article 1

In our study we demonstrated that severe COVID-19 patients had lower levels of sIFNAR1 and higher levels of sIFNAR2 in the serum, compared to asymptomatic patients. Since we measured these proteins in the serum of patients between 3-7 days after symptoms onset, we propose that they could be used as predictors of disease severity.

It could be intriguing the fact that in the same patients there are lower levels of sIFNAR1 and higher levels of sIFNAR2 in severe disease patients as they heterodimerize to form one receptor and transduce the signal. However, it is shown that in mouse tissues, the soluble and the transmembrane IFNAR2 protein might have different regulations, as both isoforms vary in expression according to the tissue (232). In addition, in multiple sclerosis patients, sIFNAR2 is increased compared to healthy donors, and it seems to be due to alternative splicing rather than proteolytic cleavage and they have a poorer response to IFN- β treatment (233). Furthermore, IFNAR1 cell surface expression depends, among others, on the posttranslational modifications as glycosylation. In some Flavivirus infections, the non-structural protein 5 (NS5) can suppress the correct glycosylation of IFNAR1 thereby interfering with IFNAR1 cell surface expression, as reviewed by Zanin *et al.* in 2021 (234). Autosomal recessive IFNAR1 deficiency has been also associated to disease severity (235). These evidences led us to think that in COVID-19 disease it is possible to have both factors: the suppression of IFNAR1 expression as well as high levels of sIFNAR2 in the same patients.

Our study, together with the presence of IFN autoantibodies and the inborn errors of type I IFN, contributes to a better understanding of the IFN response damage

during SARS-CoV-2 infection that explains 20% of the most severe forms of COVID-19 (32,228). However, new questions arise that might contribute to having the whole picture of the IFNAR role in the COVID-19. For example, it would be interesting to make a prospective study to see the dynamics of the sIFNAR1 and 2 levels in COVID-19 patients and if they can also be predictors of recovery or severity duration as well as the risk of developing a long COVID condition. As well, we could study if the severe patients in our cohort also presented the autosomal recessive IFNAR1 deficiency, or if the lower levels of sIFNAR1 are due to other mechanisms, like for example the increased ubiquitination of this protein induced by the SARS-CoV-2 virus (92). Moreover, as IFNAR and the IFN response are activated in viral infections, it could be interesting to see if sIFNAR1 and sIFNAR2 could also be predictors of disease severity in other viral infections like influenza, respiratory syncytial virus (RSV), hepatitis or human metapneumovirus (HMPV).

Since high inflammation is associated to disease severity in COVID-19 we wanted to study other predictive factors that might contribute to this phenomenon. It is known from other disease like IBD, that microbiota plays a crucial role in the developing and maintain of the inflammatory status (71,72). In that line we wanted to study the intestinal and lung microbiota in a retrospective cohort of COVID-19 patients.

Article 2: The role of IgA in intestinal dysbiosis and inflammation in COVID-19.

(In preparation)

As discussed in the Introduction section, some studies have demonstrated a significant difference in gut microbial populations between severe and asymptomatic patients during COVID-19 disease. This gut dysbiosis could play an important role in maintaining intestinal inflammation as it has been shown for other inflammatory diseases (71,72). In addition, as described for other inflammatory intestinal situations, there may be compartmentalization of the microbiota by immunoselection mediated by secretory antibodies and in particular IgA (AMIS) (72). Our team and others have demonstrated that secretory IgA may have a role, in conjunction with secretory IgM, in the binding of certain commensal intestinal bacteria and promote their tissue translocation, particularly in Crohn's disease (71). SARS-CoV-2 infection generates gastrointestinal disorders, so being able to also study and describe the role of IgA in gut dysbiosis and inflammation in COVID-19 is crucial.

To continue our study on disease predictor factors in COVID-19, we wanted to study the intestinal microbiota in COVID-19 patients and its association with IgA antibodies. We have the stools and the BAL samples from a cohort of COVID-19 patients from March to May 2020 during the first wave in France. We have three groups of patients from the CHU Nord of Saint-Etienne (critical patients in intensive care unit (ICU); severe patients in Not-ICU services; and COVID-19 negative patients).

The workflow we are using to study the total and IgA+ microbiota is as follow: as a first step, from stool and BAL samples we separated the bacteria by subsequent

filters and centrifugation steps to phenotypically characterize the microbiota population associated to IgA using flow cytometry. We then purify the IgA⁺ fraction of the faecal microbiota using magnetic separation beads (MACS) (Miltenyi Inc.). The composition of the gut microbiota (total and IgA⁺) is evaluated by sequencing the V3-V4 region of the gene encoding the RNA of the small subunit (16S) of the bacterial ribosome. The NGS (Next Generation Sequencing) service and bioinformatic analysis was contracted to the Novogene company in United Kingdom (UK). We do not perform the separation and sequencing of the IgA⁺ bacteria with BAL samples as there are a smaller number of bacteria compared to stool samples, and there are needed 10^6 bacteria to have a good sequencing step (236). However, it will be interesting to try to optimise the protocol to be able to study the IgA⁺ microbiota in upper respiratory mucosal samples. The results are very preliminary and not all the techniques and analysis are finished, therefore we do not include them in this manuscript. However, so far, we also see different microbiota profiles between the three groups of patients, even though there are bias like the antibiotic treatments that patients had during hospitalization that might modify the microbiota profile. Nevertheless, we need to better master the techniques of our protocol, specially we still need to do more set-ups to well perform the phenotypically characterisation by flow cytometry because we found a high % of IgA⁺ bacteria (>80%) in total microbiota (even in COVID-19 patients), which is no normal according to the literature and to experts in the field (237). This phenotypic characterisation is important when doing IgASeq experiments as we need to consider the probability of a specific taxa to be covered by IgA to better interpret the differences found between groups in terms of microbiota profile, as shown by Jackson et al. in 2021 (237).

In conclusion, microbiota analysis is not a trivial thing and even though there are bias that are inevitable in this type of study like population differences in daily life habits, diet, or antibiotics treatments; technical and experimental bias should be minimized as possible.

Result 2: Understanding the role of the humoral immune response in the pathophysiology of the disease.

Article 3: Systemic and mucosal immune response in COVID-19.

(Article 1: Submitted)

Several studies are nowadays published about the role of the immune response in the pathology of COVID-19 disease. However, when I started my PhD, it was the beginning of the pandemic and we rapidly saw that the immune system, notably the humoral immune response systemic and mucosal in the development of the more severe disease. We performed a retrospective cohort study with COVID-19 patients from March-May 2023 when 20A and 20C clades were the circulating ones, and no variants were described. We focused principally on the role of antibodies, and more specifically IgA as it is the most important isotype present in mucosa and their blood levels are elevated in severe disease patients compared to asymptomatic or mild patients. One of the antibody features that we wanted to explore was the FcR-dependent function and for that we used an *in vitro* test that we developed using HEK293T cells expressing either the CD16 or CD89 receptor which was published in the journal *Vaccines* in June 2022 (see Annex 2). Additionally, we develop a collaboration with CIRI's VirPath laboratory to study the neutralizing effect and other effector functions of IgA and IgG from COVID-19 patients using a respiratory epithelium model (Mucillair model). The results of this study are submitted to the journal *Science Translational Medicine*.

1 **Title: Dual function of IgA response during COVID-19**

2
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24
25 **One Sentence Summary:** Mucosal IgA antibodies are not efficient in controlling SARS-CoV-2
26 infection while systemically contributing to inflammation in severe COVID-19 patients.

27 **Abstract:** Several studies have examined the antibody response to SARS-CoV-2, with a particular
28 focus on the systemic humoral immune response and the production of immunoglobulin G (IgG)
29 antibodies. IgA plays a crucial role in protecting against respiratory viral infections, but it has also
30 been associated with the pathophysiology of COVID-19. Here we conducted a retrospective study
31 with 169 COVID-19 patients including 50 critical/severe (ICU), 47 moderates (Not-ICU), and 72
32 asymptomatic ones to explore the humoral immune response against SARS-CoV-2 infection. Our
33 results show that early systemic IgA strongly induced in severe patients does not block IgG
34 functions in terms of neutralization and it better activates the FcRs than IgG. However, despite
35 high levels of SIgA, mucosal IgA antibodies are not efficient in controlling the infection in severe
36 patients. Our study highlights the complexity of the immune response to SARS-CoV-2, with
37 elevated systemic IgA levels with highly neutralizing capacities in severe cases, as well as higher
38 IgA-FcR activation compared to asymptomatic patients, while emphasizing the need for further
39 research to fully understand the role of IgA and its structural alterations in mucosal tissues in
40 severe cases and their impact on disease progression.

42 **Main Text:**

43 **INTRODUCTION**

44 The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-
45 CoV-2) continues to pose significant global health challenges (1). Several studies have examined
46 the antibody response to SARS-CoV-2, with a particular focus on the systemic humoral immune
47 response and the production of immunoglobulin G (IgG) antibodies (2–4). In addition, licensed
48 vaccines are administered intramuscularly generating a systemic immune response but a very weak
49 mucosal immunity, while a mucosal immune response would be more appropriate for a respiratory
50 virus like the SARS-CoV-2 (5, 6). IgA plays a crucial role in protecting against respiratory viral
51 infections. For instance, IgA antibodies present in nasal secretions have been found to effectively
52 neutralize respiratory viruses, such as influenza and respiratory syncytial virus (RSV) (7, 8).
53 Recent studies have shown that IgA antibodies against SARS-CoV-2 can be detected in the saliva,
54 tears, and nasal secretions of infected individuals, suggesting that they may play a role in
55 preventing the transmission of the virus through these routes, however, they wane after 9 months
56 of hospitalization and are not induced by subsequent vaccination (6, 9–12). Moreover, patients
57 with selective IgA deficiency (sIgAD) have been shown to have a higher SARS-COV-2 infection
58 rate compared to non-sIgAD patients, and a higher rate of developing severe COVID-19 disease
59 (13, 14). However, IgA has also been associated with pathophysiology in COVID-19. Persistent
60 spike-specific IgA response in BAL samples in the later stages of the infection has been correlated
61 to non-survival patients (15). Furthermore, IgA–virus immunocomplexes (ICs) potentiate the
62 programmed cell-death pathway through which neutrophils release neutrophil extracellular traps
63 (NETosis), via Fc- α RI engagement (16). While IgG aberrant glycosylation patterns are associated
64 with a lack of FcR-dependent functions in severe COVID-19 patients and therefore a more

65 inflammatory immune response (17–19), FcR-dependent functions for IgA and their contribution
66 to pathological effects in COVID-19 disease are poorly understood.

67 In this study, we aimed to investigate mucosal and systemic humoral immunity in a cohort of
68 COVID-19 patients and shed light on the specific role of IgA and IgG antibodies in disease
69 severity. Thus, we sought to explore the dynamics and functionality of IgA and IgG antibodies in
70 severe COVID-19 patients compared to those with milder symptoms or who remained
71 asymptomatic.

72

73 RESULTS

74 Early and high levels of systemic IgA are associated to severe COVID-19

75 Here we conducted a retrospective study with 169 COVID-19 consenting patients including 50
76 critical/severe (hospitalized in an Intensive Care Unit (ICU)), 47 mild/moderate (hospitalized in
77 Not-ICU services), and 72 asymptomatic ones (see cohort description in Materials and Methods
78 section) to explore the humoral immune response against SARS-CoV-2 infection. We first
79 measured the total IgG1, IgG2, IgG3, IgG4, IgA, and IgM antibody levels in serum using a Bio-
80 Plex immunoassay (BioRad Inc.) (**Fig. 1A**). During the first 10 days post-symptoms onset there
81 were no differences in the antibody levels between ICU and Not-ICU patients which means that
82 at early stages of the disease, total antibody levels are not a differentiating marker between the
83 more severe group of patients. However, from 10 days post-symptoms onset, there were higher
84 levels of IgA in the serum of ICU patients compared to Not-ICU or asymptomatic patients [ICU:
85 2.033 g/L vs Not-ICU: 0.8357 g/L ($p < 0.0001$); vs asymptomatic: 0.5520 g/L ($p < 0.0001$)]. These
86 differences were maintained after 40 days post-symptoms onset where IgA levels were lower even
87 though still statistically higher in the ICU patients [ICU: 1.625 g/L vs Not-ICU: 0.3000 g/L
88 ($p = 0.0420$); vs asymptomatic: 0.5222 g/L ($p = 0.0036$)]. These results suggest that IgA is induced
89 earlier than other isotypes in the more severe patients and that IgA dominates SARS-CoV-2
90 specific humoral response in severe patients.

91 Therefore, we next studied the kinetics of SARS-CoV-2 specific antibody response recognizing
92 the Spike 1 subunit (S1), the Receptor-binding domain (RBD), the S1 mutated D614G, (S1-
93 D614G), the Nucleocapsid (N), the Spike 2 subunit (S2), and the polyprotein before cleavage
94 (SEM), in the serum of the same cohort of patients (**Fig. 1B**). For all targeted antigens, higher IgA
95 and IgG responses were systematically observed in severe patients. This pattern is more evident

96 against all the S1 antigens (S1, RBD, and S1D614G), confirming the highest immunogenicity of
97 the Spike protein. Both IgA and IgG response in ICU patients increase until 40 post-symptoms
98 onset and decay after, whereas for Not-ICU patients the peak is achieved around day 30 post-
99 symptoms onset. This suggests that the specific SARS-CoV-2 antibody response in the more
100 severe patients is stronger and longer in time compared to less severe patients.

101 **Early systemic IgA strongly induced in ICU patients does not block IgG functions**

102 To investigate the functionality of the humoral immune response elicited during the infection we
103 first study the neutralizing activity of antibodies. We assessed neutralizing activity against the
104 Wuhan strain with a live virus neutralization *in vitro* test. ICU patients neutralized the virus even
105 at the last tested dilution (1:1280), while asymptomatic patients started losing the neutralization
106 by the 1:320 dilutions (**Fig. 2A**).

107 Secondly, we wondered whether IgA and IgG contributed equally to the neutralizing activity in
108 the different groups of patients. To this end, we assessed if IgA could block IgG functions by
109 competition. To address this question, we used agarose beads coupled to Protein A to deplete total
110 IgA antibodies from the serum of 9 ICU patients and 12 asymptomatic patients. IgA depletion was
111 achieved at more than 95% of efficacy for all the samples. Neutralizing activity of IgA-depleted
112 and not depleted serum was evaluated with the same live virus neutralization assay as previously.
113 We found no significant differences between ICU and asymptomatic patients before and after IgA
114 depletion, suggesting that IgA is not blocking the potential IgG-neutralizing effect (**Fig. 2B**).

115 To better understand the respective contributions of IgA and IgG to SARS-CoV-2 neutralization,
116 we purified IgA and IgG from the sera of 10 patients (5 ICU and 5 asymptomatic patients) by
117 affinity chromatography using Protein A-coupled beads or Protein G-coupled beads respectively.
118 Purified antibodies were obtained with more than 98% of purity and IgG or IgA residuals were

119 checked systematically. We tested the neutralization potential of the purified antibodies at 10
120 $\mu\text{g/mL}$ in the same *in vitro* neutralization test as previously. There were no statistically significant
121 differences between the IgA neutralization activity of ICU patients compared to asymptomatic
122 ones (**Fig. 2C**). However, purified IgG from ICU patients neutralized less (13% inhibition) than
123 IgG from asymptomatic patients (42% inhibition) with statistically significant differences,
124 suggesting a loss of neutralizing function of IgG in the more severe patients. When purified IgA
125 and IgG were mixed, there were no significant differences in neutralization between ICU and
126 asymptomatic patients, indicating that the loss of function of IgG does not affect the overall
127 neutralization and that there is no competition or blocking effect between IgA and IgG. We then
128 evaluated the neutralizing activity of purified IgA and IgG in the highly predictive and
129 physiologically relevant MucilAirTM reconstituted human airway epithelium (HAE) model (**Fig.**
130 **2D**). Preincubation of SARS-CoV-2 with 2.5-10 ng/mL of purified IgA from severe patients
131 resulted in complete viral neutralization in 4/5 samples and in partial neutralization, meaning a >2
132 \log_{10} reduction in viral titers in the first 48h but with still measurable viral replication, in the
133 remaining sample. Conversely, higher levels of purified IgG from severe patients were needed to
134 achieve 1/5 complete and 2/5 partial neutralization. Complete and partial neutralization were
135 respectively 1/5 and 4/5 for purified IgA from asymptomatic patients and IgG only achieved partial
136 neutralization in 2/5 samples. Overall, neutralizing Ig concentrations from severe patients were
137 lower for IgA vs IgG, and for Ig from severe vs asymptomatic patients. (9)

138 To study the Fc receptor potential or ADCC-like functions of IgA and IgG from patients' sera, we
139 used a previously described *in vitro* test that uses HEK expressing either CD16 or CD89 as effector
140 cells (20). We observed that IgA from ICU patients was more efficient to produce an FcR-
141 dependent signal than IgA from asymptomatic patients (mean relative FcR activation fold change:
142 33 vs 15; $P < 0.01$). The inverse was observed for IgG (mean relative FcR activation fold change:

143 16 vs 30; $P < 0.05$), suggesting again a loss of function for IgG in the sera of severe patients and a
144 gain of functions for IgA when compared to asymptomatic patients (**Fig 2E**).

145 **Mucosal IgA antibodies are not efficient in controlling infection in severe patients**

146 As SARS-CoV-2 is a respiratory virus, we wanted to study the humoral immune response in the
147 mucosa. To this purpose, we compared anti-SARS-CoV-2 IgA and IgG responses in nasal swabs
148 and bronchoalveolar lavages (BAL) obtained from the same patients and normalized with the total
149 IgA or IgG antibody concentration in each sample. ICU patients had a higher anti-SARS-CoV-2
150 S1 IgG response in nasal swabs compared to Not-ICU (relative antibody concentration mean ranks:
151 58.34 vs 40.84; $p < 0.05$) or asymptomatic patients (relative antibody concentration mean ranks:
152 58.34 vs 27.99; $p < 0.0001$) (**Fig. 3A**). ICU patients also had a higher nasal S1 IgA response but
153 only compared to asymptomatic patients (relative antibody concentration mean ranks: 55.83 vs
154 30.25; $p < 0.001$) (**Fig. 3A**). In BAL samples statistically significant differences were only found
155 between ICU and COVID-19(-) patients for IgA (relative antibody concentration mean ranks:
156 35.30 vs 18.45; $p < 0.05$) and IgG (relative antibody concentration mean ranks: 34.14 vs 17.33;
157 $p < 0.01$) (**Fig. 3G**).

158 Then we compared the level of anti-spike specific secretory IgA (SIgA) using a newly developed
159 assay between the three groups of patients. In both mucosal compartments (nasal swabs and BAL),
160 we found higher levels of SIgA in ICU patients compared to Not-ICU or asymptomatic patients
161 ([nasal swabs mean ranks: ICU: 27.36; Not-ICU: 26.92; Asymptomatic: 16.10] and [BAL mean
162 ranks: ICU: 22.16; Not-ICU: 11.11; COVID-19 (-): 8.000]), and the differences were statistically
163 significant (**Fig. 3B** and **Fig. 3H**). Surprisingly, we did not find any neutralizing activity either in
164 nasal swabs or in BAL samples for any of the groups (**Fig. 3C** and **Fig. 3I**). In addition, as IgA2
165 is the most predominant IgA isotype in mucosa, we wanted to compare the levels of IgA1 and

166 IgA2 specific to SARS-CoV-2 between groups in nasal swabs (**Fig. 3D** and **Fig. 3E**) and in BAL
167 (**Fig. 3J** and **Fig. 3K**) to see if there could be a disbalance in the levels of IgA isotypes in COVID-
168 19 patients. We observed that in nasal swabs from ICU patients, there were higher levels of anti-
169 S1 IgA1 than in nasal swabs from Not-ICU patients and these differences were statistically
170 significant (nasal swabs mean ranks: ICU: 21.60; Not-ICU: 12.36; $P<0.0339$) (**Fig. 3D**). In BAL
171 samples we did not find any statistically significant difference between groups (**Fig. 3E**). However,
172 when comparing anti-S1 IgA2 we did find statistically significant differences between nasal swabs
173 from ICU patients and asymptomatic patients but not with Not-ICU patients (nasal swabs mean
174 ranks: ICU: 10.10; Not-ICU: 16.09; asymptomatic patients: 21.80; ICU vs. Asymptomatic:
175 $P<0.0339$) (**Fig. 3J**). In BAL samples there were statistically significant differences between Not-
176 ICU patients and COVID-19 (-) patients (BAL mean ranks: Not-ICU: 28.55; COVID-19 (-)
177 patients: 8.714; $P<0.0009$) (**Fig. 3K**). Even though there were no differences between ICU and
178 Not-ICU patients, there is a trend to lower levels of anti-S1 IgA2 in more severe patients, which
179 suggests that the local immune response is deficient in ICU patients. IgA in mucosal tissues is
180 found to be polymeric and monomeric IgA in mucosal tissues might not correctly protect against
181 viral infections (21), thus we wondered if the structure of the IgA found in the mucosal samples
182 of our study was altered. In nasal swabs, monomers, as well as dimers, were detected in all three
183 groups (**Fig. 3F**). However, monomer concentrations were significantly higher in the BAL from
184 ICU patients. Such a difference was not observed in BAL from Not-ICU patients (**Fig. 3L**).

185 **DISCUSSION**

186
187 Here we studied the humoral immune response against SARS-CoV-2 in a cohort of patients from
188 the first wave of the COVID-19 pandemic in France in 2020. We found a significant elevation in
189 systemic IgA levels among ICU patients compared to non-ICU or asymptomatic patients, and this
190 response is found to be more robust and enduring in severe COVID-19 cases. This response is

191 found to be polyreactive and specific to S1, RBD, S2, and N SARS-CoV-2 proteins as previously
192 described (4, 15). These findings corroborate previous studies indicating a more pronounced and
193 persistent antibody response in severe COVID-19 patients explained, at least in part, by the
194 persistence of SARS-CoV-2 proteins and mRNA in the small intestine epithelium even months
195 after the infection (22–24). In addition, high levels of IgA at the early stage of the disease (10-15
196 days post-symptoms) were already observed in a previous study, where CCR10⁺ plasmablasts
197 circulating at this stage of the disease, secret predominantly IgA (9). Such observations suggest
198 that systemic anti-spike IgA could potentially serve as an early indicator of disease severity and
199 play a pivotal role in the immune response against SARS-CoV-2.

200 When studying the functionality of the IgA and IgG response in COVID-19 patients, higher
201 neutralization activities were observed in severe patients. This result confirmed those reported
202 before (25, 26) showing that sera from severe patients better neutralized the infection *in vitro*
203 compared to asymptomatic patients. However, when comparing the individual contribution,
204 contrary to previous research (27), we did not find differences in neutralization before and after
205 IgA depletion. This difference might be due to the methods employed to evaluate viral
206 neutralization. When Davis *et al.* used an RBD-ACE2 binding inhibition multiplex bead-based
207 assay, we used an *in vitro* neutralization test which is closer to real-life conditions and therefore a
208 more complex system that not only depends on the RBD-ACE2 binding inhibition. Nevertheless,
209 consistent with prior research (9), our study found that purified IgA from sera of severe patients
210 exhibited higher neutralization potential, particularly in a more physiological model using lung
211 epitheliums, compared to IgA from asymptomatic patients. In addition, a loss of neutralizing
212 activity of purified IgG from severe patients when compared to asymptomatic patients was
213 observed. This result is consistent with others that described a loss of function for IgG in severe
214 COVID-19 patients associated with a defect of neutralization activity(9, 17). Importantly, there

215 was no evidence of IgA blocking the neutralizing effect of IgG as observed in other infections like
216 HIV (28). These results suggest, as proposed by other studies, that neutralizing IgA activity could
217 play a critical role during the acute phase of the infection (29).

218 FcR-dependent functions can also play a role in controlling disease and promoting a robust
219 immune response. In our study, we observed a loss of CD16-dependent function in IgG from
220 severe patients compared to asymptomatic patients. These findings contradict other studies that
221 have reported increased FcR activation in IgG from severe patients, and they have associated the
222 afucosylation of IgG with enhanced FcR affinity in severe patients (17, 30). Whether the observed
223 loss of function in our study is due to receptor affinity or structural differences between IgG from
224 severe and asymptomatic patients requires further investigation. We also found higher CD89-
225 dependent activity in severe patients than in asymptomatic ones. However, in another study,
226 antibody-dependent phagocytosis (ADP) was not affected after IgA depletion suggesting that IgA
227 contributes to neutralization but is not efficient in activating some FcR-dependent functions (27).
228 In contrast, our study is in line with evidence that showed higher NETosis triggered by IgA in
229 severe disease patients (31, 32). Moreover, IgA could also be structurally modified in severe
230 patients leading to an increase in FcR-dependent function. Taken together, our results show higher
231 neutralization for systemic IgA than IgG, especially in severe disease patients, and higher IgA-
232 FcR activation in severe patients than in asymptomatic patients. However, this uncontrolled IgA
233 activation and the compromised functionality of IgG antibodies in the early phase of the disease
234 could exacerbate the inflammatory response and contribute to the severity of the disease as it has
235 been widely described (17, 33).

236 We also investigated mucosal immune responses, specifically in nasal swabs and BAL samples.
237 Consistent with previous studies (34–36), we observed higher levels of anti-SARS-CoV-2 IgA and
238 IgG in nasal swabs and BAL of ICU patients compared to asymptomatic or COVID-19 free

239 patients, indicating a more robust immune response in severe cases. However, the lack of
240 neutralizing activity in both nasal swabs and BAL samples across all patient groups contradicts
241 findings from other studies, which have reported neutralizing activity in mucosal samples (9, 37,
242 38). Those studies evaluated SARS-CoV-2 neutralization using a pseudovirus *in vitro* test.
243 However, despite the advantages of this type of test (39), it only focuses on the Spike protein from
244 SARS-CoV-2, which does not provide a complete picture as in a live virus neutralization test, as
245 we used. Ruiz, *et al.* reported a loss of neutralizing activity in BAL from non-survivors at later
246 stages of COVID-19 despite the persistent levels of S1-, RBD-, S2-, and NP-specific IgG and S1-
247 specific IgA once SARS-CoV-2 was cleared from the lungs (15). This might suggest that the high
248 IgA levels found in our study, even though not-neutralizing, could activate FcR-dependent
249 functions as has been suggested by Ruiz, *et al.* via the formation of IgA immunocomplexes (ICs)
250 (15). Furthermore, in our study, we detected higher levels of sIgA in severe patients compared to
251 COVID-19 free patients which might promote human lung inflammation and fibrosis by inducing
252 fibroblast activation and enhancing the production of inflammatory cytokines (40). IgA present in
253 mucosal tissues is mostly dimeric and it is produced locally, but plasma monomeric IgA can reach
254 the airways through a receptor-independent process called transudation (41, 42). This process is
255 more likely to occur in damaged lung tissue, as observed in patients with severe COVID-19. In
256 our study, we found IgA monomers and dimers in mucosal secretions, especially more monomers
257 than dimers in BAL from severe disease patients, which is consistent with the previous study of
258 Sterlin *et. al* (9). As we found more IgA-dependent FcR activation in serum from severe patients,
259 if IgA monomers found in mucosal tissues are transudate from the serum, we might expect more
260 FcR functions in mucosal tissues and therefore more inflammation. Moreover, mIgA1 has been
261 described to activate cytotoxic CD8+ T cells (43), and an aberrant glycosylation profile increase
262 their pro-inflammatory effect which is associated to higher disease severity in autoimmunity (44,

263 45). If IgA has an aberrant glycosylation pattern in COVID-19 as it is described for IgG, remains
264 to be fully studied.

265 Our results suggest that the mucosal antibody response, at least in terms of neutralization, may not
266 be as efficient in controlling the infection as previously thought, and that FcR-dependent function
267 might contribute to the pathophysiology of the disease in the upper and lower respiratory tract.
268 Our discrepancies and contrasting findings highlight the complexity of the mucosal immune
269 response to SARS-CoV-2 and suggest that there may be variations in the immune profiles of
270 different patient populations.

271 Our study has several limitations as it is a retrospective study at the time of the Wuhan SARS-
272 CoV-2 strain circulation. The timing of the samples used in this study is not long enough to fully
273 characterize the duration of the humoral immune response against SARS-CoV-2 infection. In
274 addition, the low levels of IgA and IgG antibodies found in the serum of asymptomatic patients
275 did not allow us to restrain the quantitative exploration of all the functions as well as structural
276 properties of SARS-CoV-2 specific antibodies in our cohort of patients. Moreover, it remains to
277 be determined whether the antibody response found in mucosal secretions contributes to the
278 pathology of COVID-19 disease *via* the FcR-functions.

279 In conclusion, our study highlights the complex and varied immune response to SARS-CoV-2,
280 with elevated systemic IgA levels with highly neutralizing capacities in severe cases, as well as
281 higher IgA-FcR activation for IgA compared to asymptomatic patients. However, contradictory
282 findings regarding neutralization and FcR-dependent functions in mucosal tissues emphasize the
283 need for further research to fully understand the role of IgA and its structural alterations in severe
284 cases and their impact on disease progression.

285 MATERIALS AND METHODS

286 Study design

287 169 PCR-positive SARS-CoV-2-infected patients were included in a retrospective cohort study
288 conducted at CHU Nord de Saint-Etienne (Saint-Etienne, France) during the first wave of SARS-
289 CoV-2 infections in France (from February 2020 to May 2020). COVID-19 patients were split into
290 three groups according to the WHO classifications of disease severity (46): 72 patients with mild
291 or asymptomatic disease, 47 patients who were hospitalized but not required admission to ICU,
292 and 50 patients with severe disease requiring admission to the ICU. Serum samples were collected
293 between 1- and 67 days post-symptom onset. Nasal swabs were collected at the moment of the
294 first PCR for SARS-CoV-2 infection confirmation. Bronchoalveolar lavages were collected
295 whenever it was possible for hospitalized patients (ICU and Not-ICU) as well as for 16 PCR-
296 negative SARS-CoV-2 patients, sampled before the pandemic. Written informed consent for
297 participation was obtained from all subjects; ethics approval was obtained from CPP Ile de France
298 V (NCT04648709).

299

300 Cells

301 VeroE6 cell lines were obtained from American Type Culture Collection (ATCC), CRL-1586, (not
302 authenticated but regularly tested for mycoplasma contamination). HEK CD89⁺ and HEK CD16⁺
303 cell lines were obtained from InvivoGen and used as effector cell lines for the FcR activation assay.
304 All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) growth medium
305 containing antibiotic-antimycotic (AAT) and supplemented with 2% SVF (for VeroE6 cells) or
306 10% SVF (for HEK CD89⁺ and HEK CD16⁺ cells). Cells were harvested using trypsin/EDTA
307 solution. All cell lines were maintained at 37°C in humidified air containing 5% CO₂.

308 HEK-CD89⁺ (FcαRI) and HEK-CD16⁺ (FcγRIIIa) cell lines were obtained from InvivoGen and
309 previously described (20). All cells were maintained in Dulbecco's modified Eagle medium
310 (DMEM) supplemented with 10% FCS and 1% antibiotic-antimycotic (AAT). Cells were
311 harvested with trypsin/EDTA solution and the stable expression of FcR was checked by flow
312 cytometry before each experiment. All cell lines were maintained at 37°C, in a humidified
313 atmosphere containing 5% CO₂.

314

315 **Quantification of total immunoglobulins**

316 Serum samples were tested using the Bio-Plex Pro Human Isotyping Panel assay (Bio-Rad
317 Laboratories, Hercules, CA) according to the manufacturer's instructions. Briefly, 50 µL of diluted
318 beads were added to each well, and plates were washed with Bio-Plex wash buffer. Standards,
319 blanks, controls, and samples were added in the indicated dilutions, following an incubation step
320 at 25°C for 1h. Another washing step was performed, and detection antibodies were added for
321 30min at 25°C in the dark. Plates were washed and the streptavidin-PE diluted 1X was added and
322 incubated for 10min at 25°C in the dark. A final washing step was carried out and beads were
323 resuspended in 125 µL of assay buffer. Plates were read in a Bio-Plex Luminex 200 system. Results
324 were expressed in g/L.

325

326 **Measurement of Spike-specific IgA**

327 The IgA binding to SARS-CoV-2 Spike was evaluated using ELISAs. In brief, a Spike protein
328 solution (1 µg/ml; #40591-V08H Spike S1-RBD Sino Biologicals) was coated onto high binding
329 96-half-well plates (ThermoFisher) overnight at 4°C. After washing and blocking, serum samples
330 were added and incubated for 1h at 37°C. The plates were washed and then incubated with an anti-
331 human IgA (A0295; Sigma-Aldrich) or IgG (A6029; Sigma-Aldrich) secondary Ab conjugated to

332 horseradish peroxidase (HRP). After the addition of the HRP substrate (3,3',5,5''-
333 tetramethylbenzidine (TMB; 34021; Thermo Fisher Scientific)) and stop solution (1 M HCl),
334 optical density units were measured at 450 nm in a microplate reader (TECAN). For serum
335 samples, a ratio of OD was calculated between each sample and a negative control (pool of pre-
336 pandemic serum samples) that was added in duplicate to each run. For nasal swabs and BAL
337 samples, the IgA and IgG equivalent content was calculated using a calibration curve with a
338 recombinant anti-RBD IgA (B Cell Design #IB3C4 PV) or anti-RBD IgG (B Cell Design #
339 X30F12-PU), and the limit of detection was 0.1 ng/ml Equivalent.

340

341 **IgA depletion and immunoglobulins purification**

342 IgA depletion was performed in 21 sera (9 ICU patients and 12 asymptomatic patients). For that,
343 100 μ L of serum were diluted in 1 \times PBS and mixed with 100 μ L of peptide M/agarose column
344 (InvivoGen) after resin equilibration. Depleted IgA plasma fractions were collected by spinning
345 the resin at 1000 g for 1 min after 1h of incubation at 25°C. Purified IgA was eluted with 0.1 M
346 glycine (pH 2 to 3; Sigma-Aldrich), and pH was immediately neutralized with 1 M Tris.

347 To increase the amount of purified immunoglobulins (Igs) we diluted in 1 \times PBS 500 μ L of serum
348 and passed it through a double chromatography system as follows: first, diluted serum was loaded
349 onto protein G/agarose column (InvivoGen) after equilibration. The flowthrough was collected
350 and passed to a second column filled with peptide M/agarose (InvivoGen) to obtain the purified
351 IgA. IgA and IgG were eluted using 0.1 M glycine (pH 2 to 3; Sigma-Aldrich), and the pH was
352 immediately adjusted to 7.5 with 1 M Tris. PBS buffer exchange was performed using Amicon
353 Ultra centrifugal filters (Merck Millipore) through a 50 kDa membrane according to the
354 manufacturer's instructions. All chromatography steps were carried out at a flow rate of 0.5

355 ml/min. The purity of IgG and IgA fractions was evaluated using ELISA. There were less than 1%
356 of undesirable isotypes in the purified Ig.

357

358 **Live virus SARS-CoV-2 neutralization assays in Vero E6 cells**

359 To detect and measure neutralizing antibodies, a plaque reduction neutralization test (PRNT) was
360 employed. First, each serum specimen was diluted 10-fold in a culture medium and heated for 30
361 minutes at 56°C to prevent complement-mediated reduction of viral activity. Then, 2-fold serial
362 dilutions of each serum were mixed in equal volumes with the live SARS-CoV-2 virus (strain:
363 19A (B.38)), followed by 1h of incubation at 37°C. The mixture was then transferred to 96-well
364 microplates covered with Vero E6 cells reaching a viral concentration of 100 TCID₅₀/well and
365 incubated at 37°C in a 5% CO₂ atmosphere. The microplates were microscopically evaluated 5
366 days later and wells with the presence of cytopathic effect were compatibilized. Viral
367 quantification was performed in the supernatant by RT-PCR as described before (47) and used to
368 calculate the % of infection inhibition. For nasal swabs and BAL samples, the same protocol was
369 used except that nasal swabs were first diluted at 1:5, and BAL at 1:10 in the culture medium, and
370 the neutralizing titer was determined as the last sample dilution that infected 50% of the wells. For
371 IgA depletion, the IC₅₀ was calculated using GraphPad Prism 10.0.1 (GraphPad, San Diego, CA,
372 USA). All experiments were performed in a biosafety level 3 laboratory.

373

374 **Live virus SARS-CoV-2 neutralization assays in HAE**

375 To evaluate the neutralization capacity of purified IgA and IgG from patients, we mixed 75 µL of
376 each Ig sample (final concentrations of 2.5, 5, and 10 ng/mL) with 75 µL of a Wuhan-like 19A
377 SARS-CoV-2 virus (GISAID accession number: EPI_ISL_411218; 19A (B)) at a MOI of 0.01.

378 After incubation for 1h at 37 °C, the 150 µL-mix was inoculated on the apical side of MucilAir™
379 nasal HAE (Epithelix SARL, ref: EP02, pool ref: MP0010) maintained in the air-liquid interphase
380 and incubated for 1h at 37°C and 5% CO₂. The apical poles of HAE were gently washed twice
381 with warm OptiMEM (1X) medium (GIBCO BRL, ref. 31985-047) prior to inoculation. Samples
382 were tested in duplicate and recombinant anti-Spike and non-SARS-CoV-2 specific IgA/G were
383 used as positive and negative controls, respectively. One-hour post-inoculation (hpi), the virus-Ig
384 mix was removed, and the HAE put back in the incubator. Twenty-four hpi, the apical poles of
385 HAE were washed with warm OptiMEM, which was then collected for quantification of viral
386 nsp14 gene copies (RT-qPCR) as previously described (48). This step was repeated at 48 and 72
387 hpi.

388

389 **SARS-CoV-2 RT-PCR for viral amplification**

390 The amount of SARS-CoV-2 in the culture supernatant was measured using RT-PCR without the
391 need for nucleic acid extraction. The Luna Universal Probe One-Step RT-qPCR Kit from New
392 England Biolabs (Ref. E3006L) (49) was used for this purpose. To perform the test, 5 µL of
393 supernatant were diluted 1/10 with DNase-free and RNase-free water and mixed with the reaction
394 solution to obtain a total volume of 14 µL. The reaction solution contained 5 µL Luna® Universal
395 Probe One-Step Reaction Mix, 0.5 µL Luna® WarmStart® RT Enzyme Mix and 1.5 µL of a
396 mixture of primers at 400 nM (E_Sarbeco_F: ACAGGTACGTTAATAGTTAATAGCGT and
397 E_Sarbeco_R: ATATTGCAGCAGTACGCACACA) and the probe at a concentration of 200 nM
398 (E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ) (50). The RT-PCR was
399 initiated with a reverse transcription step at 55°C for 10 min, followed by 40 cycles of denaturation
400 at 95°C for 10s and annealing at 60°C for 60s. A viral standard curve was generated for each
401 analysis.

402

403 **SARS-CoV2 specific FcR activation assay**

404 SARS-CoV-2 specific FcR activation assay was performed as previously described (20) with the
405 following modification: SARS-CoV-2 infected VeroE6 cells were used as target cells that were
406 incubated with the patient's serums diluted at 1:100. Effector cells expressing Fc receptor (HEK-
407 CD16⁺ or HEK-CD89⁺) were co-cultured with the target cells at a 1:1 effector/target ratio for 48h
408 at 37°C. Alkaline phosphatase was measured in the culture supernatant using Quantibblue
409 (InvivoGen) at 620nm. FcR activation was calculated as follows: (OD620nm sample/ OD620nm
410 pre-pandemic pool serum) and normalized to the anti-S1 antibody concentration for each sample.

411

412 **Immunoblotting**

413 Nasal swabs and BALs were centrifuged at 200g for 5min, and total protein concentrations were
414 determined using a Bradford assay. Samples were diluted in deionized water and then in NuPAGE
415 LDS Sample buffer 4X (Ref: NP0008, ThermoFisher Scientific) to achieve a final amount of 20
416 µg of total proteins to load onto the gel. Samples were then heated at 70°C for 10 min. Proteins
417 were separated using 4 to 12% Bis-Tris polyacrylamide gel electrophoresis (Ref: NW04125BOX,
418 ThermoFisher Scientific) for 45 min at 200 V and then transferred to nitrocellulose for Western
419 blot analysis. After 16h of membrane blocking with 1× PBS with 5% nonfat milk, human IgA and
420 chain J were detected with an anti-IgA-PerCPVio700 (Ref: 130-116-885 Miltenyi) used at a
421 1:2000 dilution and an anti-chainJ-AF488 (Ref: sc-133177 AF488, Santa Cruz Biotechnology,
422 INC) used at 1:2000 dilution, respectively, for 1h at 25°C. Fluorescence was visualized with a
423 camera system (iBright 1500, ThermoFisher Scientific). All incubations were in 1× PBS with 5%
424 nonfat milk, and wash steps used 1× PBS with 0.1% Tween 20.

425

426 **Statistical analysis**

427 Statistical significances were calculated using a two-way ANOVA with Tukey or Sidak correction
428 for multiple comparisons depending on the data set. The non-parametric test Kruskal-Wallis was
429 also used with Dunn's correction for multiple comparisons. Significant *P* values are indicated as
430 described: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001. All statistical calculations
431 and graphs were generated using GraphPad Prism 10.0.1 (GraphPad, San Diego, CA, USA).

432

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619

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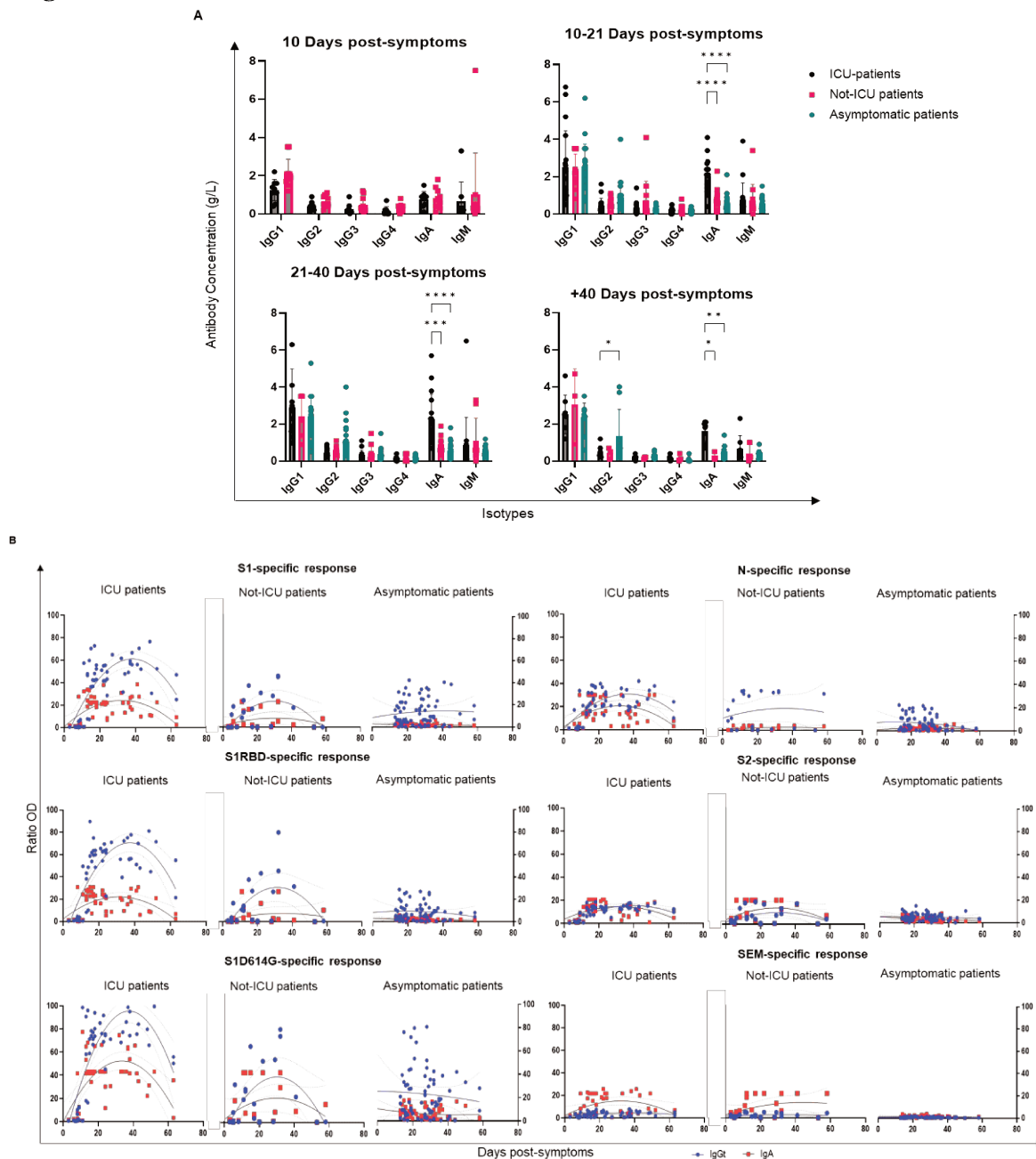
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624 **Author contributions:** Melyssa Yaugel-Novoa, Blandine Noailly, Fabienne Jospin, Bruno
625 Pozzetto, Andrés Pizzorno and Aurélien Traversier carried out the experiment. Melyssa Yaugel-
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627 Nevers analyzed the data and wrote the manuscript with support from Thomas Bourlet, Stephane
628 Paul and Manuel Rosa-Calatrava. Thomas Bourlet and Stéphane Paul supervised the project.

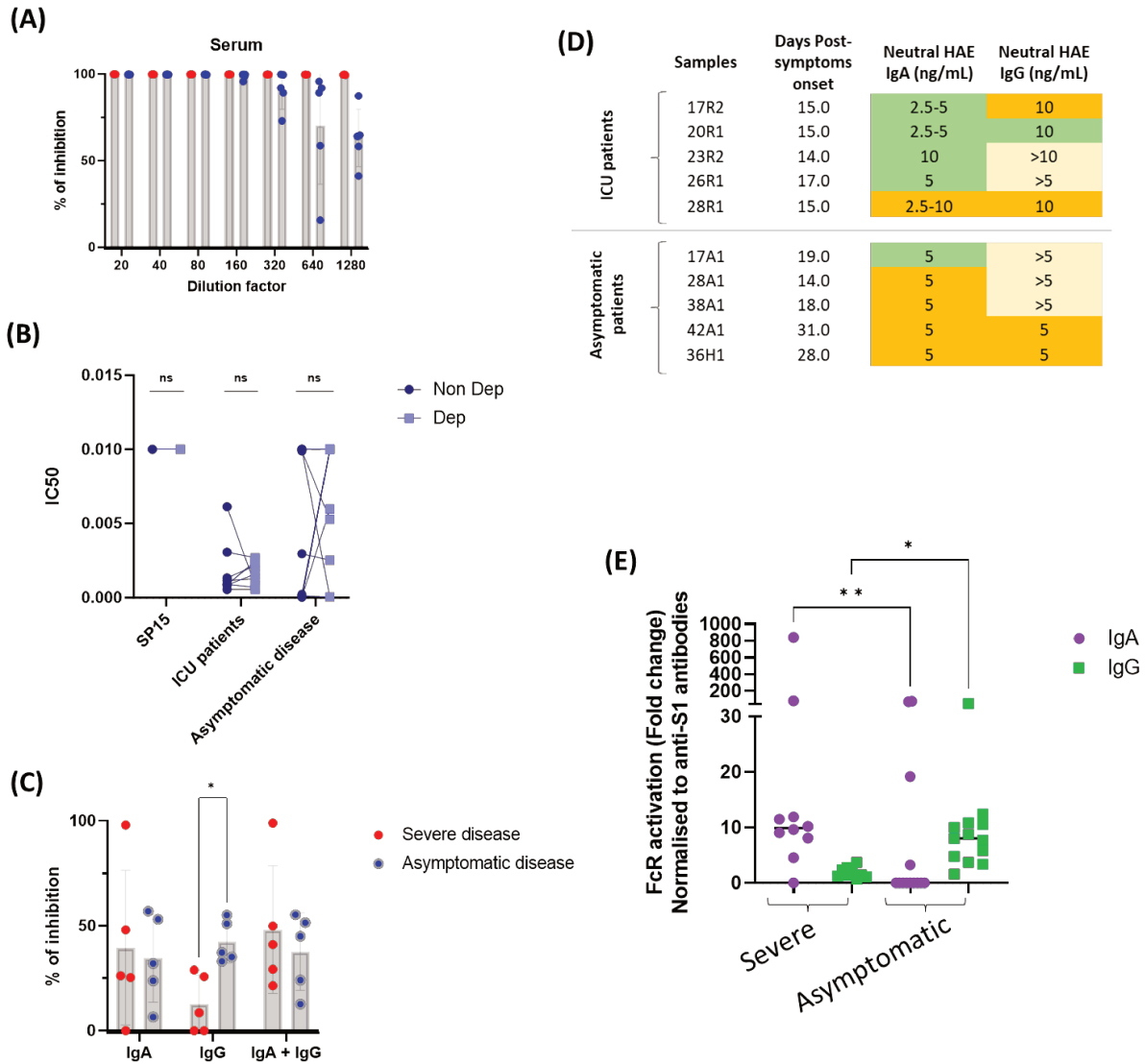
629 **Competing interests:** Authors declare that they have no competing interests.

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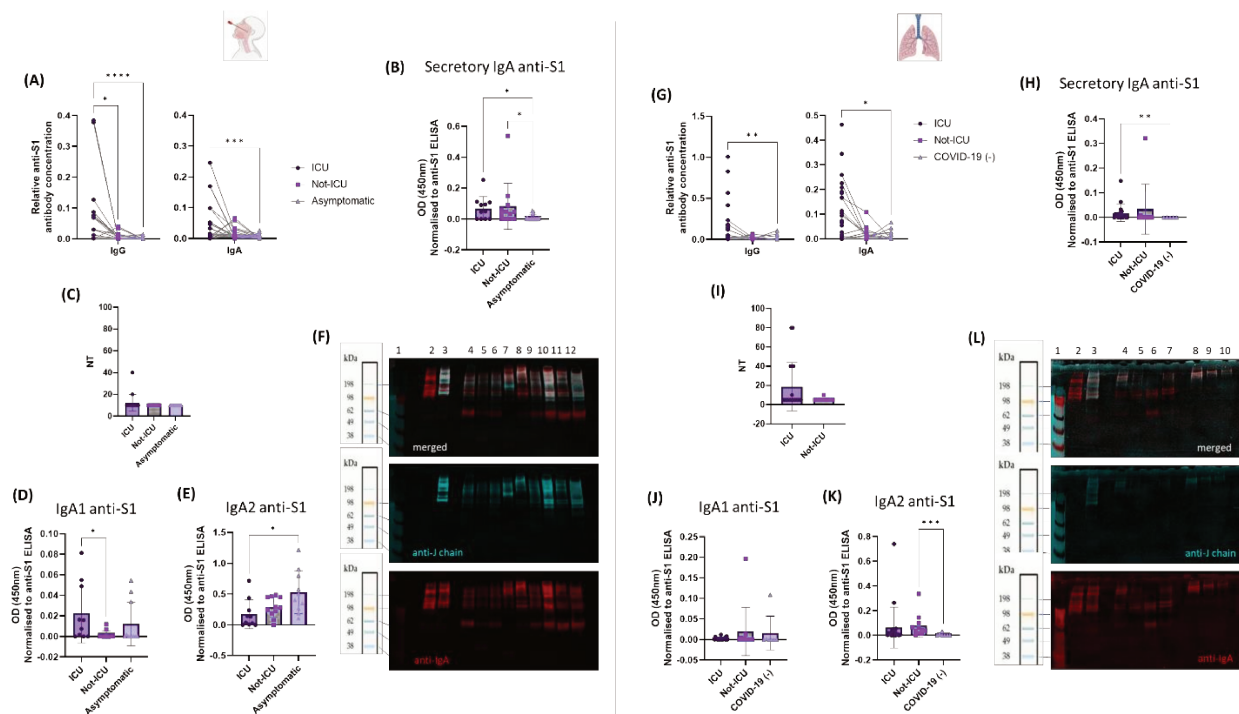


634 **Fig. 1. Kinetics of antibody response in SARS-COV-2 infection. (A)** Total IgG1, IgG2, IgG3,
 635 IgG4, IgA and IgM antibody response in the serum of 169 patients at different times post-
 636 symptoms onset. Each dot corresponds to one patient. *P* value was calculated using the Tukey test
 637 for multiple comparisons (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001). **(B)** Specific
 638 IgA and IgG anti-SARS-CoV-2 antibody response in serum against spike-1 protein (S1), spike-1
 639 receptor-binding domain (RBD), D614G mutated S1 protein (S1D614G), nucleocapsid protein
 640 (N), spike-2 protein (S2), and polyprotein spike-envelop-membrane protein (SEM), at different
 641 times post-symptoms onset. Each dot corresponds to one sample. Red squares correspond to IgA
 642 and blue circles to IgG. Dot lines represent the nonlinear fit using the least square regression with
 643 a 95% confidence interval.



644

645 **Fig. 2. Effector functions of systemic IgA and IgG anti-SARS-CoV-2 antibodies.** (A) Neutralizing activity of 10
 646 sera tested at different dilutions in a live virus neutralization test. Each dot corresponds to a sample. (B) Neutralizing
 647 activity of IgA depleted and non-depleted sera from 10 patients, tested at equivalent IgG concentrations. SP15
 648 corresponds to a pool of sera from a pre-pandemic date. P value was calculated using Sidak correction for multiple
 649 comparisons with $\alpha=0.05$. (C) Neutralizing activity of purified IgA and IgG antibodies at a concentration of 10 $\mu\text{g/mL}$
 650 from 10 sera collected between 15- and 30-days post-symptoms onset. P value was calculated using Sidak correction
 651 for multiple comparisons with $\alpha=0.05$ ($*P < 0.05$). (D) Neutralizing activity of purified IgA and IgG antibodies from
 652 10 patients was measured in reconstituted human airway epithelia (HAE). Green numbers indicate complete
 653 neutralization, orange numbers indicate partial neutralization and yellow numbers indicate no neutralization. (E) Fold
 654 change of FcR activation capacity dependent on CD89 (IgA) or CD16 (IgG) from sera of 23 patients tested in a 1:100
 655 dilution in vitro. Each dot corresponds to one sample. P value was calculated using Dunn's correction for multiple
 656 comparisons with $\alpha=0.05$ ($*P < 0.05$, $*P < 0.01$).
 657



659

Fig. 3. Mucosal immune response to SARS-CoV-2 in Nasal swabs (A-F) and BAL (G-L). (A and G) Relative IgG and IgA anti-S1 concentrations, normalized to total IgG or IgA rates, in 78 patients (A, nasal swabs) and 57 patients (G, BAL). Each dot corresponds to a different sample. P value was calculated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons with $\alpha=0.05$ ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$). (B and H) Secretory IgA anti-S1 levels, normalized to anti-S1 IgA concentrations, in 43 patients (B, nasal swabs) and 34 patients (H, BAL). Each dot corresponds to a different sample. P value was calculated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons with $\alpha=0.05$ ($*P < 0.05$, $**P < 0.01$). (C and I) Neutralizing activity of 55 nasal swabs and 29 BAL tested at 1:10 and 1:5 dilutions, respectively, measured in a live virus neutralizing test *in vitro*. (D and J) IgA1 anti-S1 levels normalized to anti-S1 IgA concentrations; in 34 patients (D, nasal swabs) and 40 patients (J, BAL). Each dot corresponds to a different sample. P value was calculated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons with $\alpha=0.05$ ($*P < 0.05$). (E and K) IgA2 anti-S1 levels, normalized to anti-S1 IgA concentrations, in 34 patients (E, nasal swabs) and 40 patients (K, BAL). Each dot corresponds to a different sample. P value was calculated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons with $\alpha=0.05$ ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (F and L) Immunofluorescent Western-Blot after an SDS-PAGE performed in non-reducing conditions for crude nasal swabs (F) (lane 1: Molecular weight marker; lane 2: IgA control without chain J; lane 3: IgA control with chain J; lane 4-6: nasal swab from 3 asymptomatic patients; lane 7-9: nasal swab from 3 Not-ICU patients; lane 10-12: nasal swab from 3 ICU patients); and BAL (L) (lane 1: Molecular weight marker; lane 2: IgA control without chain J; lane 3: IgA control with chain J; lane 4-7: BAL from 4 ICU patients; lane 8-10: BAL from 3 Not-ICU patients) respectively where IgA monomers and dimers are detected. For all the samples a total amount of 20 μ g of protein was loaded.

680

Discussion of Article 3

In this study, we investigated the humoral immune response against SARS-CoV-2 in a cohort of patients from the first wave of the COVID-19 pandemic in France (between March and May 2020). Our findings revealed a significant increase in systemic IgA levels among ICU patients compared to non-ICU or asymptomatic patients. Moreover, this response was found to be more robust and long-lasting in severe cases of COVID-19. The IgA response was polyreactive and specific to various SARS-CoV-2 proteins (S1, RBD, S2, and N), as described in previous studies (123,125,134,238). These results are consistent with earlier research that indicated a stronger and more persistent antibody response in severe COVID-19 patients, possibly due to the prolonged presence of SARS-CoV-2 proteins and mRNA in the small intestine epithelium even months after infection (124,136,139). Notably, high levels of IgA were observed during the early stage of the disease (10-15 days post-symptoms) in a previous study, where CCR10+ plasmablasts predominantly secreted IgA (137). The functionality of IgA and IgG responses in COVID-19 patients was also examined. Higher neutralization activities were observed in severe patients, confirming previous reports that severe patients' sera better neutralized the infection *in vitro* compared to asymptomatic patients (123,144,239). Purified IgA from severe patients' sera displayed higher neutralization potential, especially in a more physiological lung epithelium model, compared to IgA and IgG from asymptomatic patients, consistent with prior research (137). Importantly, there was no evidence of IgA blocking the neutralizing effect of IgG. Furthermore, FcR-dependent functions were studied, and a loss of CD16-dependent function in IgG from severe patients was observed, contrary to previous studies that reported an increased FcR activation in IgG from severe patients

associated to IgG afucosylation in these patients (206,240). Whether the observed loss of function in our study is due to receptor affinity, or structural differences between IgG from severe and asymptomatic patients, requires further investigation. CD89-dependent activity, however, was found to be higher in severe patients than in asymptomatic individuals. While contradictory with a study where IgA depletion did not affect ADP function (241), our study is in line with evidence that showed higher NETosis triggered by IgA in severe disease patients (180,182). Moreover, IgA could also be structurally modified in severe patients leading to an increase in FcR-dependent function. These findings suggest that the functionality of systemic IgA is higher than IgG, particularly in severe disease patients. Nevertheless, uncontrolled IgA activation and compromised IgG functionality in the early phase of the disease could exacerbate the inflammatory response contributing to the severity of COVID-19. We also investigated the mucosal immune responses in nasal swabs and BAL samples. While higher levels of anti-SARS-CoV-2 IgA and IgG were observed in nasal swabs and BAL of ICU patients compared to asymptomatic or COVID-19 free patients, no neutralizing activity was detected in these mucosal samples, contrary to some previous studies (137,158,170). However, those studies used a pseudovirus neutralization test which only focuses on the S protein from SARS-CoV-2 while our live-virus neutralizing test is closer to a real infection. The high IgA levels found in our study, even though not-neutralizing, could activate FcR-dependent functions as has been suggested by Ruiz, et al. via the formation of IgA immunocomplexes (ICs) (238). We found more IgA monomers (mIgA) than dimers (dIgA) in BAL from severe disease patients compared to asymptomatic ones, which is consistent with the previous study of Sterlin et. al (137). mIgA can transudate from the serum to the lung epithelium (162,163), and if they present an aberrant

glycosylation pattern that will increase the activation of FcR-dependent functions, more inflammation can occur, and disease severity might increase.

Our study has several limitations as it is a retrospective study at the time of the Wuhan SARS-CoV-2 strain circulation. The timing of the samples used in this study is not long enough to fully characterize the duration of the humoral immune response against SARS-CoV-2 infection. In addition, the low levels of IgA and IgG antibodies found in the serum of asymptomatic patients did not allow us to fully explore of all the functions as well as structural properties of SARS-CoV-2 specific antibodies in our cohort of patients. Moreover, it remains to be determined whether the antibody response found in mucosal secretions contributes to the pathology of COVID-19 disease via the FcR-functions.

In conclusion, our study provides insights into the complex humoral immune response to SARS-CoV-2, with elevated systemic IgA levels in severe cases and higher functionality for IgA compared to IgG. The conflicting findings regarding neutralization and FcR-dependent functions in mucosal tissues emphasize the need for further research to understand the role of IgA and its structural alterations in severe cases and their impact on disease progression.

Result 3: Immune response against SARS-CoV-2 elicited after vaccination

In the introduction we talked about the unprecedentedly fast development of vaccines during COVID-19 pandemic. Several questions arose after the first vaccines came out to the market and notably when new variants started to appear. These questions included (1) the efficacy of the homologous vs heterologous vaccination scheme and towards the emergence of SARS-COV-2 variants; (2) the long-term immunity after vaccination or breakthrough infections; and (3) if the vaccine elicited immunity enhances infection in naïve individuals. All these questions are matter of study of the three following articles presented in this section.

To address the first question, I was involved in a collaboration with another group in the CIRI, where we compared the immunogenicity of heterologous (ChAdOx1-BNT162b2) and homologous (BNT162b2-BNT162b2) vaccination scheme between health care workers in an observational study during 2020 and 2021.

Article 4: Heterologous vaccination against SARS-CoV-2
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Immunogenicity and efficacy of heterologous ChAdOx1–BNT162b2 vaccination

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 Check for updates

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Following severe adverse reactions to the AstraZeneca ChAdOx1-S-nCoV-19 vaccine^{1,2}, European health authorities recommended that patients under the age of 55 years who received one dose of ChAdOx1-S-nCoV-19 receive a second dose of the Pfizer BNT162b2 vaccine as a booster. However, the effectiveness and the immunogenicity of this vaccination regimen have not been formally tested. Here we show that the heterologous ChAdOx1-S-nCoV-19 and BNT162b2 combination confers better protection against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection than the homologous BNT162b2 and BNT162b2 combination in a real-world observational study of healthcare workers ($n = 13,121$). To understand the underlying mechanism, we conducted a longitudinal survey of the anti-spike immunity conferred by each vaccine combination. Both combinations induced strong anti-spike antibody responses, but sera from heterologous vaccinated individuals displayed a stronger neutralizing activity regardless of the SARS-CoV-2 variant. This enhanced neutralizing potential correlated with increased frequencies of switched and activated memory B cells that recognize the SARS-CoV-2 receptor binding domain. The ChAdOx1-S-nCoV-19 vaccine induced a weaker IgG response but a stronger T cell response than the BNT162b2 vaccine after the priming dose, which could explain the complementarity of both vaccines when used in combination. The heterologous vaccination regimen could therefore be particularly suitable for immunocompromised individuals.

The ChAdOx1-S-nCoV-19 and BNT162b2 vaccines (hereafter referred to as ChAd and BNT, respectively) have been shown to confer strong protection against SARS-CoV-2 infection and to induce robust immune responses directed against the spike protein of SARS-CoV-2 when administered in a homologous setting^{3–5}. However, the effectiveness of a ChAd–BNT combination as a prime–boost regimen has not been formally tested in vaccine trials. To compare the risk of SARS-CoV-2 infection following heterologous ChAd–BNT or homologous BNT–BNT vaccination schedules, we extracted data from the occupational medicine database of the University Hospital of Lyon (Hospices Civils de Lyon), France. Specifically, we analysed the number of individuals who received each vaccination regimen (which started in January 2021 in both groups) and the number of SARS-CoV-2 infections (documented by a positive

PCR with reverse transcription (RT–PCR) result) that occurred at least 2 weeks after the booster dose in each group (Table 1). Ten infections were identified out of 2,512 individuals (0.40%) in the heterologous vaccination group compared with 81 infections out of 10,609 individuals (0.76%) in the homologous vaccination group (Fig. 1). As the age of the individual and the vaccination regimen are known covariates, we used a multiple logistic regression model to model the probability of being infected. Our predictors were age (set as a continuous variable, considered as a possible confounder) and the vaccination regimen. Results showed that age is not associated with infection status ($P = 0.4514$). In particular, patients aged 60 years or above (715 out of 13,121) were not more infected (3 out of 715; 0.42%) than patients aged 60 years or below (88 out of 12,406 (0.71%); two-sided Fisher's exact test, $P = 0.4890$).

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Table 1 | Infection rate in vaccinated HCWs at the Lyon University Hospital

Naive HCWs		BNT-BNT	ChAd-BNT
Vaccinated participants	Numbers of participants	10,609	2,512
	Median age (minimum–maximum)	42 (19–76) years ^a	33 (19–64) years ^a
SARS-CoV-2 RT-PCR positive at least 14 days after second vaccine dose	Numbers of infected participants (%)	81 (0.76) ^b	10 (0.4) ^b
	Median age (minimum–maximum)	41 (22–65) years	32 (22–49) years

^aTwo-sided Student's t-test, $P=5\times 10^{-24}$
^bLogistic regression model, $P=0.0384$

However, the vaccination regimen was significantly associated with the probability of being infected after vaccination ($P = 0.0384$). Individuals vaccinated with BNT-BNT were twice as likely to be infected than those vaccinated with ChAd-BNT (relative risk of 2.03).

Antibodies and virus neutralization

To understand the immunological basis of this difference, we assembled a cohort of healthcare workers (HCWs) without comorbidity (Covid-Ser-Vac). Half of this HCW cohort received the homologous BNT-BNT combination within the recommended 4-week interval between the two doses, whereas the second half received the BNT booster after receiving the first ChAd dose approximately 12 weeks before. For both groups, the mean age was 41 years and 34 years, respectively, and about 70% of participants were female (Table 2). Three blood samples were drawn: before vaccination, before the second dose and

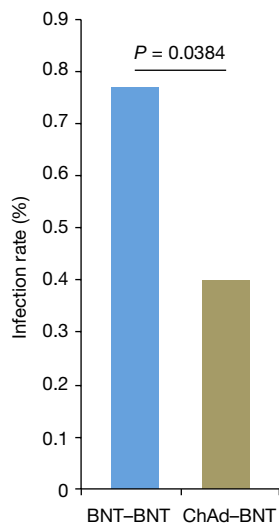


Fig. 1 | Incidence of SARS-CoV-2 infection after different vaccination regimens. Histograms show the infection rate (as documented by a positive SARS-CoV-2 RT-PCR result) among groups of HCWs who were vaccinated with the homologous BNT-BNT combination ($n = 81$ out of 10,609) within the recommended 4-week timeframe between the two doses or with the BNT booster after receiving the first ChAd dose ($n = 10$ out of 2,512) approximately 12 weeks before, as recorded by the service of occupational medicine, Hospices Civils de Lyon. Data show the infection rates that occurred 14 days after receiving booster up to the end of recording (15 August 2021). Statistical significance was calculated using a logistic regression model adjusted for age. Demographic data and other statistics are available in Table 1.

Table 2 | Clinical characteristics of patients in the Covid-Ser-Vac study

	Heterologous vaccine ($n=29$)	Homologous vaccine ($n=31$)	P value	Adjusted P value
Male sex (n (%))	9 (31.03)	8 (25.80)	0.7742	0.7742
Age in years (median (IQR))	34 (27–40)	41 (33–52)	0.0016	0.0056
Body mass index ¹ (n)	28	31		
Median (IQR)	22.25 (20.7–24.3)	23.12 (21.45–25.83)	0.2279	0.3988
Currently smoker (n (%))	5 (17.24)	8 (25.8)	0.5358	0.6251
Alcohol consumption ¹ (n (%))	7 (24.13)	5 (16.12)	0.5269	0.6251
Delay between two vaccine doses				
Median (IQR)	85 (84–85)	29 (26–31)	<0.0001	0.0007
Delay between second vaccine dose and third blood sample				
Median (IQR)	30 (28;34)	28 (27;31)	0.0245	0.057
Presence of comorbidity ^a (n (%))	0	0	NA	NA

Comorbidities include neurological disorders, cardiovascular disorders, hypertension, heart failure, diabetes, immune deficiency, liver disease, kidney disease, cancer, hypothyroid or rheumatic disease. For alcohol consumption, this was defined as consumption at least once a week. NA, not applicable.
Mann-Whitney and Fisher two-sided tests were used for quantitative and qualitative variables (in italic), respectively. Adjusted P values were calculated using the Benjamini-Hochberg method.
^aIndicates one missing data.

4 weeks after the second dose. Immunological analyses were performed longitudinally (Extended Data Fig. 1), and a linear regression model was used to compare immune parameters between groups to adjust for age differences.

As previously shown^{6,7}, the BNT vaccine induced a stronger level of SARS-CoV-2 receptor binding domain (RBD) or spike S1-specific IgG than the ChAd vaccine after the first dose. However, these IgG titres were no longer statistically different between groups after the second dose (Fig. 2a, b). Moreover, the positivity rate was 100% for both heterologous and homologous vaccinations. Serum IgA levels measured after the booster vaccination tended to be greater in the homologous than in the heterologous setting (median (interquartile range (IQR)) of 46.7 ng m⁻¹ equivalent (Eq) (36.30–78.70 ng m⁻¹ Eq) versus 37.4 ng ml⁻¹ Eq (25.40–59.80 ng ml⁻¹ Eq), respectively; equivalence was to the standard used (Methods)) (Fig. 2c).

We then measured the ability of serum samples to neutralize SARS-CoV-2 spike-pseudotyped viral particles⁸. None of the sera displayed a detectable neutralizing activity before vaccination or after the first dose. However, most of the sera displayed a neutralizing activity after the second dose, and those from the ChAd-BNT vaccinated individuals displayed a higher neutralizing efficacy than those from the BNT-BNT vaccinated individuals (median (IQR) of 62% (34–93%) versus 99% (89–100%) of neutralization, respectively; $P < 0.001$) (Fig. 2d).

To validate these results, we tested the ability of the sera to prevent Vero E6 cell infection by SARS-CoV-2 isolates belonging to various clades, including 19A (B38 lineage), Alpha (B.1.1.7 lineage), Beta (B.1.351 lineage), Gamma (P1) and Delta (B.1.617.2 lineage)⁹. Each SARS-CoV-2 isolate was

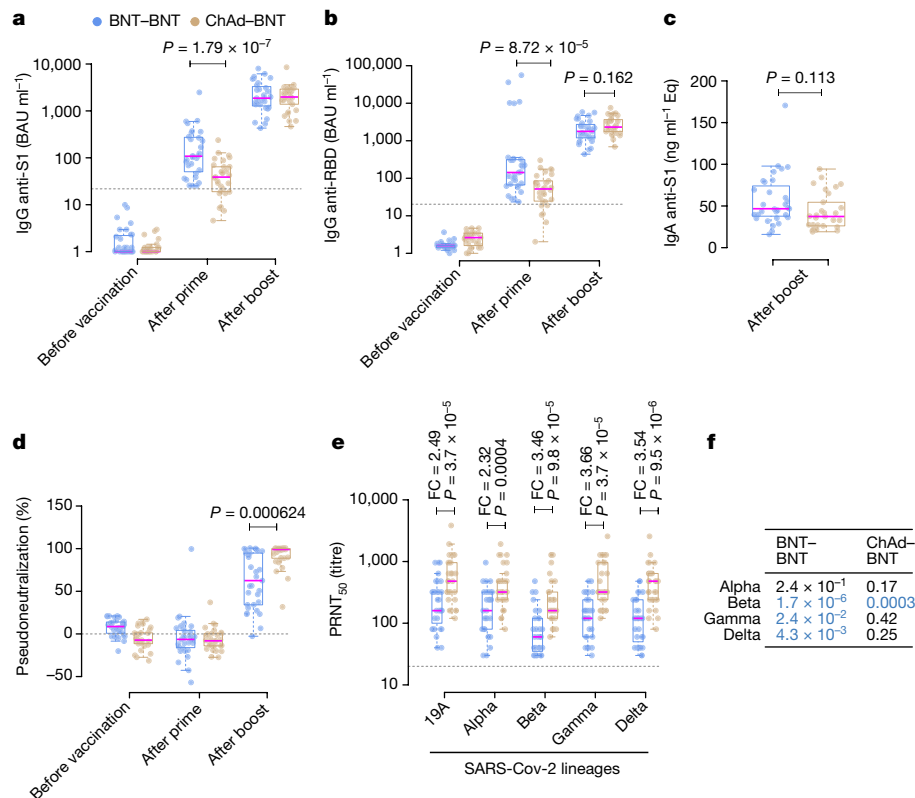


Fig. 2 | Heterologous vaccination induces higher neutralizing antibody titres. **a–c**, Sera from ChAd-BNT ($n = 29$) or BNT-BNT ($n = 31$) individuals were assayed for S1-specific IgG (**a**), RBD-specific IgG (**b**) or S1-specific IgA (**c**) levels using commercial or custom-made ELISA tests at different times during the vaccination process as indicated (**a, b**) or 4 weeks after full vaccination (**c**). In **a–c**, concentrations are expressed in binding antibody units per ml (BAU ml^{-1}) or ng ml^{-1} Eq of immunoglobulin as indicated. Each serum sample was evaluated as a single measurement (**a, b**) or in triplicate (**c**). Dotted lines in **a** and **b** indicate positive detection according to the manufacturer. **d–f**, Sera from ChAd-BNT ($n = 29$) or BNT-BNT ($n = 31$) vaccinated individuals were assayed in triplicate for their capacity to neutralize the entry of virus-like particles pseudotyped with the Wuhan strain SARS-CoV-2 envelope (**d**) or in duplicate for their capacity to

neutralize infection of Vero E6 cells by different SARS-CoV-2 strains (**e, f**), as indicated. Data show the per cent of neutralization relative to a positive control (**d**) or the 50% plaque reduction neutralization test (PRNT_{50}) (**e**), and are expressed as dot plots, with one dot corresponding to one individual. The limit of detection is shown as a dotted line in **e**. FC, fold change in the mean of the indicated groups. In all panels, box-and-whiskers plots are shown (see Methods for details), and the median is represented by the magenta middle line. A linear regression model was used to compare values between groups, and this model was corrected for age. Exact P values are shown for the indicated comparisons when significant or nearly significant. **f**, Comparison of serum neutralizing activity against the reference lineage 19A and against the variants of concern for each group. P values are shown and calculated using the linear regression model described in **e**.

sequenced and confirmed to contain the characteristic mutations of its viral clade. The ChAd-BNT vaccinated individuals exhibited 2.3-fold to 3.6-fold higher serum neutralizing antibody titres against the different variants than BNT-BNT vaccinated individuals (Fig. 2e). Moreover, in the latter group, the neutralizing responses observed against the Beta, Gamma and Delta variants were significantly reduced compared with that against the 19A clade. By contrast, the neutralizing activity detected in sera from ChAd-BNT vaccinees was similarly high against all strains except the Beta clade (Fig. 2e, f). These data demonstrate that the neutralizing potential of antibodies generated by heterologous vaccination is less affected by spike mutations appearing in variants of concern than that of antibodies induced by homologous vaccination.

Vaccine-induced memory cells

We then studied the memory B cell (mBC) compartments using fluorescently labelled tetrameric RBDs to track RBD-specific mBCs and to analyse their phenotype (the gating analysis is presented in Extended Data Fig. 2a). As shown in Fig. 3a and Extended Data Fig. 2b, which correspond to concatenated flow cytometry plots, the frequency of RBD-binding mBCs was comparable for both vaccination groups after the prime dose but was on average twofold higher for the ChAd-BNT vaccination schedule after the booster dose. Moreover,

switched ($\text{IgD}^- \text{CD27}^+$) mBCs accounted for about 67% and 47% of the RBD-specific mBC pool in the ChAd-BNT group and the BNT-BNT group, respectively (Fig. 3b, c, Extended Data Fig. 2c). The expression pattern of IgM and IgG isotypes was next used to compare the immunoglobulin isotype distribution among the switched mBCs. The results showed that the homologous vaccination schedule promoted more IgG-switched mBCs (62% versus 48%) than the heterologous vaccination schedule (Fig. 3d, Extended Data Fig. 2d). Finally, to address the activation status of mBCs, we analysed their expression of CD21 and CD11c. It is generally considered that the concurrent loss of CD21 and the upregulation of CD11c are phenotypic features associated with B cell activation independent of the developmental stage¹⁰. Resting mBCs were therefore defined as $\text{CD21}^+ \text{CD11c}^-$ cells, whereas $\text{CD21}^- \text{CD11c}^+$, $\text{CD21}^+ \text{CD11c}^+$ and $\text{CD21}^- \text{CD11c}^-$ cells were globally considered as activated mBCs. The frequencies of activated RBD-specific mBCs were significantly enhanced in the ChAd-BNT group compared with the BNT-BNT group (Fig. 3e, Extended Data Fig. 2e). In particular, the proportions of the $\text{CD21}^- \text{CD11c}^+$ subset were almost three times higher in the ChAd-BNT group. Altogether, our results indicate that compared with the homologous vaccination schedule, the ChAd-BNT combination induces significantly more RBD-specific B cells, increases the frequencies of post-switch mBCs and induces a more active mBC generation process.

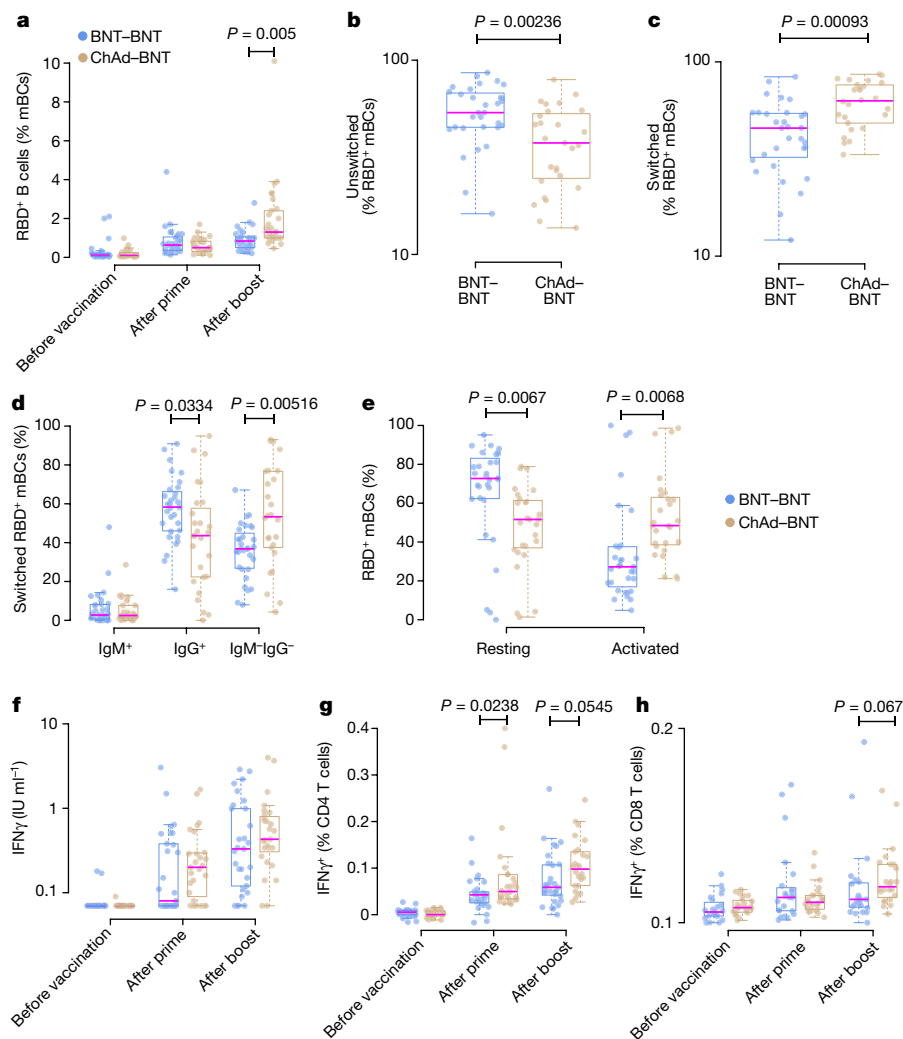


Fig. 3 | Analysis of RBD-specific memory B cells and spike-specific memory T cells in vaccinated individuals. a–e, Flow cytometry analyses of spike-specific mBCs. **a,** Frequencies of SARS-CoV-2 RBD-binding mBCs among the pool of polyclonal mBCs before vaccination, after prime and after boost injections. **b, c,** Proportions of unswitched (**b**) and switched (**c**) RBD-binding mBCs in per cent of RBD-binding mBCs. **d,** Pattern of immunoglobulin isotypes expressed by switched mBCs. **e,** Proportions of resting (CD21⁺CD11c⁻) and activated (CD21⁺CD11c⁺, CD21⁻CD11c⁻ and CD21⁻CD11c⁺) mBCs among the entire pool of RBD-binding mBCs. **f–h,** T cell responses. **f,** IFN γ release after whole-blood stimulation with RBD peptides. IFN γ was measured by ELISA in the supernatant. **g, h,** Flow cytometry analyses of the percentage of cells positive

for intracellular IFN γ expression among CD4 (**g**) or CD8 (**h**) T cells after PBMC stimulation with commercial spike peptides. In all panels, box-and-whiskers plots (see Methods for details) of $n = 29$ and $n = 31$ participants in heterologous and homologous vaccination groups, respectively, are shown. The median value is shown in each group as a purple line. A high cellular mortality rate at the thawing step led to the exclusion of a few samples in each panel; each individual dot corresponds to one participant, and each measurement was performed once. In all panels, a linear regression model was used to compare values between groups, and this model was corrected for age. Exact P values are shown for the indicated comparisons when significant or nearly significant.

We then longitudinally monitored the T cell response of vaccinees against RBD peptides using a whole-blood interferon- γ (IFN γ) release assay. The T cell response was higher after ChAd immunization than after BNT priming and was similarly increased by the BNT booster dose in both groups (median of 0.33 UI ml⁻¹ versus 0.43 UI ml⁻¹) (Fig. 3f). To confirm and consolidate these results, we measured the spike-specific CD4 and CD8 T cell response at the single-cell level by flow cytometry after stimulating peripheral blood mononuclear cells (PBMCs) from vaccinees with a pool of commercial peptides spanning the entire spike protein and then staining for intracellular IFN γ (the gating strategy is presented in Extended Data Fig. 3). Both vaccination regimens were able to induce a progressive increase in the frequency of S-reactive CD4 and CD8 T cells from the pre-vaccine to the post-booster phase (Fig. 3g, h). However, the heterologous combination resulted in a stronger CD4 T cell response, both after priming and boosting, and in an increasing trend in the CD8 T cell response after the booster dose.

Finally, to understand interrelations between immune parameters, we performed a matrix analysis (Extended Data Fig. 4). This confirmed that the neutralizing antibody activity correlated with the titres of spike-specific IgG, regardless of the variant analysed, and not correlated with IgA levels. Moreover, the neutralization activity correlated with the percentage of switched RBD⁺ mBCs and with the percentage of activated RBD⁺ mBCs. This provides further support that better neutralizing antibodies are produced as a result of sustained B cell activation.

Discussion

Heterologous prime–boost vaccinations have been reported to be more immunogenic than homologous ones in experimental settings¹¹. This has also been proven for some human vaccines^{12,13}. Moreover, studies using mice have demonstrated the strong immunogenicity of the ChAd–BNT combination¹⁴. Here we report that heterologous ChAd–BNT vaccination

confers better protection against infection—which is associated with more switched mBCs and higher virus neutralizing antibody titres—irrespective of the variant analysed. This finding is of particular importance considering the global increase in the SARS-CoV-2 Delta variant¹⁵. Other studies reported better neutralizing potential of sera from ChAd-BNT vaccinated individuals compared with BNT-BNT vaccinated individuals, but these studies only used surrogate^{16,17} or pseudovirus¹⁸ neutralization assays. Moreover, the ability of such assays to predict neutralizing activity against authentic clinical virus isolates is still debated¹⁹.

Heterologous vaccination induced antibodies with a stronger neutralization potential than homologous vaccination, but the spike-specific IgG antibody titres were comparable. This result suggests that it is the quality of the antibodies that underlies the neutralization potential of the antibody response induced by heterologous vaccination. Assuming that the secondary antibody response is primarily derived from the mBC clones generated by primo-immunization, we can postulate that ChAd and BNT formulations elicit different mBC compartments. mBCs generated by the ChAd formulation could, for example, bear antigen receptors displaying a larger breadth of epitope recognition or a better fit for the SARS-CoV-2 spike protein. This could be linked to the different conformations of the spike protein, as that of the BNT vaccine bears a mutation stabilizing the protein in its pre-fusion conformation²⁰. Our data showed that mBCs still exhibit features of activation 4 weeks after the booster dose in the heterologous regimen, which suggests that the post-boost process of mBC differentiation is more active in this schedule. This could result from a more efficient or long-lasting germinal centre reaction that facilitates an extended cycle of somatic hypermutations and possibly output of B cell clones with higher affinity antigen receptors. An enlarged pool of CD4⁺ T helper cells could contribute to an amplified or prolonged germinal centre reaction. In keeping with this notion, we report here a higher frequency of spike-specific CD4 T cells after priming with the ChAd vaccine, which has been observed in other studies^{6,7}. Moreover, stronger T cell responses may contribute to better protection independently of B cell responses²¹.

The better neutralization potential of sera from ChAd-BNT vaccinated individuals could also be linked to their relatively lower level of SI-specific IgA levels. Indeed, plasma IgA monomers specific to SARS-CoV-2 proteins are twofold less potent than IgG equivalents²². Furthermore, IgA levels persist better than IgG after infection, which coincides with a reduction in the serum neutralizing potential²³. Following a possible competition between serum IgA and IgG, the decrease in IgA levels in heterologous vaccination conditions may lead to better neutralization effects. This phenomenon has already been observed in the context of the RV144 vaccination trial against HIV-1, in which the level of envelope-specific IgA correlated with the risk of infection²⁴. In this context, IgA was also found to inhibit antibody-dependent cell cytotoxicity by competing with IgG²⁵.

Our data do not allow the discarding of the possibility that the later timing of administration of the booster dose contributed to the high efficacy of the heterologous regimen. Still, it is noteworthy that the size of the RBD⁺ mBC pool after the prime dose was comparable for both groups of vaccinees, despite the disparity in the analysis time point after the prime vaccination (4 versus 12 weeks). This observation indicates that the extended interval between prime and boost in the ChAd-BNT cohort has not been translated into a greater size of the RBD-specific mBC compartment. It has been recently demonstrated for both the ChAd vaccine²⁶ and the BNT vaccine²⁷ that long injection time intervals (12 weeks or more) provide higher binding and neutralizing antibody titres than shorter intervals (less than 6 weeks). For a comparable long vaccination interval (more than 9 weeks), the heterologous ChAd-BNT vaccination schedule induced better humoral immunity (titres and neutralization) than the homologous ChAd-ChAd combination²⁸. To our knowledge, there is only one study in which ChAd-BNT and BNT-BNT vaccination schedules were compared with the same prime-boost interval (4 weeks)⁶. That study⁶

concluded that for short intervals, the humoral responses induced by both regimens were comparable. However, a longer interval (that is, 12 weeks) between prime and boost could be needed to allow the synergy between heterologous vaccines.

Together, we present a real-world observational study of HCWs showing that the heterologous ChAd-BNT vaccination regimen confers stronger protective immunity than the homologous BNT-BNT prime-boost schedule. As hidden confounding factors might be present in our study (for example, different levels of exposure to the virus), confirmatory studies and a longer follow-up of vaccinated participants are warranted. However, our data suggest that the heterologous combination could be particularly suitable for immunocompromised individuals.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-021-04120-y>.

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Covid-Ser study group

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Methods

Ethical statement and cohort description

The use and analysis of data from the occupational health medical file were authorized after a regulatory declaration to the National Commission for Information Technology and Civil Liberties according to the reference methodology (declaration MR004 number 20-121 of 30 April 2020). The declaration of SARS-CoV-2 infection is compulsory for all staff to obtain daily allowances without loss of salary during the imposed quarantine.

For the Covid-Ser-Vac study, clinical data were recorded by a trained clinical research associate using Clinsight software (v.Csonline 7.5.720.1). Blood samples were processed and stored at the Centre de Ressource Biologique Neurobiotec, 69500 Bron. Sixty naive HCWs for COVID-19 and vaccinated with BNT and/or ChAd vaccines were included in a prospective longitudinal cohort study conducted at the Hospices Civils de Lyon. Blood sampling was performed before vaccination, before the second dose of vaccine and 4 weeks after the end of vaccination schedule. The absence of previous SARS-CoV-2 infection was confirmed using the Wantai SARS-CoV-2 Ab total assay in the pre-vaccine sample. Demographic characteristics and delays between doses are depicted in Table 2 and Extended Data Fig. 1. Written informed consent was obtained from all participants. Ethics approval was obtained from the national review board for biomedical research in April 2020 (Comité de Protection des Personnes Sud Méditerranée I, Marseille, France; ID RCB 2020-A00932-37), and the study was registered at ClinicalTrials.gov (NCT04341142).

Measurement of IgG titres

Sera were immediately stored at -80°C after blood sampling. S1-specific IgG and RBD-specific IgG were measured using Siemens Atellica IMSARS-CoV-2 IgG (sCOVG) and bioMérieux Vidas SARS-CoV-2 IgG diagnosis kits, respectively, according to the manufacturers' recommendations. For standardization of these assays to the first World Health Organization international standard, the concentrations were transformed into binding antibody units per ml (BAU ml^{-1}) using the conversion factors provided by the manufacturers.

Measurement of spike-specific IgA

ELISAs to evaluate IgA binding to the SARS-CoV-2 spike protein were performed as previously described²⁹. High-binding 96-half-well plates (2310M, Nunc) were coated with 100 μl per well of a spike protein solution ($1\ \mu\text{g ml}^{-1}$; 40591-V08H spike S1-RBD, Sino Biologicals) in PBS overnight at 4°C . Plates were washed with washing buffer containing 1 \times PBS with 0.05% Tween 20 (Sigma-Aldrich) and incubated with 170 μl of blocking buffer per well containing 1 \times PBS with 3% fat milk powder and 0.05% Tween 20 (Sigma-Aldrich) for 1 h at room temperature. Immediately after blocking, recombinant anti-RBD IgA (B Cell Design, IB3C4 PV) or serum samples diluted in PBS were added and incubated for 1 h at 37°C . Plasma samples were assayed at a 1:100 starting dilution and 7 additional 3-fold serial dilutions. Recombinant human anti-RBD IgA was used to perform a calibration curve starting at $1.5\ \mu\text{g ml}^{-1}$. Plates were washed and then incubated with anti-human IgA (A0295, Sigma-Aldrich) secondary antibody conjugated to horseradish peroxidase in blocking buffer at 1:10,000. Plates were developed by the addition of the horseradish peroxidase substrate 3,3',5,5'-tetramethylbenzidine (TMB; 34021, Thermo Fisher Scientific) for 10 min, and the developing reaction was stopped by adding 50 μl of 1 M HCl. Optical density units were measured at 450 nm in a microplate reader (Tecan). For serum samples, a positive control (serum pool from critically ill patients with COVID-19, diluted 200-fold in PBS) and a negative control (pool of historical serum samples) were added in duplicate to each run. After deduction of the background, a relative content in IgA equivalent ($\text{ng ml}^{-1}\text{Eq}$) was calculated using the calibration curve. The limit of detection of the assay was $0.1\ \text{ng ml}^{-1}\text{Eq}$.

Live-virus neutralization experiments

A plaque reduction neutralization test (PRNT) was used for the detection and titration of neutralizing antibodies. A tenfold dilution of each serum specimen in culture medium was first heated for 30 min at 56°C to avoid complement-linked reduction of viral activity. Serial twofold dilutions (tested in duplicate) of the serum specimens in culture medium were mixed in equal volume with the live SARS-CoV-2 virus. After gentle shaking and a contact of 30 min at room temperature in plastic microplates, 150 μl of the mix was transferred into 96-well microplates covered with Vero E6 cells (American Type Culture Collection (ATCC), CRL-1586, not authenticated but regularly tested for mycoplasma contamination). The plates were incubated at 37°C in a 5% CO_2 atmosphere. Infection efficiency was evaluated by microscopy 5 days later when the cytopathic effect of the virus control reached 100–500 TCID_{50} (median culture infectious dose) per 150 μl . Neutralization was recorded if more than 50% of the cells present in the well were preserved. The neutralizing titre was expressed as the inverse of the higher serum dilution that exhibited neutralizing activity; a threshold of 20 was used ($\text{PRNT}_{50}\text{ titre} \geq 20$). All experiments were performed in a biosafety level 3 laboratory. The different viral strains that were used were sequenced and deposited at GISAID (<https://www.gisaid.org/>) (accession numbers EPI_ISL_1707038 19A (B.38 lineage); EPI_ISL_1707039 Alpha (B.1.1.7 lineage); EPI_ISL_768828 Beta (B.1.351 lineage); EPI_ISL_1359892 Gamma (P.1 lineage); and EPI_ISL_1904989 Delta (B.1.617.2 lineage)).

Monitoring of T cell responses using whole-blood IFN γ release assay

Fresh blood collected in heparinized tubes was stimulated for 22 h at 37°C under 5% CO_2 with SARS-CoV-2 peptide pools (derived from the prototype Wuhan strain NC_045512.2) targeting RBD (46 peptides, $[\text{C}] = 8\ \mu\text{g ml}^{-1}$) (bioMérieux) diluted in IGRA solution (bioMérieux). The peptides (15-mer) encompassed the entire protein sequence and overlapped by five residues. The IGRA solution was used as a negative control and a mitogen as a positive control. The concentration of IFN γ in the supernatant was measured using a VIDAS automated platform (VIDAS IFN γ RUO, bioMérieux). The measuring range was 0.08–8 IU ml^{-1} and WB IGRA positivity thresholds were defined at $0.08\ \text{IU ml}^{-1}$. The IFN γ response was defined as detectable when the IFN γ concentration of the test was above threshold and the negative control was below threshold or when the IFN γ concentration of the test minus IFN γ concentration of the negative control was above threshold.

Monitoring of T cell responses by flow cytometry

Overnight-rested PBMCs were stimulated with SARS-CoV-2 overlapping peptide pools against SARS-CoV-2 spike protein (PepTivator, Miltenyi Biotec) at a final concentration of $1\ \mu\text{g ml}^{-1}$ for 1 h in the presence of $1\ \mu\text{g ml}^{-1}$ monoclonal antibodies CD28 and CD49d, and then for an additional 5 h with GolgiPlug and GolgiStop (BD Biosciences). Dead cells were labelled using Fixable Viability eFluor 780 dye (Thermo Fisher Scientific). Surface markers were stained using BV786-conjugated anti-CD3 (BD Biosciences, 565491; diluted 1:100), BUV486-conjugated anti-CD4 (BD Biosciences, 612937; 1:50), PE-Cy7-conjugated anti-CD8 (BioLegend, 301012; 1:100), APC-Cy7-conjugated anti-CD14 (BioLegend, 301820; 1:100), APC-Cy7-conjugated anti-CD56 (BioLegend, 362512; 1:100) and APC-Cy7-conjugated anti-CD19 (BioLegend, 302218; 1:100). Cells were then washed, fixed with Cytofix/Cytoperm (BD Biosciences) and stained with PE-conjugated anti-IFN γ (BioLegend). Negative controls without peptide stimulation were run for each sample. All results were acquired on a BD LSRFortessa (BD Biosciences) flow cytometer using the BD FACSDIVA v.8.01 software and analysed using FlowJo v.10.6.1 software.

SARS-CoV-2 pseudoparticle preparation and neutralization

SARS-CoV-2 spike-pseudotyped murine leukaemia virus retrovirus particles were produced as described for SARS-CoV³⁰. In brief, HEK293T

Article

cells (ATCC, CRL-1573, not authenticated but regularly tested for mycoplasma contamination) were transfected with constructs expressing murine leukaemia virus Gag-Pol, the green fluorescent protein (GFP) reporter and the SARS-CoV-2 spike protein (a gift from D. Lavillette (CAS Key Laboratory of Molecular Virology & Immunology, Institut Pasteur of Shanghai Chinese Academy of Sciences, Pasteur College, Soochow University, Jiangsu, China)). Control particles pseudotyped with the unrelated RD114 virus surface glycoprotein (from a cat endogenous virus) were generated as previously described³¹. For neutralization assays, a sample of approximately 1×10^3 pseudoparticles was incubated with a 100-fold dilution of sera or control antibodies for 1 h at 37 °C, spinoculated for 2 h at 2,500g before infection of Vero E6 cells. After 72 h of infection, the percentage of GFP-positive cells was determined by flow cytometry. As a control, the same procedure was performed using RD114 pseudoparticles. Anti-spike SARS-CoV-2 RBD (Sino Biological, 40150-V08B2) and anti-gp70 RD114 (ViroMed Biosafety Labs) antibodies were used with a 100-fold dilution as positive and negative control, respectively.

Generation of fluorescent SARS-CoV-2 RBD tetramers

Biotinylated recombinant RBD domain of SARS-CoV-2 RBD was purchased from Miltenyi Biotec (130-127-457) and tetramerized with either streptavidin-PE (Becton Dickinson (BD)) or with streptavidin-APC (BioLegend, 105243).

Flow cytometry analysis of SARS-CoV-2 RBD-specific B cells

Cryopreserved PBMCs were centrifuged and suspended in PEB buffer (PBS with 0.5% BSA and 2 mM EDTA) and incubated with Fc receptor block (Miltenyi, 130-059-901) for 15 min at 4 °C (dilution 1:10). Next, cells were washed in PEB and stained for 30 min in brilliant stain buffer at 4 °C in the dark using the following antibodies together with both the PE- and APC-conjugated recombinant RBD tetramers: anti-CD3-APC Fire 810 (BioLegend, 344858; diluted 1:100); anti-CD11c-BV785 (BioLegend, 301644; 1:50); anti-CD19-PE Vio770 (Miltenyi, 130-113-170; 1:100); anti-CD20-BV421 (BD, 562873; 1:100); anti-CD21-BUV496 (BD, 750614; 1:50); anti-CD27-PercP-Vio700 (Miltenyi, 130-113-632; 1:100); anti-CD38-Viobright FITC (Miltenyi, 130-113-433; 1:50); anti-IgM-PE-CF594 (BD, 562539; 1:50); anti-IgD-BV605 (BioLegend, 348232; 1:50); and anti-IgG-BV480 (BD, 746341; 1:50). Cells were washed in PEB and resuspended in a PEB dilution (1:500) of the fixable viability dye eFluor 780 (eBiosciences, 65-0865-18). They were next washed and fixed with 4% paraformaldehyde for 20 min at 4 °C in the dark before a final wash and resuspension for analysis. Cells were then acquired on a Cytek Aurora spectral flow cytometer equipped with five lasers operating at 355 nm, 405 nm, 488 nm, 561 nm and 640 nm using the SpectroFlo V.2.2.0 (Cytek) software. Data were analysed using FlowJo 10.6.1 software (BD).

Statistical analyses

Statistical modelling for epidemiological data. To model the probability of being infected knowing the subject age and the used vaccination regimen, we used the generalized linear model (glm) function in R to perform a multiple logistic regression model with the logit link function. The outcome variable was the infection status, whereby infected = 1 and not infected = 0. Our predictors were the age, as a continuous variable (considered as a possible confounder), and the vaccination regimen, whereby Chad-BNT = 0 and BNT-BNT = 1. On the basis of the model coefficients for the vaccination regimen ($B_0 = -5.306$ and $B_1 = 0.712$), we also assessed the relative risk (RR) as follows: $RR = (1 + \exp(-B_0)) / (1 + \exp(-B_0 - B_1))$.

Statistical analysis for immunological and virological analyses.

ChAd-BNT and BNT-BNT populations showed a significant difference in the average ages. To test for the consequence of the vaccination regimen on the different immune parameters, we fitted a multiple linear regression model, which allows for the correction of age (as a possible confounding factor) by including age and vaccination regimen simultaneously as predictors in the right explanatory side of the lm function in R (one model per immune parameter). Results of statistical significance displayed in Figs. 2 and 3 are from these adjusted models, for which age showed no effect. For box and whiskers plots, the upper and lower bounds of the box are the 75th and the 25th percentile (the third Q3 and first Q1 quartile), respectively. The IQR = $Q_3 - Q_1$; an observation was considered an outlier if it was above $Q_3 + 1.5 \times IQR$ or below $Q_1 - 1.5 \times IQR$. Upper and lower whiskers represent the maximum and the minimum values, respectively, without taking into account the outliers.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Viral sequences are available at GISAID (accession numbers EPI_ISL_1707038 19A (B.38 lineage); EPI_ISL_1707039 Alpha (B.1.1.7 lineage); EPI_ISL_768828 Beta (B.1.351 lineage); EPI_ISL_1359892 Gamma (P.1 lineage); and EPI_ISL_1904989 Delta (B.1.617.2 lineage). Source data are provided with this paper.

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Author contributions B.P., V.L., S. Djebali, V.B., M. Villard, L.P., M.Y.-N., S. Denolly, B.B., T.B., M. Valette, A.B. and K.B.-P. performed experiments and analysed the data. T.A. helped with the analysis of flow cytometry data and O.A. performed statistical analyses. N.G., J.-B.F. and A.M.-P. analysed the clinical infection data. The Covid-Ser study members collected the samples and performed experiments. B.L. supervised some of the experimental tasks. F.-L.C., S.P., T.D., J.M., T.W. and S.T.-A. designed the study, analysed the data and wrote the manuscript.

Competing interests K.B.-P. is a bioMérieux employee.

Additional information

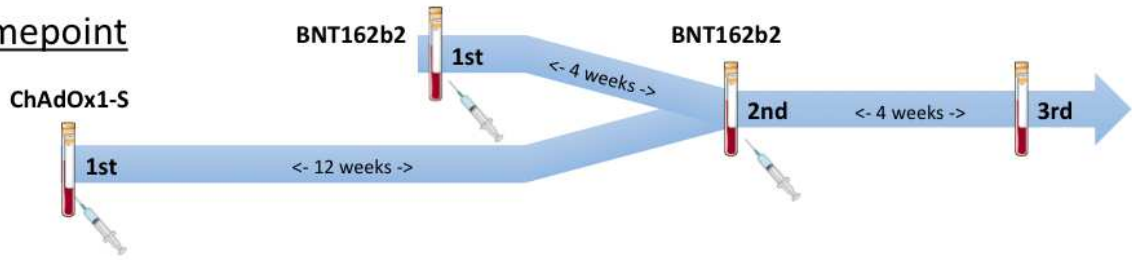
Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41586-021-04120-y>.

Correspondence and requests for materials should be addressed to François-Loïc Cosset, Stéphane Paul, Thierry DeFrance, Jacqueline Marvel, Thierry Walzer or Sophie Trouillet-Assant.

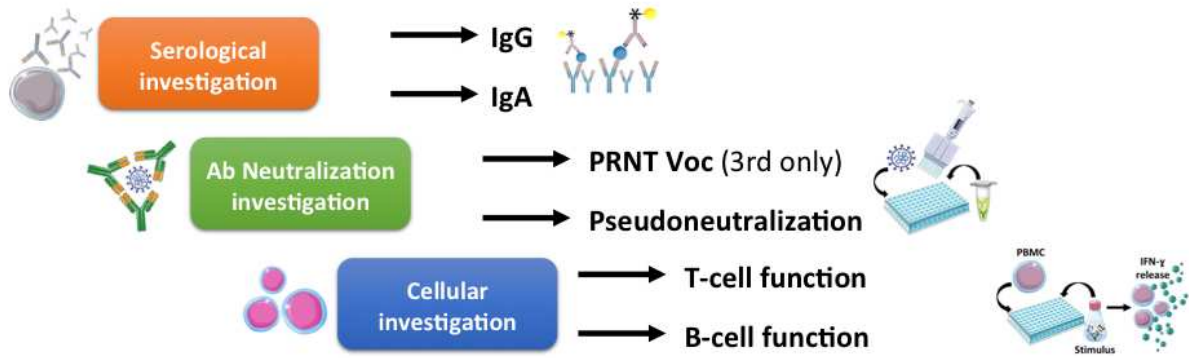
Peer review information Nature thanks Yunda Huang, Pei-Yong Shi and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Timepoint

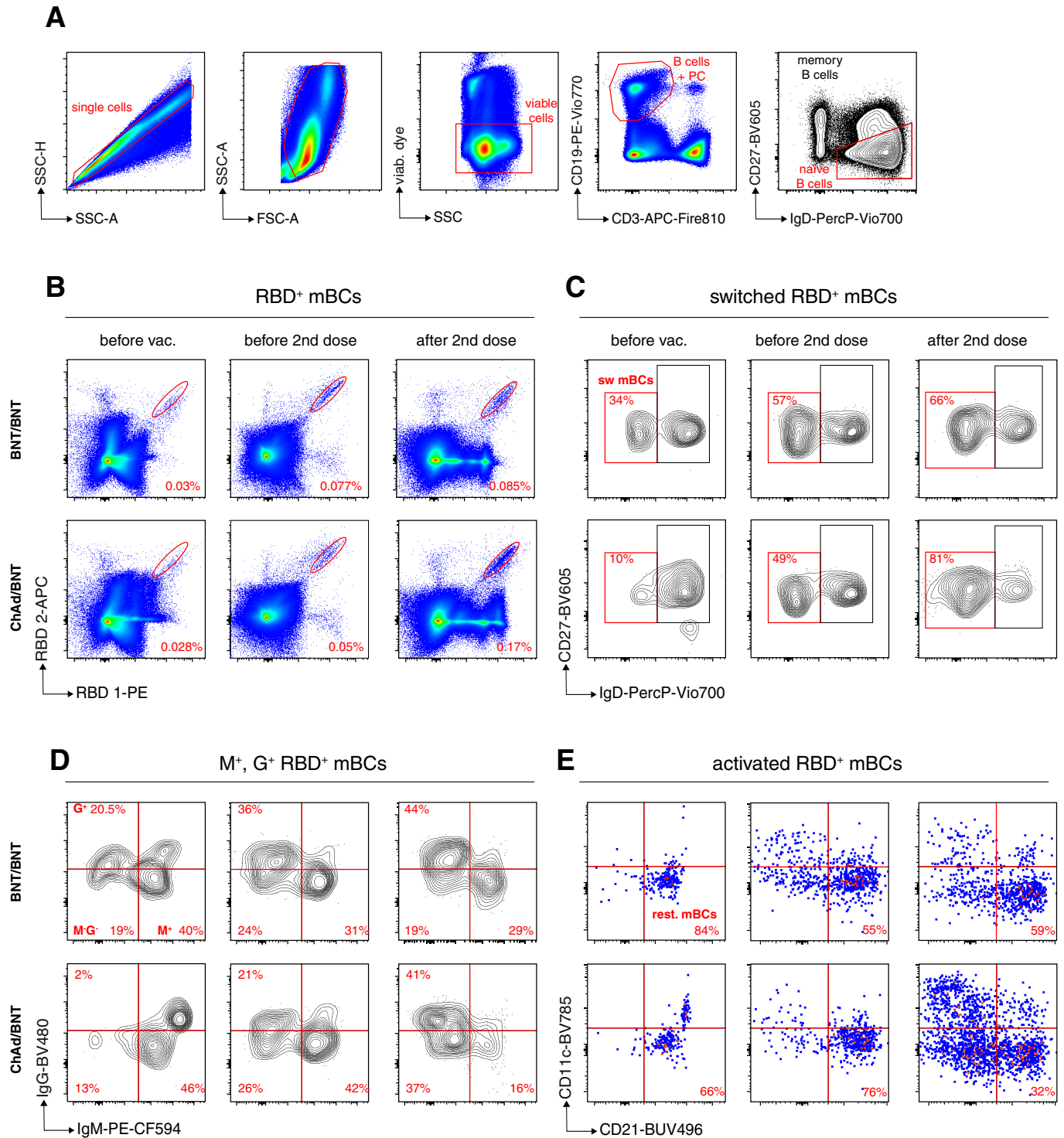


Experimental set-up



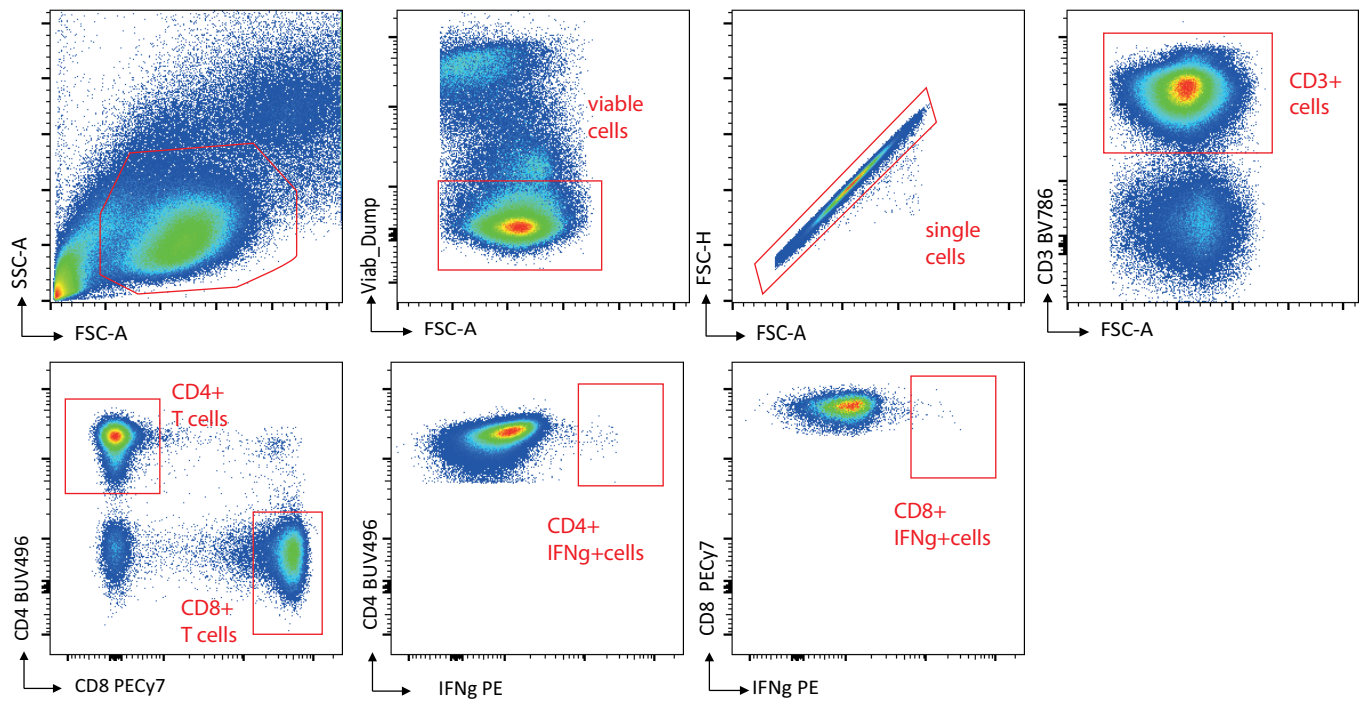
Extended Data Fig. 1 | Study design. Sixty HCWs naïve for COVID-19 and vaccinated with the Pfizer ChAd/BNT or the BNT/BNT combination were included in a prospective longitudinal cohort study. Blood sampling was performed as described, before vaccination, before the second dose of vaccine and 4 weeks after the end of vaccination schedule. Different immunological analyses were performed on the blood samples, including serological investigations (Spike-specific IgA and IgG), serum neutralization assays (both

plaque reduction neutralization test (PRNT) and pseudoneutralization), analysis of Spike-specific T cells and RBD-specific memory B cells, at various time points indicated in the text. Parts of the figure were drawn by using pictures from Servier Medical Art (<http://smart.servier.com/>), licensed under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>).



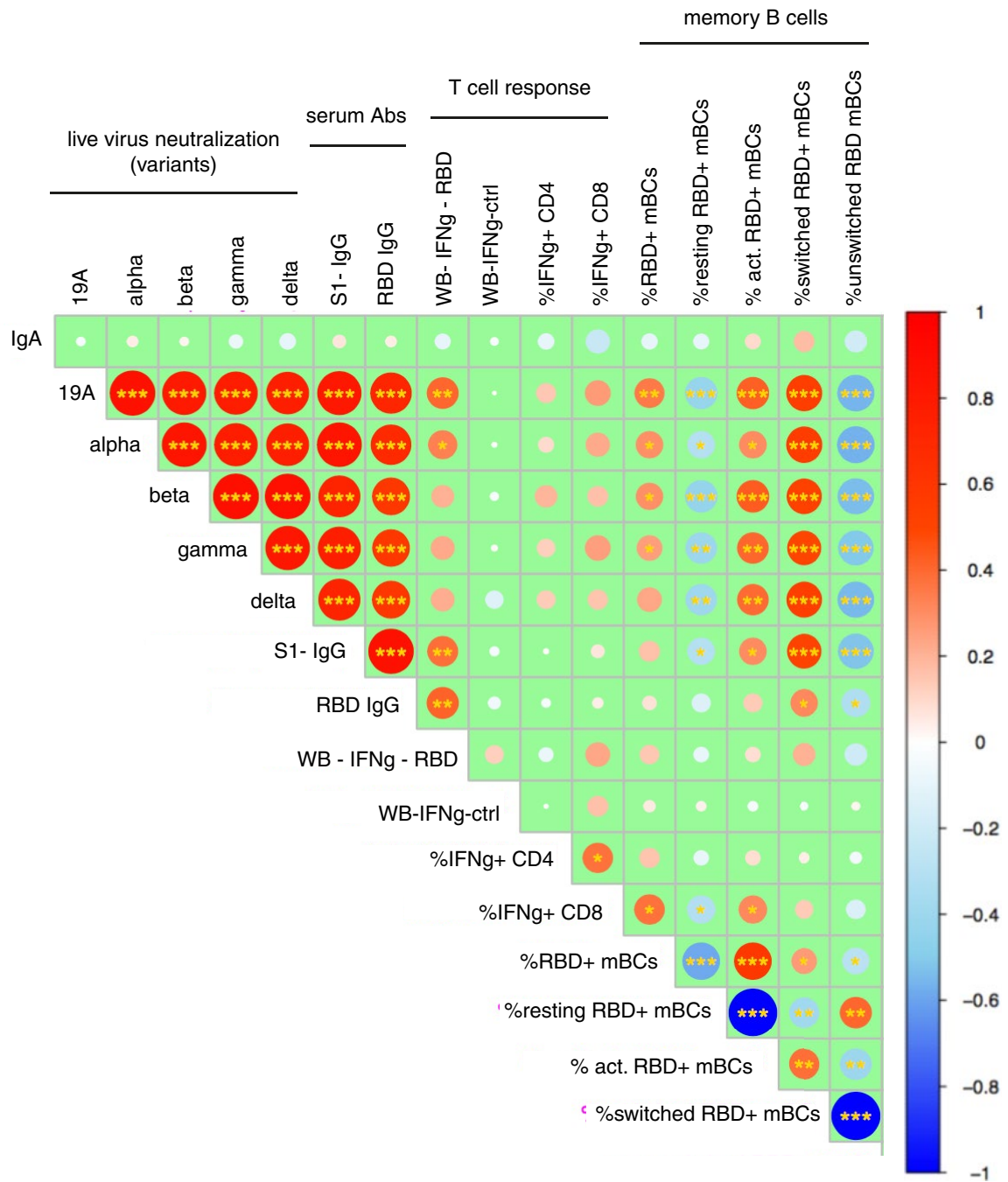
Extended Data Fig. 2 | Analysis of RBD-specific memory B cells. (A) Gating strategy. From left to right, the first three pseudo-color plots show the successive gates applied for single cells and viable cells. B cells were gated as CD19⁺/CD3⁻ cells (4th plot). Within the B cell gate, memory B cells (mBCs) were defined as non-naïve B cells on the same biparameter plot (5th plot). (B-E) Concatenated phenotypic profiles of RBD-specific mBCs for all individuals in the ChAd/BNT and BNT/BNT groups at three time points (before vaccination, before the 2nd dose, after the second dose). For each group, the phenotypic profiles correspond to the concatenation of the FACS analysis data collected

from 30 individual vaccinees. (B) Visualization of RBD-binding B cells in the mBC gate. (C) Visualization of the proportion of switched (IgD⁺) and unswitched (IgD⁻) B cells among the RBD-specific mBC pool. (D) Visualization of the pattern of Ig isotype expression by RBD-specific mBCs. (E) Visualization of the distribution of RBD-binding B cells within the resting (CD21⁺CD11c⁻) and “activated” (CD21⁻CD11c⁺/CD21⁺CD11c⁺) mBC compartments. The mean frequencies of RBD-specific mBCs falling into each quadrant are indicated in red.



Extended Data Fig. 3 | Analysis of SARS-CoV-2-specific T cells. PBMCs were stimulated as detailed in the methods and stained for CD3, CD4, CD8, CD14, CD56 and CD19 expression and with a dead cell marker. Cells were gated from left to right and top to bottom. The Viab/dump channel includes antibodies

against CD14, CD19, CD56 and a viability marker. The frequency of CD4 or CD8 T cells positive for intracellular IFNg was measured as shown in the central and right bottom panels.



Extended Data Fig. 4 | Analysis of inter-relationships between immune parameters. A matrix file with all immunological measurements for all vaccinated individuals showing the Spearman correlations and associated

p-values. The size and the color of the circles correspond to the correlation, as indicated, and the stars indicate the significance of the correlation.

Reporting Summary

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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- A description of all covariates tested
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- For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	Clinical data were recorded by a trained Clinical Research Associate using the Clinsight software (version _ Csonline 7.5.720.1). Flow Cytometry data were collected with BD FACSDIVA v8.01 or SpectroFlo V2.2.0 (Cytex)
Data analysis	Statistical analysis were performed using GraphPad Prism version 8. Flow Cytometry data were analyzed with FlowJo Software (10.6.1, FlowJo LLC, BD Life Sciences), GLM in R 4.1.0

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The data that support the findings of this study are included in the "Source data" file available online.
Viral sequences are available on GISAID (GISAID accession numbers: EPI_ISL_1707038 19A (B.38) ; EPI_ISL_1707039 Alpha (B.1.1.7) ; EPI_ISL_768828 Beta (B.1.351) ; EPI_ISL_1359892 Gamma (P.1) ; EPI_ISL_1904989 ; Delta (B.1.617.2).

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	We did not perform sample size calculations as we did not know what to expect in terms of difference for the different measurements. We tried to analyze 30 patients in each group for all immunological measurements, as, based on our experience, it was sufficient to reach good statistics for most of the measurements, given that we minimize variability by selecting patients without comorbidity with similar age range, to avoid batch effects patients from each group (ie homologous vs heterologous) were included in each experiment and the number of experiments performed to test all samples was reduced as much as technically feasible.
Data exclusions	In the T cell experiments in Figure 3G-H we had to exclude several samples in both groups that were all analyzed on the same day. Indeed, for this particular day of experiment, the thawing step was not performed properly and cells died. Because only one PBMC sample was available for each donor, we could not repeat the experiments for the corresponding individuals. No other data were excluded in this study.
Replication	For all neutralization experiments two to three replicates were performed to ensure reproducibility. For commercial Elisa kits, internal quality controls ensure reproducibility. Attempts to replicate Elisa and neutralization experiments were all successful. For flow cytometry experiments, each patient was analyzed once. Samples were run on consecutive days, and the results were consistent over the different days, ensuring the validity of the measurements.
Randomization	Participants were health care workers. The participation of the study did not modify the vaccination schedule of each participant. The intervention on participants was limited to blood sampling. They were allocated in each group on the basis of their vaccination schedule (ie homologous vs heterologous). We recruited patients after the priming step, not before (we therefore did not decide which patients would receive each vaccine). The choice of the prime vaccine was only driven by the availability (both vaccines were approved and given without preference). For immunological assays, samples were blinded and randomized.
Blinding	For all experiments in Figures 2 and 3, we used a blinded strategy to perform immunological and serological analyses: Each sample was labelled with the reference (not the name) of the patient, but not the identity of the group (ie heterologous vs homologous vaccination) and the identity was only revealed at the analysis step, after blinded data acquisition.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input type="checkbox"/>	<input checked="" type="checkbox"/> Human research participants
<input type="checkbox"/>	<input checked="" type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involved in the study
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input type="checkbox"/>	<input checked="" type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Antibodies

Antibodies used

Pseudovirus neutralization assays:
Anti-spike SARS-CoV-2 RBD (Sino Biologicals # 40150-V08B2) 1/100
anti-gp70 RD114 (ViroMed Biosafety Labs, #RD114) 1/100

Elisas
Recombinant protein Spike S1-His: SARS-CoV-2 (2019-nCoV) -Cat: 40591-V08H (Sino-biologicals) coating 1 microgr/ml
Goat Anti-human IgA (alpha chain specific)-HRP (SIGMA)--Cat: A0295-1ML dilution 1/10000
Purified form of human chimeric IgA anti-RBD of SARS-COV-2 clone IB3C4 (B Cell Design): different concentrations (standard)

Flow cytometry:

Marker	Species/targeting	Fluorochrome	Clone	Supplier	Cat number	Dilution
CD28	mouse anti-human	purified	CD28.2	BioLegend	302933	1:100
CD49d	mouse anti-human	purified	9F10	BioLegend	304339	1:100
CD3	mouse anti-human	BV786	UCHT1	BD Biosciences	565491	1:100
CD4	mouse anti-human	BUV496	SK3	BD Biosciences	612937	1:50
CD8	mouse anti-human	PECy7	RPA-T8	BioLegend	301012	1:100
CD19	mouse anti-human	APC Cy7	HIB19	BioLegend	302218	1:100
CD14	mouse anti-human	APC Cy7	M5E2	BioLegend	301820	1:100
CD56	mouse anti-human	APC Cy7	5.1H11	BioLegend	362512	1:100
IFN γ	mouse anti-human	PE	B27	BD Biosciences	554701	1:50
Fixable Viability Dye		eF780		eBioscience	65-0865-14	1:500
CD3	mouse anti-human	APCFire810	SK7	Biolegend	344858	1:100
CD11c	mouse anti-human	BV785	3.9	Biolegend	301644	1:50
CD19	mouse anti-human	PE Vio770	LT-19	Miltenyi	130-113-170	1:100
CD20	mouse anti-human	BV421	2H7	BD Biosciences	562873	1:100
CD21	mouse anti-human	BUV496	B-ly4	BD Biosciences	750614	1:50
CD27	mouse anti-human	PercP-Vio700	M-T271	Miltenyi	130-113-632	1:100
CD38	mouse anti-human	Viobright FITC	REA572	Miltenyi	130-113-433	1:50
IgD	mouse anti-human	BV605	IA6-2	Biolegend	348232	1:50
IgM	mouse anti-human	PE-CF594	G20-127	BD Biosciences	562539	1:50
IgG	mouse anti-human	BV480	G18-145	BD Biosciences	746341	1:50

Biotinylated recombinant RBD domain of SARS Cov 2 RBD was purchased from Miltenyi Biotech (# 130-127-457) and tetramerized either with streptavidin-PE (BD Biosciences, # 554061) or with streptavidin-APC (Biolegend, # 105243).

Validation

All antibodies used in this study are commercially available, and all have been validated and quality checked by the manufacturers and used in other published works (For references, refer to the supplier's websites :

<https://wwwbdbiosciences.com/en-eu>

<https://www.biolegend.com/>

<https://www.thermofisher.com/fr/fr/home/life-science/antibodies/ebioscience.html>

<https://www.miltenyibiotec.com/FR-en/>.

We also have personal experience of all these antibodies and based on this experience, we can assess the validity of the antibodies. We titrated these antibodies according to our own staining conditions of human PBMCs (usually 1-2 millions cells/100 microliters, 30 min at 4°C).

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)

Vero E6 cells (ATCC CRL-1586) and HEK293T cells (ATCC CRL-1573)

Authentication

We did not authenticate the cell lines, but such authentication is performed by the supplier (ATCC) as part of the quality control

Mycoplasma contamination

All cell lines were regularly screened and tested negative for mycoplasma using a commercial kit (Lonza MycoAlert kit) # LT07-418

Commonly misidentified lines
(See [ICLAC](#) register)

no commonly misidentified cell lines were used in the study

Human research participants

Policy information about [studies involving human research participants](#)

Population characteristics

Health care workers for COVID-19 vaccinated with Pfizer BNT162b2 and/or AstraZeneca ChadOx01ncov-19 vaccines were included in a prospective longitudinal cohort study conducted in the Hospices Civils de Lyon (Lyon, France). Demographic characteristics are depicted in Table 2.

Recruitment

For the epidemiological investigation in Figure 1 (N=13121), all subjects (health care workers) who were vaccinated with both regimens were included, without exclusion criteria. For the 60 subjects included in the immunological study, we selected 60 subjects who had not been infected by SARS-CoV-2 before vaccination, without comorbidity, and who gave their consent for the study. Written informed consent was obtained from all participants.

Ethics oversight

The use and analysis of data from the occupational health medical file was authorized after a regulatory declaration to the National Commission for Information Technology and Civil Liberties (CNIL) according to the reference methodology (declaration MR004 n° 20-121 of 30 april 2020).

Covid-ser-vac: ethics approval was obtained from the national review board for biomedical research in April 2020 (Comité de Protection des Personnes Sud Méditerranée I, Marseille, France; ID RCB 2020-A00932-37)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about [clinical studies](#)

All manuscripts should comply with the ICMJE [guidelines for publication of clinical research](#) and a completed [CONSORT checklist](#) must be included with all submissions.

Clinical trial registration	NCT04341142
Study protocol	We amended the first version of protocol (https://bmjopen.bmj.com/content/10/11/e041268) with the aim to include vaccinated HCWs _ favorable amendment obtained 15th January 2021 from the comité de Protection des Personnes Sud Méditerranée I, Marseille, France; ID RCB 2020-A00932-37).
Data collection	For analyses of infections (Figure1), Health Care Workers at the Lyon University hospital (HCL) who received each vaccination regimen were monitored for infections starting in January 2021 in both groups. Data in Figure 1 show the infections that occurred after the 14-days postboost period, up to the end of the recording (08/15/2021) For immunological analyses (Figures 2-3), clinical data were collected using the Clinsight software, during January-April 2021. . Blood samples were processed and stored at the Centre de Ressource Biologique Neurobiotec, 69500 Bron. Serological and immunological analyses were performed at the Lyon-Sud hospital or at the Centre International de recherche en infectiologie (CIRI) in Lyon.
Outcomes	For figure 1 (analyses of infections) the primary outcome was the infection status ie not infected vs infected. This was assessed by performing RT-PCR for SARS-CoV-2 upon contact with confirmed cases or upon symptoms onset. For figures 2 and 3, (covid-ser study), the primary outcome was the positivity of the SARS-Cov-2 serological test at different time points, and the secondary outcome was the serum level of IgM and IgG titers and the serum neutralization capacity.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	For T cell stimulation and staining: Cryopreserved PBMCs were thawed and rested overnight in RPMI 1640 medium supplemented with 10% FBS in 5% CO2 at 37°C. On the second day, cytokine-producing T cells were identified by intracellular cytokine staining (ICS): Briefly, the PBMCs were stimulated with individual peptides for 1 h in the presence of 1 µg/mL monoclonal antibodies against human CD28 (Biolegend) and CD49d (Biolegend) then for an additional 5h with GolgiPlug (brefeldin A, BD Biosciences), GolgiStop (monensin, BD Biosciences). Then a standard antibody staining was carried out: dead cells were first labelled with LIVE/DEAD™ Fixable eF780 dye (ThermoFisher) and then followed by surface antibody staining. Subsequently, Cytofix/Cytoperm kit (BD Biosciences) was used for permeabilizing the cells before staining the cells with antibodies against molecules expressed intracellularly. For SARS-CoV-2 RBD-specific B cells analysis : PBMC staining were clearly described in the methods section of the manuscript. Briefly, cryopreserved PBMCs were centrifuged and resuspended in PEB Buffer (PBS + 0.5% BSA and 2 mM EDTA) and incubated with Fc receptor block (Miltenyi 130-059) for 15 min at 4°C. Next, cells were washed in PEB and stained for 30 min in brilliant stain buffer at 4°C in the dark using surface antibodies with both the PE- and APC-conjugated recombinant RBD tetramers. Then, cells were washed in PEB, and resuspended in a PEB dilution of the fixable viability dye eFluor 780 (ebiosciences 65-0865-18). They were next washed and fixed in 4% paraformaldehyde (PFA) for 20 min at 4°C in the dark before a final wash and resuspension for flow cytometry analysis.
Instrument	BD LSRFortessa 5L (T cell) or Cytek Aurora spectral flow cytometer 5L (B cell)
Software	Flow cytometry data were collected with BD FACSDIVA v8.01 or SpectroFlo V2.2.0 (Cytek) and analyzed with FlowJo software version 10.7.1 (FlowJo LLC)
Cell population abundance	Bulk PBMC were used. No cell sorting was performed.
Gating strategy	Peptide-specific T cell subsets were identified via the following gating strategy: Viable lymphocytes were addressed by successive gating in SSC-A/FSC-A plot followed by excluding DUMP positive cells (dead cells, CD14, CD19, CD56) in a FSC-A/DUMP plot. Then, singlets were gated in a FSC-A/FSC-H plot and CD3+ T cells were gated in CD3/FSC-A plot. From CD3+ T cells, CD4+ and CD8+ T cells were gated in CD4/CD8 plots. Next, peptide-specific CD4 T cells were gated by plotting CD4/IFNγ and peptide-specific CD8 T cells were gated by plotting CD8/IFNγ. Gating strategy for RBD-specific B cell analysis is provided in the supplementary information file and cell population are defined. Briefly, successive gating were applied for single cells and viable cells. Then, B cells were gated as CD19+/CD3- cells.

Within the B cell gate, memory B cells (mBCs) were defined as non-naïve/non transitional B cells on the same biparameter CD27/IgD plot. Then, PE- (RBD#1) and APC- conjugated (RBD #2) SARSCoV2 RBD probes in B cell gate and memory B cell gate were gated in RBD#1/ RBD#2 plot. Unswitched (IgD+CD27+), and switched (csM, IgD-CD27+) RBD-specific memory B cells were defined. Next, IgG+ and IgG-/IgM switched RBD-binding memory B cells were defined based on the IgM/IgG plot.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Discussion of Article 4

Here we reported that heterologous vaccination induces a better immune response against SARS-CoV2 in terms of neutralizing antibody titers, and more switched memory B cells irrespective of the variant analyzed at that moment. Other studies published later have also corroborated our results and they included recent variants like Omicron (242–244). Even though heterologous vaccination is better, neutralizing activities against Omicron subvariants are negligible, and breakthrough infections after 3 months are high independent of the vaccination schedule used (244). It has been shown that Fc effector functions are different between patients who had a breakthrough infection and patients who had not, after homologous vaccination with ChAdOx1 vaccine, and this difference was correlated with a different antibody glycosylation pattern (245). We also wanted to study in our *in vitro* model the Fc-effector activation in our cohort of vaccinees. We show that IgA from heterologous vaccinated individuals is better activating FcR than IgA from homologous vaccinated individuals (**Fig. 18**) (unpublished data).

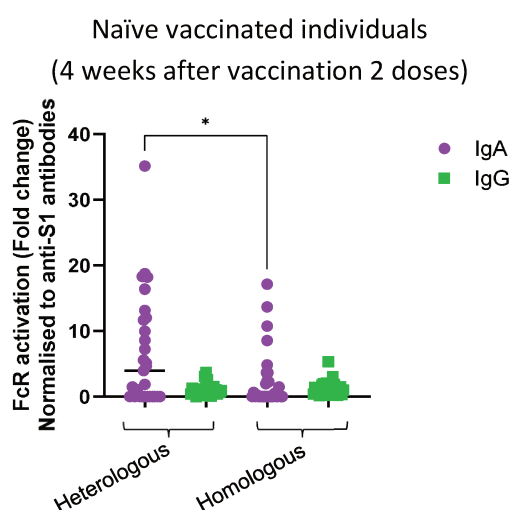


Figure 18. IgG or IgA dependent FcR activation from vaccinated individuals with a homologous or heterologous scheme.

Fold change was calculated with a baseline from a pre-pandemic pool of samples. Results were normalized by the anti-S1 IgG or IgA concentrations in each sample. Non-parametric Mann-Whitney test for unpaired comparisons (between vaccination groups) was performed using GraphPadPrism software (v. 9.4.1). Significance $p < 0.05$.

We found no differences in the IgG-dependent FcR-activation between both groups of vaccinees. This suggests that IgA are more functional in the heterologous scheme.

It would be interesting to make a follow up study with similar vaccinees to see the incidence of breakthrough infections and the correlation with Fc-effector functions. Whether some vaccinees are predisposed to generate particular antibody functional profiles more effectively or whether some vaccinees simply failed to generate anti-inflammatory FcR-binding antibodies remains unclear.

We also showed that memory B Cells (mBCs) still exhibit features of activation 4 weeks after the booster dose in the heterologous regimen, which suggests that the post-boost process of mBCs differentiation is more active in this schedule. In line with our results, later studies have reported that ChAdOx1:mRNA-1273 heterologous immunization boost the prime immunofocused responses onto epitopes expressed on prefusion-stabilized S and displayed overall higher binding affinities and increased breadth of reactivity against VOCs (242).

We further wanted to study breakthrough infections in vaccinated individuals and the long-term humoral immune response, especially in the context of Delta and Omicron variants.

Article 5: Breakthrough infections in COVID-19
(Brief communication published in NPJ Vaccines in April 2023)

BRIEF COMMUNICATION OPEN



Long-term humoral response following Delta and Omicron BA.1 co-infection

Carla Saade ¹, Bruno Pozzetto ^{1,2}, Melyssa Yaugel-Novoa ¹, Guy Oriol ³, Laurence Josset ^{1,4,5}, Bruno Lina ^{1,4}, Stéphane Paul ^{1,2}, Antonin Bal ^{1,4,5} and Sophie Trouillet-Assant ^{1,3}✉

This study reports the 6-month humoral immune response in vaccinated patients concomitantly infected with Delta and Omicron BA.1 variants of SARS-CoV-2. Interestingly, the simultaneous exposure to the Delta and BA.1 S proteins does not confer an additional immune advantage compared to exposure to the BA.1 S protein alone.

npj Vaccines (2023)8:57; <https://doi.org/10.1038/s41541-023-00652-8>

Bivalent vaccines containing two different sequences of the Spike (S) protein have been recently approved. First studies evaluating the short-term immune response after the administration of bivalent vaccines have reported contradictory results^{1–5}. Moreover, the long-term immunity outcomes of this bivalent vaccine need further investigation, especially given the waning of the humoral response. Others and we have reported cases of simultaneous infection by the Delta and BA.1 variants, both having caused major COVID-19 epidemic waves worldwide^{6,7}. The present report describes the long-term humoral response of patients infected at the same time with the Delta and BA.1 variants. The main goal of the study was to compare breakthrough infections with only one or two variants simultaneously.

We included two groups of vaccinated individuals with a breakthrough infection (BA.1 in the first group [$n = 9$] and Delta and BA.1 co-infection in the second group [$n = 9$, Supplementary Flow Chart]). Co-infection refers to the simultaneous detection of genomes belonging to two different SARS-CoV-2 variants as determined by sequencing⁶. All infected individuals experienced a mild form of COVID-19. We included 9 COVID-19-naïve individuals who received three doses of the BNT162b2 vaccine as a control group (Supplementary Table 1 for demographic data and Supplementary Table 2 for individual immunization/infection history). All individuals received the BNT162b2 vaccine to minimize differences between vaccination schemes. Blood sampling for humoral immunity investigation was performed 6 months after breakthrough infection for infected individuals or 6 months after the third vaccine injection for COVID-19-naïve individuals to take into account waning of the humoral immune response (Supplementary Table 1). Details regarding the interval between last immunization (infection for individuals with a breakthrough infection and vaccination for fully vaccinated individuals) and blood sampling for each participant, as well as immunization scheme are provided in Supplementary Table 2.

There was no significant difference in anti-receptor binding domain (RBD) IgG and anti-S1 IgA levels among individuals with a breakthrough infection caused by Delta and BA.1 or by BA.1 only; however, these levels were 4.13 and 10.83 -fold lower,

respectively, among COVID-19-naïve individuals than in those with hybrid immunity (Fig. 1a, b).

Neutralizing antibody titers were significantly lower among COVID-19-naïve individuals for all viral variants compared to BA.1-infected and Delta-BA.1-co-infected individuals. Compared to COVID-19-naïve individuals, the median neutralizing antibody titer of BA.1-infected individuals was 8-fold higher for the 19A isolate and 12-fold higher for the BA.5 isolate, and it was 8-fold higher for the 19A isolate and 16-fold higher for the BA.5 isolate in Delta-BA.1 co-infected individuals. Interestingly, no significant difference was observed between the two groups of individuals with a breakthrough infection (Fig. 1c).

In addition, we calculated the geometric mean titer (GMT) ratio between the three groups for all the tested parameters (Supplementary Table 3). These ratios were similar between the Delta-BA.1 co-infected and BA.1 infected individuals, but a marked difference was seen when compared to the COVID-19-naïve fully vaccinated individuals (ratios ranged from 4 to 20). Moreover, a multiple linear regression analysis was performed with two adjustment variables, i.e., interval between last immunization and blood sampling for humoral investigation, and age at blood sampling. This analysis found that these variables did not influence the humoral response (Supplementary Data).

Results reported herein show an enhanced humoral response after breakthrough infections caused by one or two variants. These results confirm the advantage conferred by so-called hybrid immunity that has already been shown to induce a more potent long-term humoral immune response against SARS-CoV-2 in comparison to that induced by vaccination only⁸. In addition, the absence of significant difference in anti-S total antibodies and neutralization capacity between individuals with a single or a dual breakthrough infection would suggest that a simultaneous exposure to the Delta and Omicron BA.1 variants does not confer an additional immune advantage in terms of humoral immunity.

The present study does, however, have certain limitations. The most evident is the small size of the effective in each group due to the rarity of simultaneous infection with two different SARS-CoV-2 variants; another limitation is that comorbidities were more

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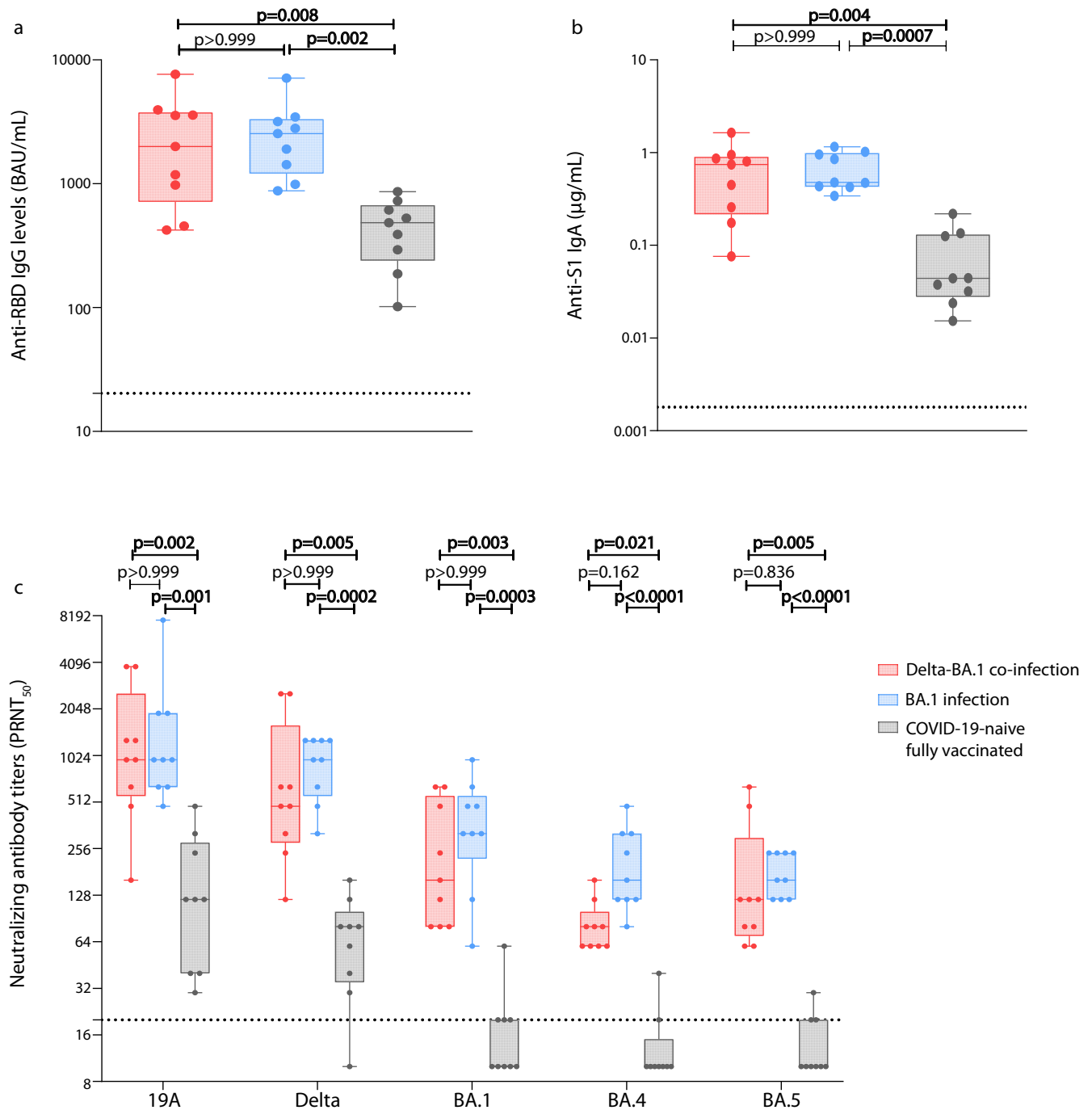


Fig. 1 Humoral immune response 6 months after last immunization in individuals vaccinated and then co-infected with Delta and BA.1, or vaccinated and then infected with BA.1, or in COVID-19-naïve fully-vaccinated individuals. Blood samples were obtained 6 months after infection for the first two groups or 6 months after the third injection for the third group. Anti-RBD IgG levels were measured using the commercially available bioMérieux Vidas SARS-CoV-2 IgG diagnosis kit according to manufacturer's recommendations and expressed in binding antibody unit (BAU)/mL. The dotted line represents the threshold of positivity (≥ 20.33 BAU/mL; **a**). Anti-S1 IgA levels were measured using an ELISA test and expressed in $\mu\text{g/mL}$. The dotted line represents the positivity threshold (≥ 0.0018 $\mu\text{g/mL}$; **b**). Neutralizing antibody titers against live SARS-CoV-2 isolates using a 50% plaque reduction neutralization test (PRNT_{50}). The isolates used for these experiments were 19A, Delta, Omicron BA.1, BA.4, and BA.5; their GISAID accession numbers are EPI_ISL_1707038, EPI_ISL_1904989, EPI_ISL_7608613, EPI_ISL_12396843, and EPI_ISL_12852091, respectively. The dotted line represents the positivity threshold (titer ≥ 20 ; **c**). Data are represented as box and whiskers plot; in each plot, the dots indicate individual samples, the upper and lower limits of the box plot represent the interquartile range [IQR] and the middle line represents the median. Whiskers represent the maximum and minimum value in each plot. All box and whiskers plots represent $n = 9$ biologically independent samples. A Kruskal–Wallis test followed by Dunn's multiple comparison tests were performed to assess differences between the three groups.

frequent in the COVID-19-naïve vaccinated group, even if these exhibited only one comorbidity each (Supplementary Table 1).

In conclusion, the results reported herein suggest the possibility of an immune imprinting in the context of anti-SARS-CoV-2 humoral response, also known as original antigenic sin. This phenomenon is in reference to a limited immune response against a new antigen variant after an exposure to the initial one^{2,3}. However, extrapolations of these findings to bivalent vaccines should be made with caution since a spontaneous infection is not equivalent to vaccination in terms of route of exposure as well as antigen load and diversity. It is also of note that contradictory results have been reported in terms of the additional protection provided by these new vaccines²⁻⁵; long-term studies using bivalent vaccines, which are now the standard of care, will give better insight into their effectiveness.

METHODS

Study design and ethics

We included two groups of vaccinated individuals with a breakthrough infection (BA.1 in the first group [$n=9$] and Delta and BA.1 co-infection in the second group [$n=9$, Supplementary Flow Chart]). We included 9 COVID-19-naïve individuals who received three doses of the BNT162b2 vaccine as a control group. Blood sampling for humoral immunity investigation was performed 6 months after breakthrough infection for infected individuals or 6 months after the third vaccine injection for COVID-19-naïve individuals. Participants were included from two clinical studies registered on ClinicalTrials.gov (NCT05060939, NCT04341142). Written informed consent was obtained from all participants and approval was obtained from the regional review board in April 2020 (*Comité de Protection des Personnes Sud Méditerranée I*, Marseille, France; ID-RCB 2020-A00932-37; ID-RCB 2021-A01877-34).

Laboratory methods

VIDAS® SARS-COV-2 IgG II (9COG, ref 424114) is an automated semi-quantitative assay for use on the VIDAS® family of instruments, for the detection of immunoglobulin G (IgG) specific for the SARS-CoV-2 receptor-binding domain (RBD) of the spike protein in human serum or plasma (lithium heparin) using the Enzyme Linked Fluorescent Assay (ELFA) technique. The VIDAS® SARS-COV-2 IgG II (9COG) is a commercialized validated assay with a CE marking. The test was carried out according to the protocol recommended by the manufacturer and expressed in binding antibody units (BAU)/mL.

Regarding the IgA ELISA assay, the test was validated with negative and positive samples as well as a calibration range with a recombinant IgA. In brief, high binding 96-half-well plates (#2310 M; NUNC) were coated with 100 μ L per well of a spike protein solution (1 μ g/mL; #40591-V08H Spike S1-RBD Sino Biologicals) in PBS overnight at 4 °C. Plates were washed with washing buffer containing 1X PBS with 0.05% Tween 20 (Sigma-Aldrich) and incubated with 170 μ L of blocking buffer per well containing 1X PBS with 3% fat milk powder and 0.05% Tween 20 (Sigma-Aldrich) for 1 h at room temperature. Immediately after blocking, recombinant anti-RBD IgA (B Cell Design #IB3C4 PV) or serum samples diluted in PBS were added and incubated for 1 h at 37 °C. Plasma samples were assayed at a 1:100 starting dilution and seven additional threefold serial dilutions. Recombinant human anti-RBD IgA was used to perform a calibration curve starting at 1.5 μ g/mL. Plates were washed and then incubated with anti-human IgA (A0295; Sigma-Aldrich) secondary Ab conjugated to horseradish peroxidase (HRP) in blocking buffer at 1:10000. Plates were developed by addition of the HRP substrate, 3,3',5,5'-tetramethylbenzidine (TMB; 34021; Thermo Fisher Scientific), for 10 min, and then the developing reaction was stopped by

adding 50 μ L of 1 M HCl. Optical density units were measured at 450 nm in a microplate reader (TECAN). For serum samples, a positive control (serum pool from critical COVID patients, diluted 200-fold in PBS) and a negative control (pool of historical serum samples) were added in duplicate to each run. After deduction of the background, a relative content in IgA Equivalent (ng/ml Eq) was calculated using the calibration curve. The limit of detection of the assay was 0.1 ng/mL Eq. All serum samples were tested as duplicates.

A live virus neutralization test measuring neutralizing antibodies titers against 19A, Delta and Omicron BA.1, BA.4, and BA.5 isolates and their GISAID accession numbers are EPI_ISL_1707038, EPI_ISL_1904989, EPI_ISL_7608613, EPI_ISL_12396843, and EPI_ISL_12852091 respectively. Viral variants used for these experiments were cultured on Vero-E6 cells. Each serum tested was diluted 1:10 and serial twofold dilutions were mixed with an equal volume (100 μ L) of virus. After gentle shaking and an incubation for 30 min at room temperature, 150 μ L of each mixture was transferred to 96-well microplates covered with Vero-E6 cells. Then, the plates were incubated at 37 °C in an atmosphere containing 5% CO₂. Measurements were obtained microscopically 5–6 days later when the cytopathic effect of the virus control reached ~100 50% tissue culture infectious dose (TCID₅₀)/150 μ L. The serum was considered to have protected the cells if >50% of the cell layer was preserved. The neutralizing titer was expressed as the inverse of the higher serum dilution that protected the cells. All serum samples were tested as duplicates.

Statistical analyses

To assess the differences in anti-RBD IgG levels, anti-S1 IgA and neutralizing antibody titers between the three groups, a Kruskal–Wallis test followed by Dunn's multiple comparison tests were performed. A multiple linear regression was performed to assess the impact of two adjustment variables, (i) delay between last immunization and blood sampling and (ii) age at blood sampling, on the humoral response described. Statistical analyses were performed using the GraphPad Prism® software (version 8; GraphPad software, La Jolla, CA, USA) and R software, version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. GISAID accession numbers for the 19A, Delta, BA.1, BA.4, and BA.5 strains used are EPI_ISL_1707038, EPI_ISL_1904989, EPI_ISL_7608613, EPI_ISL_12396843, and EPI_ISL_12852091, respectively.

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AUTHOR CONTRIBUTIONS

S.T.A. and A.B. conceived the study. C.S., B.P., S.P., and M.Y.N. performed and interpreted experiments. B.L., A.B., and L.J. performed metagenomics analysis to identify viral strains. G.O. analyzed and interpreted data. C.S. wrote the initial draft, performed statistical analysis and generated figures. All authors read, edited and approved the final manuscript.

COMPETING INTERESTS

G.O. is a bioM erieux employee. The other authors report no potential conflict of interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41541-023-00652-8>.

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Supplementary material

Supplementary Table 1: Demographic and clinical characteristics of the 27 healthcare workers included in the study.

	Delta-BA.1 co-infection (n=9)	BA.1 Infection (n=9)	COVID-19-naïve individuals (n=9)
Female, n (%)	3 (33)	6 (66)	6 (66)
Age at blood sampling, years, median [IQR]	32 [23-40]	49 [29-54]	51 [48-64]
Interval between last immunization ¹ and blood sampling for humoral immunity investigation, days, median [IQR]	203 [197-212]	193 [208-166]	179 [178-183]
Comorbidities, n (%)	1 (11)	1 (11)	6 (66)
- hypertension	0	0	1 (11)
- diabetes	0	0	1 (11)
- hypothyroidism	0	0	1 (11)
- vascular disease	0	1 (11)	0
- chronic lung disease	1 (11)	0	2 (22)
- heterozygous sickle cell disease	0	0	1 (11)
Vaccination scheme, n (%)			
- 3 injections of BNT162b2 ²	2 (22)	3 (33)	9 (100)
- 2 injections of BNT162b2	6 (66)	6 (66)	0
- 1 injection of BNT162b2	1 (11)	0	0
Previous infection, n (%)	2 (22)	2 (22)	0

IQR: interquartile range.

¹ Last immunization corresponds to the infection for both groups with a breakthrough infection and the third vaccine injection for the COVID-19-naïve fully vaccinated group.

² BNT162b2 corresponds to the Pfizer-BioNTech monovalent SARS-CoV-2 vaccine.

Supplementary Table 2. Individual characteristics of the 27 healthcare workers included into the study.

Subject	Last immunization ¹	Age at blood sampling, years	Sex	Immunization scheme	Date of first vaccine injection	Date of second vaccine injection	Date of third vaccine injection	Date of previous infection	Date of breakthrough infection diagnosis	Date of blood sampling for humoral investigation	Delay last immunization ¹ - blood sampling for humoral investigation, days
Delta-BA.1 co-infection (n=9)											
Subject-1	Simultaneous Delta and BA.1 breakthrough infection	26	Male	2 injections of BNT162b2	07/27/2021	08/18/2021	-	-	12/20/2021	07/23/2022	215
Subject-2	Simultaneous Delta and BA.1 breakthrough infection	20	Male	2 injections of BNT162b2	06/2021	07/28/2021	-	-	12/27/2021	07/22/2022	207
Subject-3	Simultaneous Delta and BA.1 breakthrough infection	43	Female	3 injections of BNT162b2	03/2021	04/08/2021	12/01/2021	-	12/27/2021	07/25/2022	210
Subject-4	Simultaneous Delta and BA.1 breakthrough infection	49	Male	3 injections of BNT162b2	06/2021	07/12/2021	12/12/2021	-	12/27/2021	08/01/2022	217
Subject-5	Simultaneous Delta and BA.1 breakthrough infection	38	Male	2 injections of BNT162b2	08/18/2021	09/2021	-	-	12/31/2021	07/21/2022	202
Subject-6	Simultaneous Delta and BA.1 breakthrough infection	18	Female	2 injections of BNT162b2	09/2021	10/07/2021	-	-	01/03/2022	07/25/2022	203
Subject-7	Simultaneous Delta and BA.1 breakthrough infection	26	Female	2 injections of BNT162b2 and previous breakthrough infection	06/2021	07/2021	-	12/16/2021	01/04/2022	07/21/2022	198
Subject-8	Simultaneous Delta and BA.1 breakthrough infection	35	Male	2 injections of BNT162b2	07/2021	07/22/2021	-	-	01/17/2022	07/23/2022	187
Subject-9	Simultaneous Delta and BA.1 breakthrough infection	32	Male	Previous infection followed by 1 injection of BNT162b2	07/07/2021	-	-	11/2020	01/18/2022	08/03/2022	197
BA.1 infection (n=9)											
Subject-1	BA.1 breakthrough infection	58	Male	3 injections of BNT162b2	09/01/2020	02/01/2021	10/14/2021	-	01/16/2022	06/21/2022	156
Subject-2	BA.1 breakthrough infection	26	Male	2 injections of BNT162b2	01/16/2021	02/25/2021	-	-	12/18/2021	07/21/2022	215
Subject-3	BA.1 breakthrough infection	33	Male	2 injections of BNT162b2	12/01/2020	02/08/2021	-	-	12/19/2021	06/30/2022	193
Subject-4	BA.1 breakthrough infection	49	Female	2 injections of BNT162b2 and previous breakthrough infection	06/01/2021	07/01/2021	-	10/01/2021	01/03/2022	06/23/2022	171
Subject-5	BA.1 breakthrough infection	25	Female	3 injections of BNT162b2	01/22/2021	02/22/2021	11/24/2021	-	01/24/2022	08/04/2022	192
Subject-6	BA.1 breakthrough infection	54	Female	3 injections of BNT162b2	06/01/2021	07/01/2021	10/01/2021	-	01/18/2022	08/22/2022	216
Subject-7	BA.1 breakthrough infection	54	Female	2 injections of BNT162b2	09/15/2021	10/15/2021	-	-	01/16/2022	07/28/2022	193
Subject-8	BA.1 breakthrough infection	51	Female	2 injections of BNT162b2	07/16/2021	08/17/2021	-	-	01/16/2022	08/05/2022	201

Subject-9	BA.1 breakthrough infection	35	Female	Previous infection followed by 2 injections of BNT162b2	04/01/2021	11/01/2021	-	03/01/2020	01/23/2022	07/04/2022	162
COVID-19-naïve individuals (n=9)											
Subject-1	Third injection of BNT162b2	49	Female	3 injections of BNT162b2	01/20/2021	02/26/2021	10/29/2021	-	-	04/26/2022	179
Subject-2	Third injection of BNT162b2	51	Female	3 injections of BNT162b2	01/20/2021	02/22/2021	11/29/2021	-	-	05/30/2022	182
Subject-3	Third injection of BNT162b2	77	Male	3 injections of BNT162b2	01/20/2021	02/17/2021	11/25/2021	-	-	05/23/2022	179
Subject-4	Third injection of BNT162b2	67	Female	3 injections of BNT162b2	01/21/2021	02/18/2021	09/14/2021	-	-	03/21/2022	188
Subject-5	Third injection of BNT162b2	58	Female	3 injections of BNT162b2	01/21/2021	02/19/2021	11/19/2021	-	-	05/17/2022	179
Subject-6	Third injection of BNT162b2	62	Female	3 injections of BNT162b2	01/22/2021	02/26/2021	11/19/2021	-	-	05/16/2022	178
Subject-7	Third injection of BNT162b2	50	Male	3 injections of BNT162b2	01/25/2021	02/22/2021	11/19/2021	-	-	05/12/2022	174
Subject-8	Third injection of BNT162b2	48	Female	3 injections of BNT162b2	02/01/2021	02/22/2021	10/22/2021	-	-	04/21/2022	181
Subject-9	Third injection of BNT162b2	38	Male	3 injections of BNT162b2	01/26/2021	02/16/2021	11/27/2021	-	-	05/31/2022	185

¹ Last immunization corresponds to the infection for both groups with a breakthrough infection and the third vaccine injection for the COVID-19-naïve fully vaccinated group.

Supplementary Table 3. Results of the serological tests in the three groups of participants.

Group	Test	GMT ¹ (95% CI) ²	GMT ratio		
			1 vs 2	1 vs 3	2 vs 3
Anti-RBD IgG (BAU/mL)					
1	Delta-BA.1 co-infected	1771 (813-3855)			
2	BA.1 infected	2220 (1336-3689)	0.80	4.53	5.68
3	COVID-19-naive fully vaccinated	391 (230-664)			
Anti-S1 IgA (µg/mL)					
1	Delta-BA.1 co-infected	0.47 (0.22-1.00)			
2	BA.1 infected	0.62 (0.44-0.88)	0.77	9.00	11.76
3	COVID-19-naive fully vaccinated	0.05 (0.03-0.10)			
Neutralizing antibodies (titer)					
19A					
1	Delta-BA.1 co-infected	1010 (472-2162)			
2	BA.1 infected	1194 (625-2280)	0.85	8.92	10.55
3	COVID-19-naive fully vaccinated	113 (54-240)			
Delta					
1	Delta-BA.1 co-infected	563 (260-1221)			
2	BA.1 infected	855 (579-1262)	0.66	9.76	14.81
3	COVID-19-naive fully vaccinated	58 (30-109)			
BA.1					
1	Delta-BA.1 co-infected	198 (100-392)			
2	BA.1 infected	318 (166-612)	0.62	12.87	20.70
3	COVID-19-naive fully vaccinated	15 (10-25)			
BA.4					
1	Delta-BA.1 co-infected	80 (61-104)			
2	BA.1 infected	186 (117-294)	0.43	6.31	14.73
3	COVID-19-naive fully vaccinated	13 (9-18)			
BA.5					
1	Delta-BA.1 co-infected	132 (68-256)			
2	BA.1 infected	174 (136-223)	0.76	10.02	13.21
3	COVID-19-naive fully vaccinated	13 (9-18)			

¹ GMT: Geometric mean titer

² 95% CI: 95% confidence interval

NB: GMT results were rounded to the unit for the first and the third parameters tested and to two digits after unit for the second one. GMT ratios were rounded to two digits after the unit for all parameters.

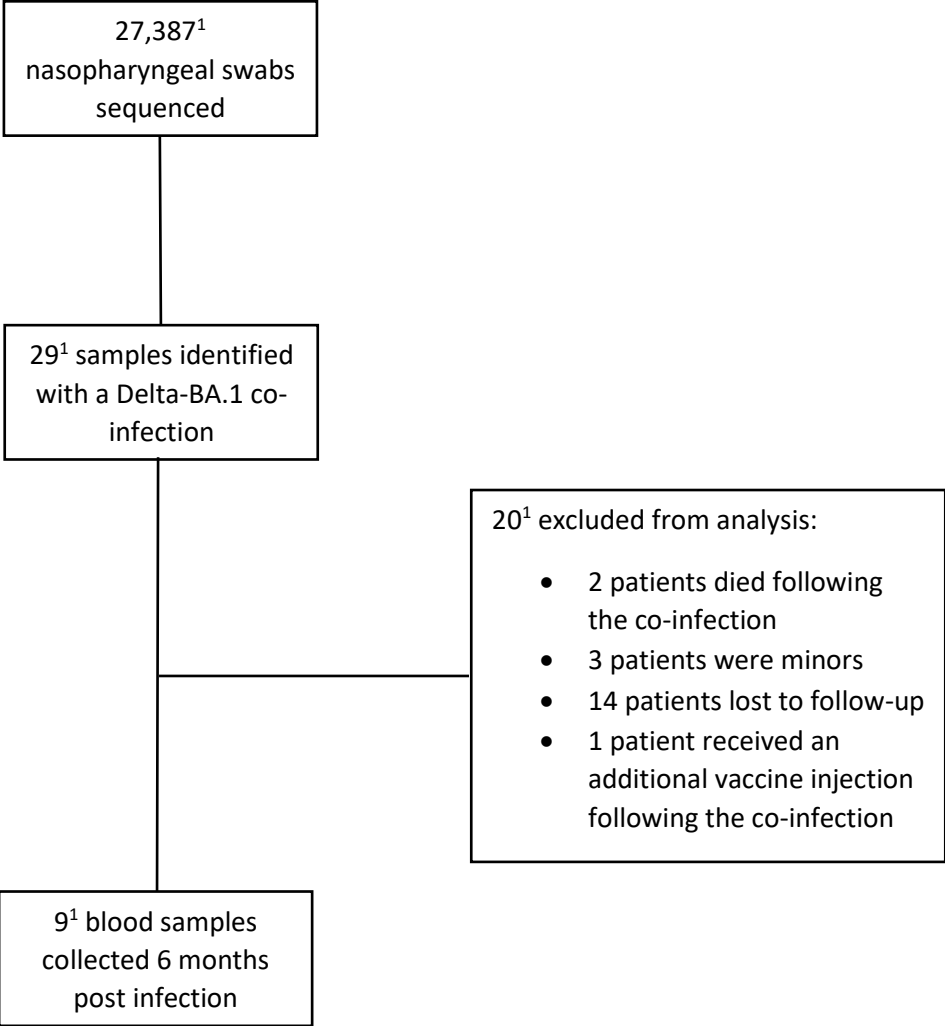
Supplementary Data:

Since demographical parameters were not similar between the three groups we performed a multiple linear regression to assess the impact of the two adjustment variables, i) delay between last immunization and blood sampling and ii) age at blood sampling, on the humoral response described. This analysis highlights that these variables could not solely explain the lack of significant difference observed in the humoral response between individuals with a Delta-BA.1 co-infection and those with a BA.1 infection or the significant difference observed between both of these groups with the COVID-19-naïve fully vaccinated group.

	Anti-RBD IgG levels (BAU/mL)	Anti-S1 IgA (µg/mL)	PRNT₅₀-19A	PRNT₅₀-Delta	PRNT₅₀-BA.1	PRNT₅₀-BA.4	PRNT₅₀-BA.5
Delay between last immunization¹ and blood sampling for humoral investigation	p-value= 0.4159	p-value= 0.4459	p-value=0.0412	p-value=0.9511	p-value=0.7228	p-value=0.3340	p-value=0.8880
Age at blood sampling	p-value=0.6509	p-value=0.8831	p-value=0.3221	p-value=0.9040	p-value=0.9775	p-value=0.9641	p-value=0.9028

¹ Last immunization corresponds to the infection for both groups with a breakthrough infection and the third vaccine injection for the COVID-19-naïve fully vaccinated group.

Supplementary Flow Chart: Flow chart detailing the inclusion process of the 9 Delta-BA.1 co-infected individuals.



¹ Patients were first included in the *Bal A et al., Nat Commun, 2022⁶*, study where researchers identified 29 cases of Delta-BA.1 co-infections

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The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. GISAID accession numbers for the 19A, Delta, BA.1, BA.4 and BA.5 strains used are EPI_ISL_1707038, EPI_ISL_1904989, EPI_ISL_7608613, EPI_ISL_12396843, and EPI_ISL_12852091, respectively.

Human research participants

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Reporting on sex and gender	Sex information of participants has been collected and presented in supplementary table S2
Population characteristics	Population characteristics such as sex, age distribution and comorbidities of the study participants are presented in table S2.
Recruitment	Participants were included 6 months following the diagnosis with a breakthrough infection caused by either Delta and BA.1 simultaneously or BA.1 only and after informed consent was obtained. As for vaccinated individuals, they were included 6 months after their third vaccine injection and after informed consent was obtained.
Ethics oversight	Approval was obtained from the regional review board in April 2020 (Comité de Protection des Personnes Sud Méditerranée I, Marseille, France; ID-RCB 2020-A00932-37; ID-RCB 2021-A01877-34).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

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Sample size	We did not perform sample size calculations as we were only able to include 9 individuals co-infected with Delta and BA.1 due to the rarity of such infections. We included 9 individuals infected with only BA.1 and 9 vaccinated with three doses of the BNT162b2 vaccine in order to be homogeneous among the three groups
Data exclusions	No data were excluded from the analyses
Replication	For the measurement of anti-RBD IgG levels, samples were tested as triplicate since we used validated commercial ELFA kits and internal quality controls ensure reproducibility. As for the anti-S1 IgA measurement assay and the neutralizing antibody assay, samples were tested in duplicates
Randomization	They were allocated in each group on the basis of their immunization scheme (ie Delta-BA.1 co-infection vs BA.1 infection vs COVID-19-naive with three vaccine injections). For immunological assays, samples were blinded and randomized.
Blinding	Coded samples were used for all experiments thus investigators were blinded to group allocation.

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Materials & experimental systems

n/a	Involved in the study
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<input type="checkbox"/>	<input checked="" type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input checked="" type="checkbox"/>	<input type="checkbox"/> Animals and other organisms
<input type="checkbox"/>	<input checked="" type="checkbox"/> Clinical data
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Methods

n/a	Involved in the study
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Antibodies

Antibodies used	Elisas Recombinant protein Spike S1-His: SARS-CoV-2 (2019-nCoV) -Cat: 40591-V08H (Sino-biologicals) coating 1 microgr/ml Goat Anti-human IgA (alpha chain specific)-HRP (SIGMA)--Cat: A0295-1ML dilution 1/10000
Validation	All antibodies used in this study are commercially available, and all have been validated and quality checked by the manufacturers and used in other published works

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	Vero E6 cells (ATCC CRL-1586)
Authentication	We did not authenticate the cell lines, but such authentication is performed by the supplier (ATCC) as part of the quality control
Mycoplasma contamination	All cell lines were regularly screened and tested negative for mycoplasma using a commercial kit (Lonza MycoAlert kit) # LT07-418
Commonly misidentified lines (See ICLAC register)	no commonly misidentified cell lines were used in the study

Clinical data

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Clinical trial registration	NCT05060939, NCT04341142
Study protocol	Study protocol is available upon request
Data collection	Participants with a breakthrough infection caused by Delta-BA.1 simultaneously or BA.1 only blood sampling was performed between june and august 2022. As for individuals who are COVID-19 naive and triple vaccinated, blood sampling was performed between march and may 2022.
Outcomes	The primary outcome was the positivity of the SARS-Cov-2 serological test and quantification of anti-RBD IgG and anti-S1 IgA, and the secondary outcome was the serum neutralization capacity.

Discussion of Article 5

As exposed in the introduction, SARS-CoV-2 evolves very quick and new variants appeared influencing the epidemiology, clinical symptoms, and immunology print in the population. Several variants might be circulating at the same time, and vaccinated individuals presented breakthrough infections. Therefore, we wanted to study the immune response after these new infections in vaccinated individuals. The results presented in this study demonstrate an increased humoral response following breakthrough infections caused by one or two SARS-CoV-2 variants. These findings support the concept of hybrid immunity, which has previously been shown to elicit a more robust and durable humoral immune response against SARS-CoV-2 compared to vaccination alone (246). Even though rare, simultaneous infections with two different SARS-CoV-2 can occur and herein we found that there is no difference in the levels of anti-S total antibodies and neutralization capacity between individuals with a single breakthrough infection and those with a dual infection involving the Delta and Omicron BA.1 variants. This suggests that simultaneous exposure to SARS-CoV-2 variants does not confer additional advantages in terms of humoral immunity. However, in this study, we only evaluated the systemic humoral immune response, but SARS-CoV-2 is a respiratory virus. Therefore, studying the mucosal immune response is necessary to better understand protection and long-term immunity and it should be matter of future studies.

It would be interesting to expand this study to a larger cohort of patients and to new variants. It might even be interesting to study if there could be triple or more simultaneous infections in some individuals. These phenomena may be more relevant in immunosuppressed individuals as viral recombinants might form in this

population. The study of their immunity after multiple infections could also be interesting to evaluate in order to give a correct follow-up and treatment to this type of patients.





Even though here we saw no differences between mono or bivalent infection, this should not be taken into consideration when talking about the bivalent vaccines, as both immunizations are not equivalent in terms of antigen exposure, diversity, and load. In addition, contradictory results regarding the additional protection provided by these new vaccines have been reported (247–250). Therefore, it could be interesting to do long-term studies using bivalent vaccines to better understand their effectiveness. Moreover, cellular immune response, including resident memory T cells, should be studied to understand their role in breakthrough infections.

With the appearance of new variants, and as seen that this is something that will continue to appear in SARS-CoV-2 evolution, the question about the possible ADE effect of vaccination or even a previous infection, it was arose since the beginning of the vaccination campaigns. We wanted to address this issue using an *in vitro* test that we previously developed in the lab (251) with some modifications for SARS-COV-2 infection. Results are in the following article of this manuscript (Article 6).

Article 6: ADE in COVID-19
(Brief report published in Vaccines in March 2023)

Brief Report

Prior COVID-19 Immunization Does Not Cause IgA- or IgG-Dependent Enhancement of SARS-CoV-2 Infection

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Abstract: Antibody-dependent enhancement (ADE) can increase the rates and severity of infection with various viruses, including coronaviruses, such as MERS. Some in vitro studies on COVID-19 have suggested that prior immunization enhances SARS-CoV-2 infection, but preclinical and clinical studies have demonstrated the contrary. We studied a cohort of COVID-19 patients and a cohort of vaccinated individuals with a heterologous (Moderna/Pfizer) or homologous (Pfizer/Pfizer) vaccination scheme. The dependence on IgG or IgA of ADE of infection was evaluated on the serum samples from these subjects (twenty-six vaccinated individuals and twenty-one PCR-positive SARS-CoV-2-infected patients) using an in vitro model with CD16- or CD89-expressing cells and the Delta (B.1.617.2 lineage) and Omicron (B.1.1.529 lineage) variants of SARS-CoV-2. Sera from COVID-19 patients did not show ADE of infection with any of the tested viral variants. Some serum samples from vaccinated individuals displayed a mild IgA-ADE effect with Omicron after the second dose of the vaccine, but this effect was abolished after the completion of the full vaccination scheme. In this study, FcγRIIIa- and FcαRI-dependent ADE of SARS-CoV-2 infection after prior immunization, which might increase the risk of severe disease in a second natural infection, was not observed.

Keywords: SARS-CoV-2; ADE; IgA; Delta; Omicron; vaccines



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1. Introduction

Antibody-dependent enhancement (ADE) is known to increase infection and severe disease rates due to various viruses, including coronaviruses, such as MERS (Middle East respiratory syndrome). Two types of ADE have been described: intrinsic and extrinsic ADE. The first one refers to the cellular mechanisms that increase viral replication and release of new virions, while the second one refers to the elements relative to virus–receptor interaction and viral cell entry [1]. The possibility of ADE in COVID-19 (Coronavirus disease) has been a matter of debate. Some studies show an ADE effect in vitro (refs. [2–7]), but clinical and epidemiological studies show that prior immunization does not increase the likelihood of severe disease [7–13]. The ADE of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection has been reported to occur via various routes in vitro. For example, sera from patients convalescing from COVID-19 can enhance infection via the FcγR or C1q protein [2–4]. Furthermore, sera from convalescent individuals, but not from vaccinated individuals, has been reported to enhance virus uptake by monocytes in vitro without the production of viral particles. However, pyroptosis is triggered, which can increase inflammation in COVID-19 [14]. The antibodies responsible for ADE have

been reported to target epitopes different from those targeted by antibodies capable of fully neutralizing infection [5–7,15]. The NTD (N-terminal domain) region of the spike protein is one of the ADE epitopes identified, with monoclonal antibodies targeting this region reported to increase SARS-CoV-2 infection in vitro in ACE2 (angiotensin-converting enzyme 2)-overexpressing cells [5,15]. However, other in vitro studies with sera from patients convalescing from COVID-19 have found no evidence of ADE, even at subneutralizing concentrations [16,17].

We investigated the possible ADE effect of sera from vaccinated individuals and from SARS-CoV-2-infected patients with severe or asymptomatic COVID-19 following challenges with the Delta (B.1.617.2 lineage) or Omicron (B.1.1.529 lineage) variants. We investigated whether ADE of SARS-CoV-2 infection occurred, and its possible dependence on IgG or IgA, using an in vitro model based on HEK-293 cells expressing the CD16 and CD89 receptors, respectively.

2. Materials and Methods

2.1. Cohorts

Twenty-six individuals who had never had COVID-19 and had been vaccinated with the Pfizer BNT162b2 and/or Moderna mRNA-1273 vaccines were included in a prospective longitudinal cohort study conducted at CHU Nord de Saint-Etienne (Saint-Etienne, France). Blood was collected before and two weeks after administration of the booster dose of vaccine. We also studied a cohort of twenty-one PCR-positive SARS-CoV-2-infected patients with the B.1 strain. COVID-19 patients were split into two groups according to disease severity: 10 patients with mild or asymptomatic disease and 11 patients with severe disease requiring admission to the ICU. Serum samples were collected between 14 and 30 days post-symptom onset (PSO). Written informed consent for participation was obtained from all subjects; ethics approval was obtained from the CPP Ile de France V (NCT04648709).

2.2. Cell Models

HEK-CD89+ (Fc α RI), HEK-CD16+ (Fc γ RIIIa), and HEKWT cell lines were obtained from InvivoGen and previously described [18]. All cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% SVF and 1% antibiotic-antimycotic (AAT). Cells were harvested with trypsin/EDTA solution and the stable expression of FcR was checked by flow cytometry before each experiment. All cell lines were maintained at 37 °C, in a humidified atmosphere containing 5% CO₂.

2.3. In Vitro Assay for Antibody-Dependent Enhancement (ADE)

The in vitro ADE assay was performed with cell lines expressing FcR (HEK-CD16+ or HEK-CD89+). Serum samples from vaccinated individuals and COVID-19 patients (1:100 dilution) were incubated with 0.7 MOI of Delta (B.1.617.2 lineage) or Omicron (B.1.1.529 lineage) variants for 1 h at 37 °C. HEK-CD16+, HEK-CD89+ or HEKWT cells were incubated with the mixture of serum plus virus for 120 h at 37 °C. Viral amplification was assessed by RT-PCR on the culture supernatant. We then calculated and compared the ratios of the amount of virus in each sample to that in a viral control without serum. Two monoclonal antibodies were used as positive controls: an anti-RBD IgG (Ref: G_SAR2-RBD_X30F12-PU; BCell Design, Limoges, France) was used for the CD16 test, and an anti-S IgA (Ref: MAB12439-100; Native Antigens, Kidlington, United Kingdom) was used for the CD89 test. Eight human serum samples from 2005 were used as the negative control, and the mean value for these samples plus twice the standard deviation was used as the cut-off for enhancement.

2.4. SARS-CoV-2 RT-PCR for Viral Amplification

SARS-CoV-2 in the culture supernatant was quantified by RT-PCR without nucleic acid extraction, with the Luna Universal Probe One-Step RT-qPCR Kit (Ref. E3006L, New England Biolabs, Ipswich, MA, USA) [19]. Briefly, 5 μ L of supernatant was diluted 1/10

with Dnase-free and Rnase-free water and mixed with the reaction solution to obtain a total volume of 14 μ L. The reaction solution contained 5 μ L Luna[®] Universal Probe One-Step Reaction Mix, 0.5 μ L Luna[®] WarmStart[®] RT Enzyme Mix and 1.5 μ L of a mixture of primers at 400 nM (E_Sarbeco_F: ACAGGTACGTTAATAGTTAATAGCGT and E_Sarbeco_R: ATATTGCAGCAGTACGCACACA), and the probe at a concentration of 200 nM (E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ) [20]. The RT-PCR was initialized with a reverse transcription step at 55 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 60 s. A viral standard curve was obtained for each analysis.

2.5. Live Virus Neutralization Experiments

Virus neutralization was performed as previously described [21]. Briefly, serial 2-fold dilutions (tested in duplicate, starting at 1:10) of the serum specimens in culture medium were mixed at equal volume with the live SARS-CoV2 virus. After incubation of the mix for 30 min at room temperature, 150 μ L of the mix was transferred into 96-well microplates covered with a monolayer of Vero E6 cells to achieve a viral concentration of 100 TCID50/well. The plates were incubated at 37 °C in a 5% CO₂ atmosphere for 5 days. After this time, the cytopathic effect was evaluated, and neutralization was recorded if less than 50% of the cells were damaged. The neutralizing titer was expressed as the inverse of the higher serum dilution that exhibited neutralizing activity; a threshold of 20 was used (PRNT50 titer \geq 20). All experiments were performed in a biosafety level 3 laboratory. The different viral strains that were used were sequenced and deposited on GISAID (GISAID accession numbers: EPI_ISL_1707038, 19A (B.38); EPI_ISL_1904989, Delta (B.1.617.2); and EPI_ISL_7608613, Omicron (B.1.1.529)).

2.6. Statistical Analysis

The statistical significance of the difference between groups for each set of conditions was determined by one-way ANOVA with Tukey's test for multiple comparisons. Statistical significance was defined as $p < 0.05$. All statistical calculations were performed and graphs were generated with GraphPad Prism 9.5.0 (GraphPad, San Diego, CA, USA).

3. Results

Prior Immunization Does Not Enhance SARS-CoV-2 Infection

We investigated whether prior immunization could enhance a new SARS-CoV-2 infection by studying two cohorts: a cohort of patients infected with SARS-CoV-2 (B.1 lineage) and a cohort of individuals who had been vaccinated with either a heterologous (Moderna mRNA1273/Pfizer BNT162b2) or homologous (Pfizer BNT162b2/Pfizer BNT162b2) vaccination scheme. The characteristics of the study population are shown in Table 1.

Table 1. Characteristics of the subjects studied, by group.

Characteristics	Groups		
	Severe Disease (<i>n</i> = 19)	Mild/Asymptomatic Disease (<i>n</i> = 36)	Vaccinated (<i>n</i> = 26)
M/F sex ratio	3.75	0.29	0.30
Age (years), median (IQR)	70 (61.5–71.5)	34 (29–40.3)	85 (29–86)

The ADE of infection was evaluated in vitro with either the Delta or Omicron variant of SARS-CoV-2. Our model consisted of HEK-293 cells expressing the FcR for either IgG (CD16) or IgA (CD89), to facilitate FcR-dependent infection. Firstly, HEK-293 cells without CD16 or CD89 expression were used to control non-FcR specific effects of sera (Figure S1 (Supplementary Materials)). None of the serum samples from vaccinated or SARS-CoV-2-infected individuals enhanced the infection of HEK-293 cells with the Delta variant via either the CD16 or CD89 receptor (Figure 1). For both homologous and heterologous

vaccination schemes, negative results for ADE were obtained after both the second and booster dose (Figure 2).

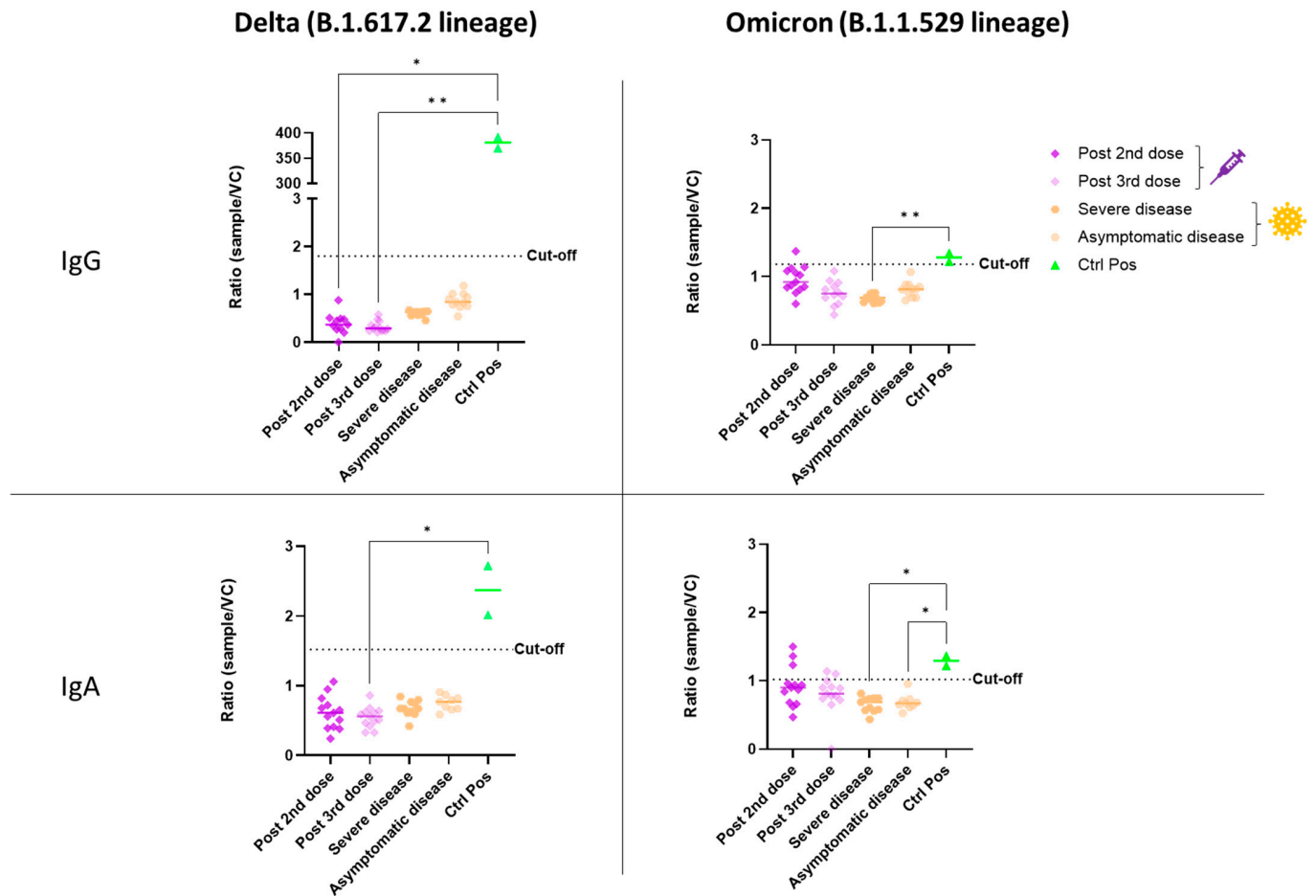


Figure 1. In vitro ADE of SARS-CoV-2 infection from vaccinated (syringe symbol) or COVID-19 patients (virus symbol) against Delta and Omicron variants. The ADE effect was evaluated using HEK-293 CD16+ or CD89+ cells. Each sample was tested simultaneously in both models (CD16 and CD89) and against both viral variants. The amount of virus present in the supernatant was determined after five days of infection by a SARS-CoV-2-specific RT-PCR. The ratio of the amount of virus in each sample and that in a viral control without serum (sample/VC) is shown. Each dot corresponds to the mean of duplicate values from a single patient. An anti-RBD IgG was used as the positive control for the CD16 test, and an anti-S IgA was used for CD89. Sera were used at a 1:100 dilution. The cut-off was determined as the mean value for eight pre-pandemic serum samples plus two standard deviations. The statistical significance of differences between groups in a Kruskal–Wallis test with Dunn’s tests for multiple comparisons is shown for each set of conditions (* $p < 0.05$; ** $p < 0.01$).

In evaluations of ADE of infection with the Omicron variant, a positive signal was obtained for some vaccinated individuals relative to the eight pre-pandemic serum samples used to define the cutoff. However, sera from COVID-19 patients did not enhance infection in vitro (Figure 1). After the second dose of the homologous vaccination scheme, more serum samples tested positive for ADE of infection dependent on the CD89 receptor than for ADE of infection dependent on CD16, suggesting that IgA in the serum may enhance infection with the Omicron variant (Figure 2). Those patients were as aged as the others and had positive levels of QUANTIFERON and anti-S IgG antibodies (Figure S2). However, they had no neutralizing antibodies against the Omicron variant (Figure 3). Nevertheless, after administration of the booster dose (a third administration of a half dose of mRNA vaccine),

most serum samples tested negative for ADE (Figure 2). Those who were still positive for ADE did not develop neutralizing antibodies two weeks after the third dose of the vaccine (Figure 3). In addition, ADE IgG-dependence is negatively correlated with neutralizing titers and anti-SARS-CoV-2 antibody levels when facing the Delta variant (Figure S3a,b). However, ADE IgA-dependence was only negatively correlated with neutralizing activity and anti-SARS-CoV-2 antibody levels when facing the Omicron variant (Figure S3c,d). These results highlight the importance of a good neutralizing response to avoid the ADE phenomenon. There was no significant difference between doses, but there was a clear trend towards lower rates of ADE after the booster dose, emphasizing a decrease in the risk of possible disease aggravation due to immunity to a prior SARS-CoV-2 variant.

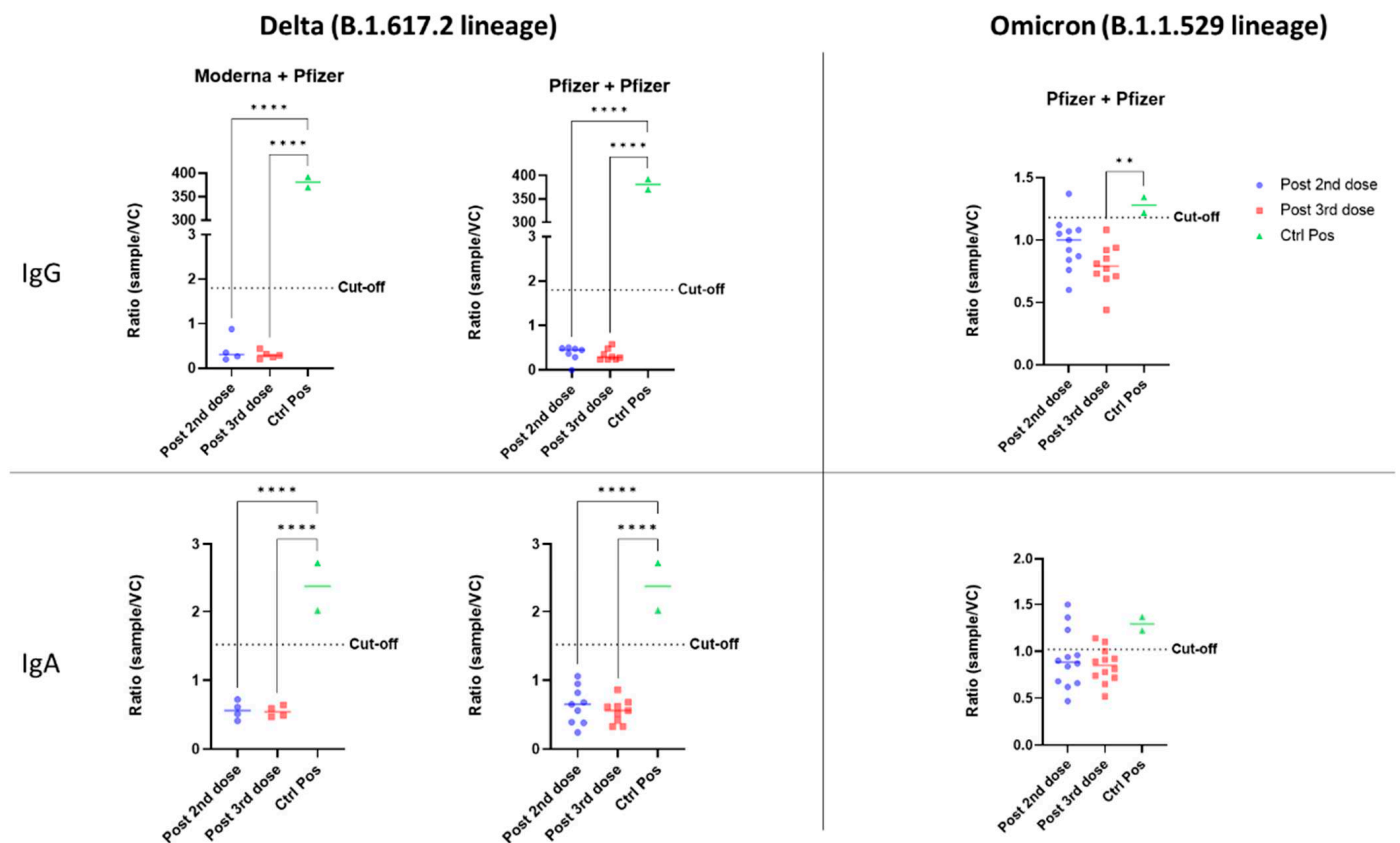


Figure 2. In vitro ADE of SARS-CoV-2 infection from individuals vaccinated with different vaccination schemes (heterologous: Moderna/Pfizer; or homologous: Pfizer/Pfizer). The ADE effect was evaluated with the Delta and Omicron variants, and the involvement of IgG and IgA antibodies was assessed using HEK-293 CD16+ or CD89+ cells. Viral quantification was measured by a SARS-CoV-2-specific RT-PCR. The ratio of the amount of virus for each sample to that for a viral control without serum (sample/VC) is shown. Each dot corresponds to a single patient. An anti-RBD IgG was used as a positive control for the CD16 test, and an anti-S IgA was used for CD89. Sera were used at a 1:100 dilution. The cut-off was determined as the mean for eight pre-pandemic serum samples plus two standard deviations. The statistical significance of differences between groups in ANOVA with Tukey tests for multiple comparisons is shown for each set of conditions (** $p < 0.01$, **** $p < 0.0001$).

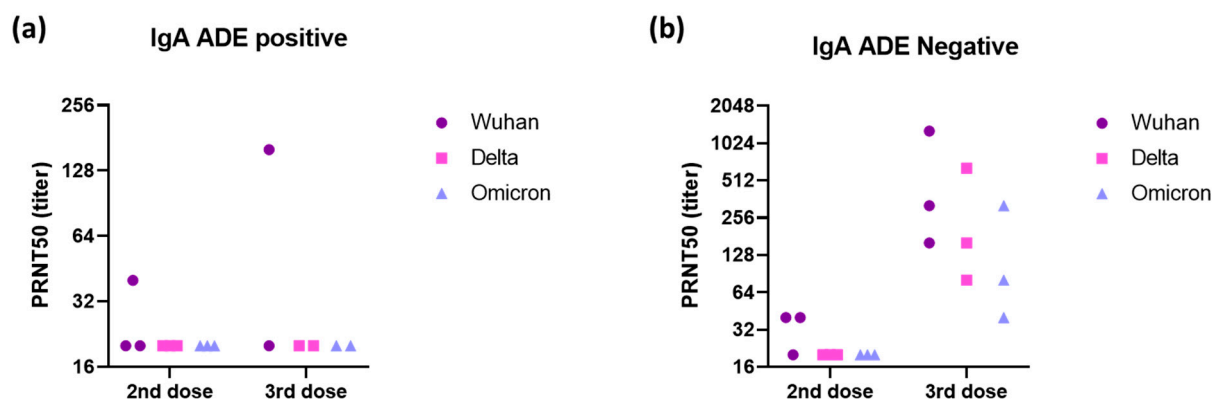


Figure 3. Neutralizing activity of (a) three positive and (b) three negative sera to IgA-dependent ADE against the Omicron variant (sera dilution starting at 1:20) was evaluated against Wuhan, Delta, and Omicron strains using a plaque reduction neutralization test (PRNT) with VeroE6 cells. Each dot corresponds to one patient.

4. Discussion

ADE has been a major subject of concern in different viral diseases such as Dengue, Zika, and respiratory syncytial virus (RSV) [1,22]. It has been suggested that ADE may contribute to SARS-CoV2 infection in vitro, but we show here that infection is not enhanced after vaccination or prior natural infection. These results contrast with those of previous studies in which patients with severe disease or convalescent serum enhanced SARS-CoV-2 infection in vitro via FcγR [2,4,23]. For instance, Shimizu et al. showed that the serum of patients with severe disease patients increased viral infection as well as IL-6 production in a myeloid cell line, suggesting that serum antibodies can contribute to the cytokine storm present in COVID-19 disease [23]. In a more recent study, the same group reported ADE of infection in some monoclonal antibodies as well as in sera from vaccinated patients [24]. However, ADE was seen within a narrow window of antibody and serum concentrations. The time match between this antibody concentration and an effective viral quantity seems unlikely during natural infection. Furthermore, we must not forget that the cellular immune response is also playing a role against SARS-CoV-2 infection and that the ADE of infection seen in vitro might be subclinical during natural infection. On the other hand, our study is in line with other studies showing the absence of ADE in SARS-CoV-2 infection [16,17]. Clark et al. showed that neutralizing titers of convalescent plasma did not change in the presence of ACE2 and FcαR or FcγRIIA [16]. Garcia-Nicolas et al. did not find evidence for ADE of infection in human monocyte-derived macrophages (hMDM) or pro-inflammatory cytokine responses when evaluating convalescent serum [17].

The mild ADE effect observed in our study when sera from vaccinated individuals were incubated with the Omicron variant can be explained by the mutations in the Omicron spike protein relative to the strain used to produce the vaccine. However, we also found that after full vaccination (two full doses plus a half-dose booster), the ADE effect in vitro decreased substantially. This finding is consistent with the observation that, despite the lower neutralizing efficacy of antibodies against the Omicron variant, the number of patients with severe disease is not higher after vaccination [12,13]. Furthermore, preclinical and clinical studies have suggested that no ADE occurs in vivo [7]. For example, vaccination/challenge studies in non-human primates have reported no increase in disease severity in this animal model [8,9]. In addition, treatment with convalescent plasma did not increase the risk of severe disease in COVID-19 patients [10,11,25].

This study has some limitations. Even though CD16 is a known FcγR by its ADCC activity, notably in NK cells, it is not the only FcγR expressed in human immune cells. Therefore, results might be different if other FcγRs are tested. Nevertheless, as discussed earlier, some studies have tested the ADE of infection in vitro with other FcγRs and no ADE has been seen, for example, with the FcγRIIA [16]. In addition, the HEK-293 tested

here were ACE2 negatives and it has been previously shown that the ADE of SARS-CoV-2 infection might require ACE2 as a co-receptor [3].

Overall, in this study, the Fc γ RIIIa- and Fc α RI-dependent ADE of SARS-CoV-2 infection after prior immunization, which might increase the risk of severe disease in a second natural infection, was not observed.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/vaccines11040773/s1>, Figure S1: Analysis of the ADE of SARS-CoV-2 infection in vitro with serum samples from individuals vaccinated or COVID-19-positive patients; Figure S2: Specific antibody response anti-SARS-CoV-2. Figure S3: Specific antibody response anti-SARS-CoV-2.

Author Contributions: M.Y.-N., B.N., A.-E.B. and F.J. performed the experiments. M.Y.-N., E.B.-N., S.L., L.W., B.N. and S.P. wrote the manuscript with support from T.B.; S.P. supervised the project. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee CPP Ile de France V (NCT04648709).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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Delta (B.1.617.2 lineage)

Omicron (B.1.1.529 lineage)

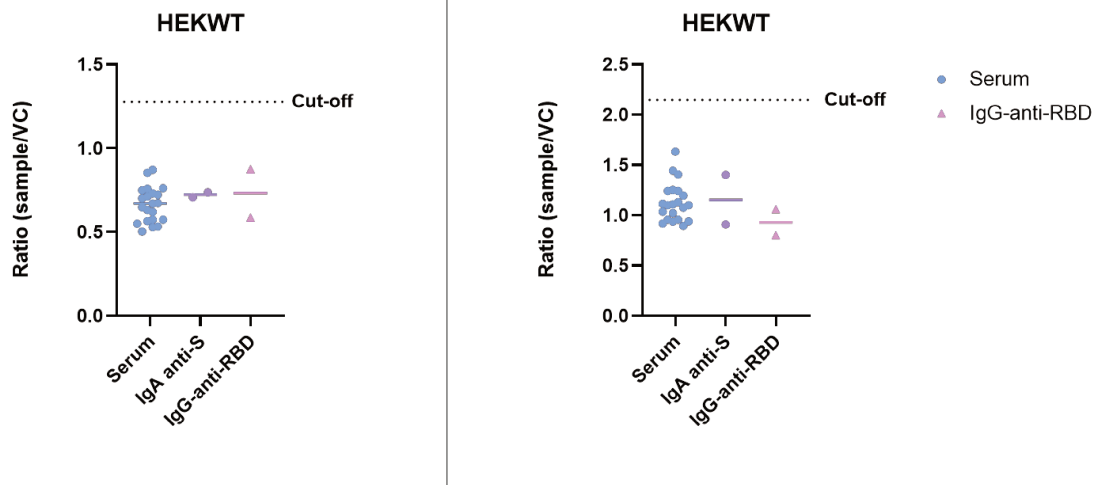


Figure S1. Analysis of the ADE of SARS-CoV-2 infection *in vitro* with serum samples from individuals vaccinated or COVID-19-positive patients. The anti-RBD IgG and anti-S IgA used as positive controls in the CD16/CD89 tests were also tested. The ADE effect was evaluated with HEK wild-type cells (HEKWT) against the delta and omicron variants. The ratio of the amount of virus for each sample to that for a viral control without serum (sample/VC) is shown. Each dot corresponds to a single patient. The cut-off was determined as the mean for eight pre-pandemic serum samples plus two standard deviations.

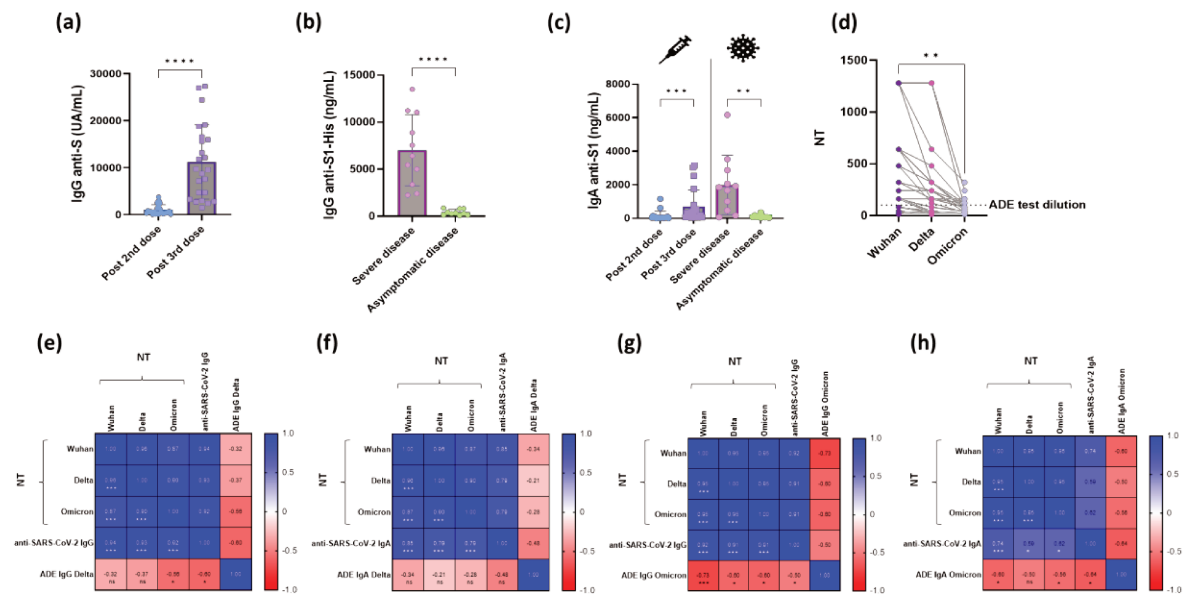


Figure S2. Specific antibody response anti-SARS-CoV-2. IgG (a and b) and IgA (c) against spike-1 (S1) were measured using ELISA in vaccinated individuals

(designated with a syringe symbol) and COVID-19 patients (designated with a virus symbol). *P* value was calculated using an unpaired t-test. **(d)** Neutralizing titers (NT) for each tested serum against Wuhan, Delta, and Omicron variants were measured by a live-virus neutralization test. *P* value was calculated using a Kruskal-Wallis test with Dunn's tests for multiple comparisons. Each dot represents one individual. **(e-h)** Correlation matrices between ADE, viral neutralizing titers, and anti-SARS-CoV-2 Ab concentrations. Correlation coefficients were calculated using Spearman's correlation tests and they were plotted using GraphPad Prism v.9.5.1. for panels A-C (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001).

Discussion of Article 6

ADE has been a major subject of concern in different viral diseases such as Dengue, Zika, and (RSV) (252,253). It has been suggested that ADE may contribute to SARS-CoV-2 infection *in vitro*, but we show here that infection is not enhanced after vaccination or prior natural infection. These results contrast with those of previous studies in which patients with severe disease or convalescent serum enhanced SARS-CoV-2 infection *in vitro* via FcγR (212,214,254). Furthermore, we must not forget that the cellular immune response is also playing a role against SARS-CoV-2 infection and that the ADE of infection seen *in vitro* might be subclinical during natural infection. On the other hand, our study is in line with other *in vitro* studies showing the absence of ADE in SARS-CoV-2 infection even in the presence of ACE2 and other FcRs (217,218). Our findings are consistent with the observation that, despite the lower neutralizing efficacy of antibodies against the Omicron variant, the number of patients with severe disease is not higher after vaccination (201,225). Furthermore, preclinical and clinical studies have suggested that no ADE occurs *in vivo* (219).

It will be interesting to include in our *in vitro* model other FcγRs as CD16 is not the only FcγR expressed in human immune cells. As well as include ACE2 as it has been previously shown that the ADE of SARS-CoV-2 infection might require ACE2 as a co-receptor (213).

General Discussion and perspectives

Since the beginning of the pandemic in December 2019, there has been a huge increase of published studies about the SARS-CoV-2 infection. In PubMed, there are 206,078 results when searching for “SARS-CoV-2 COVID-19” in just three years, while for other viral diseases like “HIV AIDS” there are 166,585 results in 40 years of research. It is now well accepted that COVID-19 is an inflammatory disease that, rather than being killed by the virus, is our immune system that is dysregulated creating excessive inflammation leading to tissue damage and organ failure. However, what makes some patients develop an uncontrolled inflammation and not all, is still under investigation as it is a multifactorial process.

During my Ph.D., we contribute to the understanding of the immune response during SARS-COV-2 infection and after vaccination. The innate immune response is the first response to pathogens that helps mount the adaptive response which is more specific and has the capacity to generate a memory response for subsequent infections (255). If the innate response is dysbalanced and the signals to trigger the adaptive response are not well established or delivered, the adaptive response will also be defective, and susceptibility to infections or disease severity increases. For example, NEMO or MyD88 are TLR downstream signalling molecules that induce the activation of transcription factors like NF- κ B which regulates the expression of pro-inflammatory cytokines including IL-1, IL-6, tumor necrosis factor (TNF)- α , and IL-12 (256). Defects in one of these molecules could hamper the correct activation of a protective immune response. Innate immunodeficiencies including mutations in these molecules have been correlated with herpes simplex virus encephalitis, viral infections, and mycobacterial disease (257,258). Moreover, innate immunodeficiencies in the IFN-related genes have been correlated with increased disease severity in COVID-19 (31,33,228,259,260). For example, X-linked recessive TLR7 deficiency, and IRAK4 or

MyD88 deficiency, are associated to critical COVID-19 pneumonia, and plasmacytoid dendritic cells (pDCs) are dependent on IRAK4 and TLR7 for SARS-CoV-2 sensing and type I IFN production. Autosomal recessive IFNAR1 and IFNAR2 deficiencies are also associated to COVID-19 severity (261) We show for the first time that sIFNAR1 and sIFNAR2 are also correlated with disease severity in COVID-19 and that they can serve as predictor markers of the severity of the disease. Even though no significant, sIFNAR1 is inversely correlated with disease severity, while we found significantly higher levels of sIFNAR2 in the same patients. These altered levels of the sIFNAR in the most severe patients can damper the IFN antiviral response for example by sequestering the type I IFN in the serum as the sIFNAR2 cannot transduce the signal. We still need to study by which mechanisms these receptors are altered in COVID-19 patients, but alternative splicing modifications or even viral inhibition of post-translational modifications might be involved. These mechanisms have been described for other disease like multiple sclerosis or in Flavivirus infections, therefore it might also be present in SARS-CoV-2 infection (233,234). Whether the patients in our study present some genetic modifications interfering with the correct expression of the IFNAR1 and IFNAR2 molecules, whether they also present recessive autosomal deficiencies in TLRs hampering the correct activation of the IFN antiviral response, or whether they also present auto-antibodies against type-I IFN, could be a matter of study for future projects. So far, it is not known neither if sIFNAR1 and sIFNAR2 levels are associated with a better recovery prognosis or a higher predisposition to develop long COVID. It would not be weird to think that they might be associated with a higher risk of developing this condition because as a part of the IFN response is damaged, it might be less viral clearance, therefore more sustained inflammation, and maybe viral reservoir as it has been suggested for long COVID patients (53). In addition, our results

open the door to study the potential role of sIFNAR1 and sIFNAR2 as predictor factors of disease severity in other severe viral infections like influenza, RSV or HMPV.

I also demonstrate that the adaptive immune response is also altered in COVID-19 severe patients. We, as others (35,91,122,137,152,160,206,262,263), show that there are higher levels of anti-SARS-CoV-2 antibodies in the serum of severe patients than in asymptomatic ones, and as early as the first week after symptoms onset. These antibodies are directed principally against the S protein but also against the N protein. We also showed that anti-SARS-CoV-2 IgA antibodies are detectable earlier than IgG in the most severe patients. With these results, one of our first hypotheses was that IgA might block IgG functions as it has been described for other viral infections like HIV (264). At least in our *in vitro* models with VeroE6 cells and with the lung epithelium model, systemic IgA from severe patients neutralizes better the SARS-CoV-2 virus than IgG, and better than IgA and IgG from asymptomatic patients. This suggests that there is no competition between IgA and IgG for neutralization and that IgA is a more potent SARS-CoV-2 neutralizer than IgG as shown by others (137,241). However, neutralizing activity is not the only functional property of antibodies during a viral infection. FcR-dependent functions are also important in controlling the infection. It has been shown that IgG antibodies elicited in severe patients are structurally different compared to those in asymptomatic patients (205,206,265–267). Those antibodies are less fucosylated and sialylated which makes them more active in some FcR-dependent functions like ADCC, and ADCD generating more immune system activation and inflammation (205,206,265–267). This phenomenon has also been described for other pathologies like MS (multiple sclerosis) and RA (268). In our study, we observed a loss of CD16-dependent function in the serum from severe patients compared to asymptomatic patients. These findings contradict those studies which have reported

an increased FcR activation in sera from severe patients, associated with afucosylated IgG which has an enhanced FcR affinity (206,240). Whether the observed loss of function in our study is due to receptor affinity, or structural differences between IgG from severe and asymptomatic patients, requires further investigation. Recently a study showed that serum IgM antibodies in severe COVID-19 patients have also a distinct glycosylation profile compared to moderate disease patients (269). They found increased di- and tri-sialylated glycans and altered mannose glycans in total serum IgM in severe patients, as well as elevated levels of antigen-specific IgM ADCD (269). While there are studies about the IgG-specific FcR-dependent functions in COVID-19, less is known about the IgA-specific FcR-dependent functions. We demonstrated that serum IgA antibodies from severe patients have higher CD89 activation compared to asymptomatic patients. So far there are no studies about the glycosylation profile or post-translation modifications in the IgA antibodies of COVID-19 patients. This was one of the lines of study we wanted to study during my PhD project but unfortunately, the amounts of IgA that we were able to purify from the sera were not enough, especially for the asymptomatic patients as they present lower levels of antibodies than severe patients. It would be very interesting to make this study in the short term, as it has been shown for example in RA that modifications in the glycosylation profile of IgA in serum can modify the effector functions potentiating NETosis and therefore increasing inflammation (268). It is known that in COVID-19 there are higher levels of neutrophils and NETosis induction as well as longer persistence of NETs in the tissues and blood stream, and IgA might be behind this hyperactivation of neutrophils (180,182,270). In addition, IgA glycosylation modifications can increase complement deposition and the formation of inclusion bodies, as is the case in the IgA nephropathy (271). Small-vessel vasculitis driven by deposits of IgA-C3 ICs (184), or IgA vasculitis,

formerly known as Henoch–Schönlein purpura (HSP), has also been reported in children with COVID-19, suggesting that SARS-CoV-2 infection may be a trigger (185). In our project, we started a collaboration with CYNBIOSE to study ICs deposition in the lungs of non-human primates infected with SARS-CoV-2. Unfortunately, we had limited access to the samples, and we could not make a correct setup of the immunofluorescence technique.

In addition, we show that the humoral mucosal response, at least in terms of neutralization, may not be enough/efficient in controlling the infection in the most severe patients. We evidence this showing that severe patients present higher levels of anti-S IgA and IgG antibodies in nasal swabs and BAL samples, but we did not find any neutralizing activity. This contradicts some studies that detected higher neutralization titers in samples from severe patients, however, those studies employed pseudovirus or RBD-ACE2 inhibition techniques to measure neutralizing activity (158,170). Despite the advantages of this type of assays (272), it only focuses on the Spike protein from SARS-CoV-2, which does not provide a complete picture as in the live-virus neutralization test we used, being our test closer to a real infection situation. Nevertheless, another study showed a loss of neutralizing function in mucosal antibodies from non-survivor patients despite the persistence of anti-SARS-CoV-2 antibodies, suggesting that mucosal IgA might not be able to control the infection by neutralization activity but activating different FcR-dependent mechanisms that may increase inflammation (238). According to this, we show higher levels of mIgA in the BAL of severe patients that might come from the systemic circulation *via* transudation mechanisms. These IgA have been described to be more potent than dIgA or SIgA in the activation of FcR-dependent functions and therefore being more pro-inflammatory. Their presence in the lower mucosa can increase inflammation and therefore tissue

damage, increasing disease severity, but it is still unknown if, at the same time, they can block the neutralizing effect of the mucosal SIgA. Our discrepancies and contrasting findings highlight the complexity of the mucosal immune response to SARS-CoV-2 and suggest that there may be variations in the immune profiles of different patient populations. This emphasizes the need for further research to fully understand the role of IgA and its structural alterations in severe cases and their impact on disease progression.

In addition, we show that a heterologous vaccination scheme is more immunogenic and efficient in developing a good immune response against different variants of SARS-CoV-2 than the homologous scheme. This was something not surprising, as in other respiratory infections like influenza, the heterologous prime-boost vaccination scheme has also been shown to be more immunogenic and generate a broader antibody response (273,274). In Dengue infection, subsequent infections with different viral serotypes generate a broader immune response targeting more conserved epitopes between serotypes, and symptomatic or severe infections after a third or fourth infection are very rare, evidencing that heterologous immunizations are the better way to fight a viral agent (275). It is noteworthy that current COVID-19 mRNA vaccines are administered intramuscularly, and this vaccination route do not create a mucosal immune response when administered as a primary immunization they rather boost the mucosal immune response generated with a primo infection. Therefore, mucosal vaccines should be prioritized for respiratory pathogens. In COVID-19 several studies have shown that the combination of infection and vaccination generates a stronger immune response than vaccination alone (198,201,242–244,276). However, viral variants put in danger this immune response elicited after vaccination, but in our study of breakthrough infections we showed that hybrid immunity induces a more

potent long-term humoral immune response against SARS-CoV-2 and that simultaneous infections do not confer an additional advantage in terms of immunity. A limitation of this study is that we did not measure the mucosal immune response. Nevertheless, there are shreds of evidence that mucosal S-specific IgA antibodies are correlated with Omicron variants breakthrough infection (277,278). This is not the case for serum or mucosal S-specific IgG antibodies which is a piece of evidence that mucosal IgA should be taken as a correlate of protection against infection rather than serum IgG and more importantly because the mucosa is the port of entry of the SARS-CoV-2 virus (277,278). We also showed that a prior infection or vaccination in naïf individuals does not enhance SARS-CoV-2 infection as is the case in other infections like Dengue virus (275,279,280). However, ADE of disease has been reported by others. For example, Junqueira *et al.* found that monocytes and macrophages can be infected by the SARS-CoV-2 *via* FcR (CD16) while they express little to non ACE2 receptor (216). The infection was not producing new viruses but was inducing cell death by pyroptosis, which releases inflammatory cytokines (IL-1 β) that recruit other immune cells, therefore, amplifying inflammation and tissue damage (216). And they found these dying cells in the lung also. Nevertheless, this study was in the context of a primo infection and not in immunized patients. Clinical data shows that the appearance of new variants did not increase the number of severe patients, suggesting that neither ADE of infection nor ADE of disease is a characteristic of COVID-19, which is consistent with our results and other studies (217,218).

In the current epidemiological situation where most of the population has been infected or vaccinated, or both, fewer cases of severe disease are reported, therefore it is complicated to determine the triggers of disease severity, however, studying the trained immunity and the strength of mucosal immune response after hybrid

immunization will help to develop new vaccine strategies to better protect against subsequent infections with new viral variants.

In summary, with my PhD project, we corroborate that starting with damages in the innate immune response and following with a defective adaptive response, in COVID-19 disease there is a dysbalanced immune response towards a hyper-inflammatory environment where multifactorial mechanisms intervene. With this work, we can see the limitations of retrospective studies but also the importance of a well-designed study to follow up on a pandemic. In addition, the importance of vaccination and its protective role against severe disease has been also highlighted during this project. In the future, it will be important to better understand the mucosal immune response to fight against respiratory pathogens, as well as the correct characterization of the initial cohort in a pandemic.

Annexes


Annexe 1: Review about the humoral immune response in COVID-19 and
after vaccination.

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REVIEW ARTICLE



Role of the humoral immune response during COVID-19: guilty or not guilty?

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Systemic and mucosal humoral immune responses are crucial to fight respiratory viral infections in the current pandemic of COVID-19 caused by the SARS-CoV-2 virus. During SARS-CoV-2 infection, the dynamics of systemic and mucosal antibody infections are affected by patient characteristics, such as age, sex, disease severity, or prior immunity to other human coronaviruses. Patients suffering from severe disease develop higher levels of anti-SARS-CoV-2 antibodies in serum and mucosal tissues than those with mild disease, and these antibodies are detectable for up to a year after symptom onset. In hospitalized patients, the aberrant glycosylation of anti-SARS-CoV-2 antibodies enhances inflammation-associated antibody Fc-dependent effector functions, thereby contributing to COVID-19 pathophysiology. Current vaccines elicit robust humoral immune responses, principally in the blood. However, they are less effective against new viral variants, such as Delta and Omicron. This review provides an overview of current knowledge about the humoral immune response to SARS-CoV-2, with a particular focus on the protective and pathological role of humoral immunity in COVID-19 severity. We also discuss the humoral immune response elicited by COVID-19 vaccination and protection against emerging viral variants.

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INTRODUCTION

In December 2019, a new coronavirus emerged, attracting considerable attention worldwide. The SARS-CoV-2 virus (severe acute respiratory syndrome coronavirus-2), a member of the betacoronavirus family, went on to cause the current COVID-19 pandemic¹. More than 460 million confirmed cases of COVID-19 have been reported to date, with more than six million deaths². COVID-19 disease is highly variable in terms of clinical outcome, ranging from asymptomatic or mild disease, resembling a common cold, to more severe disease, including acute respiratory distress syndrome (ARDS) requiring hospitalization with oxygen therapy, or even death³.

The genome of SARS-CoV-2 encodes structural proteins (the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins) essential for virion formation. S protein has two subunits (S1 and S2) separated by a furin cleavage site crucial for viral infection and distinguishing this virus from its relative, SARS-CoV⁴. The S protein contains the receptor-binding domain (RBD), which binds to angiotensin-converting enzyme 2 (ACE2) in host cells. ACE2 acts as the viral receptor, mediating virus entry and triggering an immune response in the host to eliminate the virus. S protein is, therefore, one of the principal targets of antibody-based immunotherapies and vaccines. S protein mutations have generated a wide spectrum of emerging variants, (variants of concern (VOC)) with different phenotypes affecting transmission and antibody sensitivity.

The immune response to pathogens is characterized by the activation of innate and adaptive responses, and their humoral and

cellular components. Antibodies play a crucial role in protection against viral diseases via various mechanisms involving both their Fab and corresponding Fc portions. The Fab-mediated mechanisms include neutralization, in which the entry of the virus into the host cell is sterically blocked. Fc mechanisms include complement activation, antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent phagocytosis (ADP). However, antibody effector functions can also exacerbate inflammation and generate more damage, as in the antibody-dependent enhancement (ADE) observed in dengue disease⁵.

This review summarizes current knowledge about the humoral immune response to SARS-CoV-2, highlighting the differences and points in common between systemic and mucosal humoral immune responses to the virus. We focus on the role of the antibody response, particularly the mucosal response, and its possible involvement in determining COVID-19 severity. We discuss the humoral immune response elicited by COVID-19 vaccination, together with protection against emerging viral variants. The modulatory effect of pre-existing immunity to other coronaviruses on the immune response to SARS-CoV-2 is also considered.

SYSTEMIC HUMORAL RESPONSE IN COVID-19

The proportions of total IgA, IgM and IgG antibodies and of total IgG subclasses are not modified following SARS-CoV-2 infection⁶. However, anti-SARS-CoV-2 antibody production varies with disease severity and depends on patient characteristics, such as sex and age (Fig. 1). Indeed, many studies have reported that

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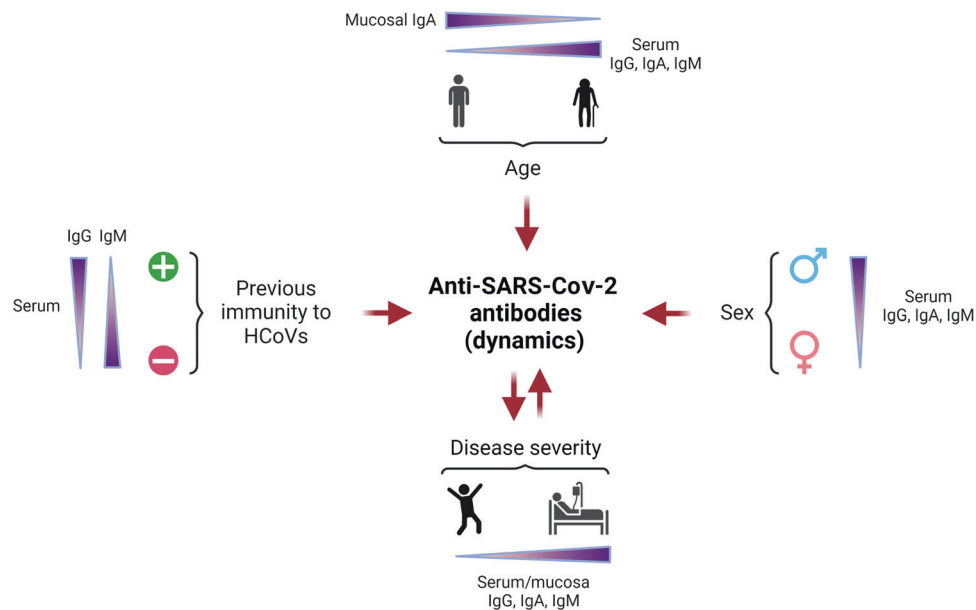


Fig. 1 Factors affecting the dynamics of anti-SARS-CoV-2 antibodies. The characteristics of the patient can modulate the anti-SARS-CoV-2 antibody response. Specific IgG, IgA and IgM levels in serum are high in elderly individuals, whereas the mucosal IgA levels are low in these individuals, contrasting with the situation in young patients, who have lower anti-SARS-CoV-2 antibody titers in serum and higher levels of mucosal IgA. Titers of specific IgG, IgA, and IgM in serum are higher in male than in female patients. Patients previously infected with another human coronavirus (HCoV) (represented by the “+” symbol) develop more anti-SARS-CoV-2 IgG than IgM in serum, whereas the contrary occurs in patients with no previous immunity to HCoV (represented by the “-” symbol). Disease severity can also modulate specific responses in patients, as a more severe disease is associated with higher titers of anti-SARS-CoV-2 IgG, IgA, and IgM in serum. The quality and quantity of these antibodies can also modify disease severity. (Created with www.Biorender.com).

specific antibody titers (even for antibodies directed against non-structural proteins and accessory proteins) are higher in patients with moderate/severe disease than in patients with asymptomatic/mild disease over the course of the infection^{7–17}. Antibody levels are also higher in men than in women^{8,18,19}, an observation that can be accounted for by the higher levels of ACE2 expression in men than in women²⁰, rendering men more susceptible than women to SARS-CoV-2 infection. Similar differences between the sexes have been reported for other viruses, including MERS, SARS-CoV, Epstein Barr virus, HBV, HCV, and West Nile virus²¹. The humoral immune response to SARS-CoV-2 can also be modulated by the patient’s age. Indeed, older patients with severe disease present more anti-spike IgG, IgM and IgA antibodies than younger patients^{15,22}, but this may also be due to confounding factors, such as comorbid conditions²³. Furthermore, immunosenescence²⁴ and inflammaging²⁵ may also contribute to the more severe disease observed in older individuals. In addition, adults may have an ineffective, dysregulated innate immune response, leading to uncontrolled pro-inflammatory cytokine production (cytokine storm), and tissue injury²⁶. The serum immune responses of children to the SARS-CoV-2 virus are limited to anti-spike IgG antibodies, whereas adults also produce anti-N IgG antibodies²². This observation is consistent with the milder course of infection in younger individuals, resulting in the release of smaller amounts of N protein from cells infected with the virus. Nevertheless, after mild COVID-19, children and adults have similar levels of anti-RBD IgG antibodies with similar abilities to inhibit RBD-ACE2 interactions¹⁸.

Serum IgG, IgA and IgM antibodies are elicited principally against the spike (S1, S2 and RBD domains) and nucleocapsid proteins^{10,12,14,16,22,27–33} (Fig. 2). Interestingly, both symptomatic and asymptomatic patients produce antibodies directed principally against S2³⁴. The fusion peptide (FP) is the region most frequently targeted by specific IgM, IgG and IgA in both groups of patients, but antibodies against this region are underrepresented in the specific IgG and IgA epitope repertoires in symptomatic

patients compared to asymptomatic ones³⁴. Conversely, the anti-SARS-CoV-2 IgA antibodies in the serum of symptomatic patients better recognize the N-terminal domain (NTD) and RBD in S1 than those in the serum of asymptomatic patients³⁴. These differences in antibody repertoire can modulate the efficacy of the immune response and, therefore, disease outcome³⁴. In addition, the affinity of the antibodies against the SARS-CoV-2 prefusion spike detected in the serum and nasal washes has been shown to be significantly higher in asymptomatic adults than in symptomatic COVID-19 patients³⁴, suggesting that the antibody response in asymptomatic patients is more effective at controlling the infection than that in symptomatic patients. Some studies have reported higher levels of IgG avidity maturation in patients with severe disease, indicating the presence of larger numbers of memory B cells and/or long-lived plasma cells, which could be rapidly restimulated to prevent reinfection^{11,16}. However, even though reinfection with SARS-CoV-2 is a rare event, occurring in less than 1% of COVID-19 cases, patients with a previous severe infection and those over the age of 65 years are more prone to reinfection^{35,36}, suggesting that natural immunity cannot be relied upon for protection.

By contrast to other infections, IgM are not the first antibodies to appear in the blood of patients infected with SARS-CoV-2 patients³⁷, probably due to prior immunity to other coronaviruses, as discussed below. Indeed, specific IgA is the predominant isotype in the first week post-symptom onset (PSO); there is then a peak of anti-SARS-CoV-2 IgM between 10 and 15 days PSO and an IgG peak around day 20 PSO^{11,13,30,32,33,38,39} (Fig. 2). IgM levels decrease significantly one-month PSO³³, but specific IgA and IgG levels in the blood remain stable more than 6 weeks PSO^{39,40} and IgG can be detected for up to 1 year PSO^{11,12,29,30,41–43}. However, asymptomatic patients have lower levels of anti-SARS-CoV-2 antibodies and they, therefore, lose their specific IgG antibodies faster and more frequently than symptomatic patients¹⁴. The spike-specific IgG1 and IgG3 subclasses predominate over IgG2 and IgG4 in SARS-CoV-2-infected patients^{40,44–46}, as in other viral

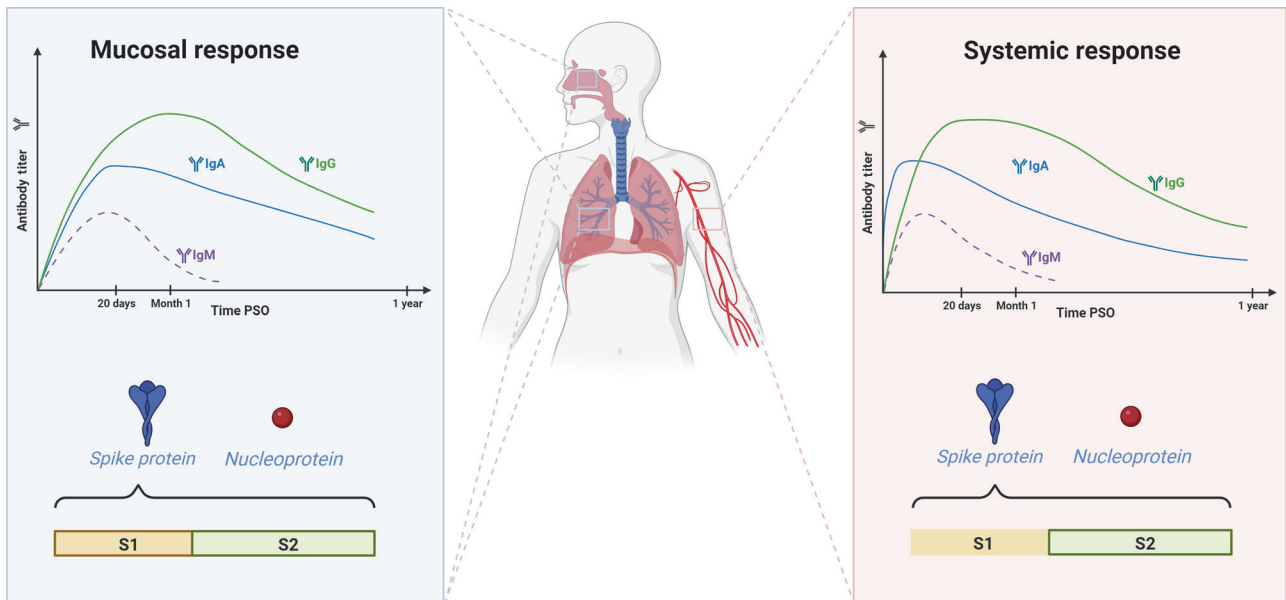


Fig. 2 Mucosal and systemic humoral immune responses to SARS-CoV-2. Both mucosal and systemic humoral immune responses to SARS-CoV-2 are characterized by a transient IgM response detectable until 1 month post-symptom onset (PSO). The mucosal response is dominated by IgG and IgA, whereas the systemic response is initially dominated by IgA. Specific IgA and IgG antibodies are detected in mucosal tissues even at 9 months PSO, and such antibodies are detected in the serum until 1-year PSO. Both responses are directed against the spike protein and nucleoprotein. Despite the targeting of epitopes in the S1 and S2 subunits of the spike protein in both responses, the mucosal response is more diverse (represented by the line around the S1 and S2 rectangles) than the systemic response, in which the epitopes targeted lie principally in the S2 subunit (marked with the line only in the S2 rectangle). (Created with www.Biorender.com).

infections, with HIV⁴⁷ or H1N1 influenza virus, for example⁴⁸. The presence of anti-SARS-CoV-2 IgA in serum is associated with gastrointestinal symptoms in COVID-19, whereas no such association has been found for IgG³². SARS-CoV-2 can replicate in human enterocytes⁴⁹ and may activate the local production of anti-SARS-CoV-2 IgA, contributing to an increase in specific IgA levels in the blood.

Antibodies from the serum of COVID-19 patients have been shown to cross-react with the spike proteins of the four seasonal human coronaviruses (HCoVs: 229E, HKU1, NL63, and OC43)⁵⁰. It remains a matter of debate whether prior immunity to HCoV is protective against SARS-CoV-2 infection. Several studies have reported that the levels of specific IgG and IgA against HCoVs are significantly higher in asymptomatic than in symptomatic COVID-19 patients, suggesting that prior HCoV infection can modulate COVID-19 severity^{51–54}. By contrast, other studies found no significant correlation between prior anti-HCoV immunity and COVID-19 severity^{27,55,56}. Anti-SARS-CoV-2 IgG titers are higher in patients with prior seasonal coronavirus immunity⁵⁷ (Fig. 1), suggesting that the SARS-CoV-2 specific response is a recall-type response and should be protective. Cross-reactivity between OC43- and 229E-specific antibodies and SARS-CoV-2 proteins may be higher in children than in adults⁵⁸, probably due to the high frequency of respiratory illnesses during childhood^{59,60}. It has also been suggested that prior HCoV immunity in children is protective against SARS-CoV-2 infection, as children develop less severe forms of COVID-19 than adults⁶¹. Several regions in the S2 subunit of the spike protein are homologous between HCoVs and SARS-CoV-2, but there is no homology in the RBD region⁶² and specific antibodies directed against HCoVs cannot neutralize SARS-CoV-2 in vitro⁵⁷. Moreover, SARS-CoV-2 infection can occur in children regardless of their prior HCoV immunity⁶³ and specific antibodies against HCoVs in children are unable to block SARS-CoV-2-RBD-ACE2 interaction⁵⁸. Most of these observations were made with serum samples, but it is also very important to understand whether the mucosal response is induced similarly in all types of COVID-19 patients.

Box 1. Humoral immune response to other coronaviruses

To date, seven coronaviruses infecting humans have been identified. Four of these viruses (human coronavirus (HCoV) -229E, -NL63, -OC43, and -HKU1) circulate as endemic strains and cause relatively mild common cold symptoms¹⁶⁵. However, infection with these viruses can lead to the hospitalization of immunocompromised, elderly, or very young individuals^{59,166}. Children may be protected during the first three months after birth by anti-HCoV antibodies transferred from the mother⁵⁹. Anti-S IgG and IgM antibodies against the four HCoVs can be detected in children, their frequency increasing with age to a plateau at 6 years of age, whereas no anti-S IgM is detected in healthy adults, suggesting that most HCoV infections occur in infancy or early childhood^{59,167–169}. Nevertheless, binding, and neutralizing antibody titers are higher in older adults¹⁷⁰, in whom there is more anti-HCoV IgG in serum than specific IgA in nasal washes¹⁷¹. Antibody responses to HCoVs are not well maintained, and reinfections are common within 12 months¹⁷². Antibody repertoires against HCoVs differ qualitatively between children and adults. In children, anti-HCoV IgG antibodies target functionally important and structurally conserved regions of the spike, nucleocapsid, and matrix proteins¹⁷³.

The other three coronaviruses—the Middle East respiratory syndrome coronavirus (MERS-CoV), SARS-CoV, and SARS-CoV-2—can cause life-threatening respiratory infections^{165,174,175}. Serum from SARS-CoV-convalescent patients contains cross-reactive antibodies against other HCoVs^{176–178}, but not cross-neutralizing antibodies¹⁷⁹. Anti-N SARS-CoV-specific IgA, IgM and IgG antibodies in serum can be detected within 1 week of the onset of illness, and they peak at about day 15 post-symptom onset (PSO)^{180–182}. SARS-CoV-specific IgM antibodies are detected until 7 months PSO, but titers decline after the first month. By contrast, neutralizing antibody and SARS-CoV-specific IgG antibody titers remain stable over this period^{181–183}. Anti-SARS-CoV IgA avidity remains low in a proportion of patients, even during late convalescence, whereas IgG avidity increases with time until 9 months PSO¹⁸².

MUCOSAL HUMORAL IMMUNITY IN COVID-19

SARS-CoV-2 displays pulmonary tropism. Mucosal immune responses therefore probably play a crucial role in preventing the entry and spread of the virus Box 1. Only a few studies have investigated the kinetics and quality of the mucosal immune response to SARS-CoV-2. Mucosal responses, particularly anti-spike IgM, are inversely correlated with the viral load in nasopharyngeal swabs, indicating that a strong early nasal antibody response may play a key role in limiting disease by initiating or facilitating early

viral clearance⁶⁴. An association has also been reported between a strong nasal antibody response, specifically anti-RBD IgA, and the resolution of systemic symptoms, such as fatigue, fever, headache, dizziness, joint or muscle pain, and swollen lymph nodes⁶⁴. Early control of viral replication in the upper respiratory tract, reducing the spread of the virus to the periphery, thereby limiting systemic symptoms, might account for this association. Specific anti-SARS-CoV-2 antibodies have been detected in the saliva^{31,42,65}, nasopharynx^{6,38,64,66–69}, bronchoalveolar lavages (BAL)³¹ and trachea of children⁷⁰, and these antibodies are directed against S, RBD and N proteins^{6,31,42,64,65,68}. Limited data are available for stool samples, with only one study reporting minimal differences in anti-S IgA and IgG antibodies between infected and non-infected patients⁷¹, and another study detecting anti-RBD IgA antibodies in 11% of patients, particularly those with the most severe disease or presenting diarrhea⁷². Total IgM and IgG (including IgG subclasses) levels in nasal fluid, like those in serum, are similar in healthy donors and COVID-19 patients. By contrast, total IgA levels increase with disease severity⁶. As in the systemic humoral immune response, specific antibody titers in mucosal tissues are higher in moderate/severe disease than in asymptomatic/mild disease, over the course of the infection^{6,7,66} (Fig. 1). Interestingly, anti-SARS-CoV-2 IgA titers in mucosal tissues are inversely correlated with age in mild disease patients with low or absent specific serum response³⁸. This can be explained, as suggested by Cervia et al. in 2021, that the extent and the duration of clinical symptoms modulate the immune response³⁸ and that a good mucosal immune response may be sufficient to control viral replication when presenting mild or asymptomatic disease. However, the mucosal immune response provides insufficient protection against infection with the Omicron variant. SARS-CoV-2-infected children have very low levels of neutralizing antibodies in the lower respiratory tract⁷⁰. Ravichandran et al. used genome fragment phage display library (GFPDL) technology to study epitope recognition by specific IgM, IgG and IgA antibodies in nasal secretions³⁴. They found that IgM, IgA and IgG responses generally targeted a broader spectrum of epitopes in the NTD, RBD, FP, heptad repeat 1 (HR1) and HR2 regions distributed throughout the S1 and S2 subunits of the spike protein, whereas the systemic response was essentially restricted to S2³⁴. Interestingly, both monomeric and dimeric IgA were detected in the BAL of COVID-19 patients³¹ suggesting that anti-SARS-CoV-2 antibodies in mucosal tissues may have different origins. Most of the dimeric IgA probably arises locally, but plasma monomeric IgA can reach the airways through a receptor-independent process called transudation, which is more likely to occur in damaged lung tissue, as observed in patients with severe COVID-19^{73,74}.

As described for other infections⁷⁵, the IgG/IgA ratio follows a gradient down the respiratory tract, with higher titers of IgA than of other isotypes in saliva, but higher IgG titers than IgA titers in BAL³¹. IgG has been described as the predominant immunoglobulin in the saliva at the start of the disease⁷ (Fig. 2). Indeed, anti-SARS-CoV-2-specific IgG levels peak between days 31–45 PSO, and disappear at about day 106–115 PSO, whereas IgM and IgA appear at about day 20 PSO but their levels decrease more rapidly than those of IgG⁶⁵. Specific anti-RBD IgA levels were found to be higher in saliva than in serum after day 49 PSO³¹, whereas airway-specific IgA and IgG levels declined significantly within three months of infection⁶⁹. However, these antibodies remained detectable until nine months PSO^{42,64}. As in serum, the milder the disease, the shorter the life of the specific antibodies detected in mucosal tissues⁴².

In conclusion, systemic and mucosal responses vary according to disease severity and infection kinetics. When studying the humoral immune response to SARS-CoV-2 infection, we are monitoring the strength of the immune response triggered by the infection rather than the quality of this response to protect

patients against the more severe disease. Even though there could be detrimental features of the individual immune response that could hamper the good evolution of the disease, what is the ultimate role of antibodies during the infectious process?

ANTIBODY-DEPENDENT PROTECTIVE FUNCTIONS IN COVID-19

Fc-effector functions, such as ADP, have been associated with protection against other coronaviruses or HIV. Shiakolas et al. showed that six monoclonal antibodies from SARS-CoV patients that cross-reacted with SARS-CoV-2 virus-induced ADP but not neutralization in vitro, and that this Fc-effector function was associated with milder hemorrhagic disease in mouse lungs⁷⁶. Furthermore, in a nonhuman primate model, Spencer et al. showed that the bNAb 10E8v4 displayed ADP reducing HIV viremia in non-neutralizing conditions⁷⁷.

In vivo studies of SARS-CoV-2 infection have suggested that the humoral immune response is protective. For instance, a mixture of S309 and S2M11 monoclonal antibodies isolated from convalescent individuals and with different mechanisms of binding to the SARS-CoV-2 spike protein yielded additive neutralizing effects and elicited robust ADCC and ADP in hamsters^{78,79}. In an FcγR-humanized mouse model, Yamin et al. showed that the REGN monoclonal antibody cocktail (currently in clinical use) induced protection against lethal SARS-CoV-2 challenge and that this protection was dependent on Fc-FcγR interaction, suggesting that Fc-effector functions improve the efficacy of REGN treatment⁸⁰. Furthermore, McMahan et al. showed that, following the passive transfer of an IgG pool from rhesus macaques convalescing from SARS-CoV-2 infection to naïve macaques, IgG1 levels, neutralizing titers, ADCC and ADP were associated with protection against viral challenge⁸¹.

Anti-SARS-CoV-2 antibody levels gradually decrease in the serum of patients, but both neutralizing antibody titers and robust specific memory B-cell responses are detected at 5 and 9 months PSO in patients who have had moderate and severe COVID-19, and they remain detectable at 1 year PSO, probably contributing to protection against reinfection^{14,30,39,42,82} (Fig. 3). During SARS-CoV-2 infection, the quality of antibody effector functions is correlated with their magnitude⁶⁷, their specificity for RBD in both serum and nasal secretions^{6,14}, and with disease severity⁷¹. Different studies have shown that there is a dysfunction of NK cells in severe COVID-19 preventing the correct ADCC effect to occur^{40,83}. In patients with milder infections, as in women, NK-dependent ADCC activity is overrepresented relative to neutralization. The opposite situation is observed in patients with more severe disease and in men⁴⁰, suggesting that antibody effector functions other than neutralization contribute to better protection. In particular, Witkowski et al. demonstrated that the uncontrolled TGF-β secretion by infected cells in severe patients, inhibits the expression of the integrin-β2 in NK cell surfaces making them unable to bind and kill infected cells⁸³. Still, more studies are needed to decipher why there is an increase in TGF-β levels in severe COVID-19 patients and not in mild or asymptomatic patients.

Lee et al. showed that convalescent patients presented a significant decline in plasma S-specific antibody-dependent ADCC and ADP activity over time, but that these responses remained detectable for longer than neutralization⁸⁴, suggesting the involvement of a different antibody repertoire. Studies with RBD-specific monoclonal antibodies isolated from convalescent or infected patients (such as the S2H13, S2H14, S2X35, and S309), have shown that because of the different orientation of the complex spike-monoclonal antibody relative to the FcR, only S309 and S2H13 were capable to produce effective Fc-dependent functions¹⁶. Anti-S1 and anti-RBD specific antibodies from hospitalized COVID-19 patients elicit higher levels of antibody-dependent complement deposition (ADCD) and ADCC, but

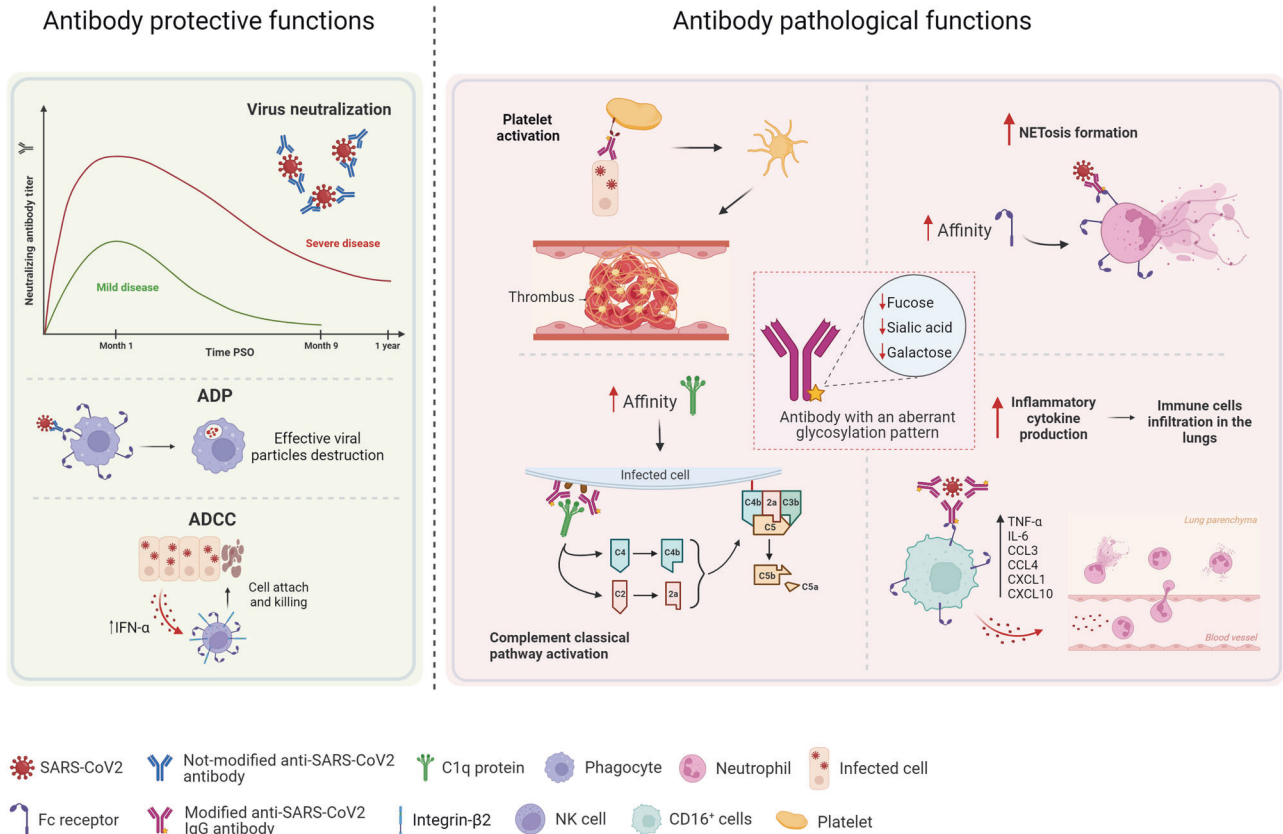


Fig. 3 Protective and pathological functions of SARS-CoV-2 antibodies. Anti-SARS-CoV-2 antibodies can exert protective functions, such as neutralization, antibody-dependent phagocytosis (ADP) and antibody-dependent cellular cytotoxicity (ADCC). Neutralizing antibody titers are higher in patients with severe disease than in those with mild disease patients, and these antibodies are detected until 1-year post-symptom onset (PSO); however, their levels decrease more rapidly in patients with mild disease. Serum from non-hospitalized patients and convalescent plasma from patients who have recovered display more Fc-dependent effector functions, such as ADP and ADCC, than serum from hospitalized patients. The pathological effects of antibodies in COVID-19 are related in part to aberrant glycosylation patterns, which are observed in the anti-SARS-CoV-2 IgG antibodies of patients with severe, but not mild disease. These structural modifications can trigger inflammatory processes, such as cytokine production, immune cell infiltration into the lungs, or platelet-mediated thrombosis. These aberrant glycosylation patterns increase the affinity of antibodies for the C1q protein, thereby leading to activation of the classical complement pathway, and increases in affinity for Fc γ R enhance Fc-dependent functions, such as neutrophil extracellular trap formation (NETosis), which is associated with higher levels of inflammation. (Created with www.Biorender.com).

lower levels of ADP than antibodies from non-hospitalized and convalescent COVID-19 patients^{85,86} (Fig. 3). In parallel, higher levels of ADCC are associated with higher levels of systemic inflammation, whereas higher levels of ADP are associated with milder systemic inflammation during COVID-19⁸⁵. In addition, Adeniji et al. reported an absence of differences in anti-S or anti-RBD IgG antibody levels likely to account for the differences in ADCC or ADCC between hospitalized and non-hospitalized patients⁸⁵. Overall, these data indicate that as far as SARS-CoV-2 IgG antibodies are concerned, it is quality rather than quantity that determines disease outcome.

Less is known about the Fc-effector functions of antibodies against SARS-CoV-2 in mucosal tissues, and most studies to date have focused on antibody neutralization capacity. Robust neutralization and ADP were detected in nasal washes from convalescent individuals who had experienced mild or severe disease^{67,86}. In COVID-19 patients, mucosal neutralization has been shown to be associated with nasal^{67,72} and saliva-specific IgA³¹, and with RBD-specific IgM⁷¹. Anti-RBD IgA antibodies are more neutralizing than IgG in BAL, suggesting that IgA is more important for this function in the lung³¹. In addition, Butler et al. showed that depleting IgG antibodies from the nasal washes of convalescent patients decreased ADP but did not affect the neutralization of the virus. By contrast, IgA depletion decreased

neutralization capacity without modifying ADP activity⁶⁷. These observations provide support for the notion that IgA is a key factor in the neutralization of the SARS-CoV-2 virus in the upper respiratory tract, as reported for other coronaviruses in human and animal models^{87,88}. In breast milk, anti-spike IgA titers and neutralization capacities are strongly positively correlated and are sustained for up to 10 months PSO^{89–91}, which suggests that breastfeeding may protect newborns, consistent with observations that breastfed newborns rarely experience severe COVID-19^{92,93}.

Questions have been raised about the protective functions of antibodies in the face of the increasing number of variants during the SARS-CoV-2 pandemic. Studies in both animal models and humans have shown that neutralizing antibodies elicited against one variant have a lower capacity to neutralize other variants. In a Syrian hamster model, Mohandas et al. showed that infection with the Omicron (B.1.1.529) variant generated antibodies that were less effective for the neutralization of Alpha (B.1.1.7) strains and unable to neutralize the Beta (B.1.351) and Delta (B.1.617.2) strains⁹⁴. Some studies in humans have also reported a lower capacity of convalescent plasma from the first wave of the pandemic to neutralize later SARS-CoV-2 variants, such as Beta (B.1.351) and Delta (B.1.617.2)^{34,41,86,95,96}. This effect can be accounted for by the differences in spike mutations between

variants. For example, Laurie et al. demonstrated that variants with mutations at the E484 position of the spike protein (such as Zeta (P.2), Iota (B.1.526), B.1.351, or B.1.617.2) were better neutralized by serum from E484K-exposed patients than other variants⁹⁷. However, sequential boosters with different variants strengthen the neutralizing capacity of the antibodies elicited. In vitro studies showed that six monoclonal antibodies isolated from convalescent patients (2K146, S2X324, S2N28, S2X259, sotrovimab, and S2H97) cross-react and neutralize the Omicron variant as well as SARS-CoV virus⁹⁸. Even though these antibodies target different epitopes, they are all highly conserved between viral variants⁹⁸, indicating that a broader response can overcome antigenic shift. Accordingly, the Omicron variant is better neutralized in vitro by serum from convalescent vaccinated individuals, or from individuals inoculated with three doses of vaccine^{97–99}. Similar observations have been reported for other viral infections, such as dengue, in which the cross-reactive antibodies elicited after a secondary heterologous infection have a higher avidity and protect against a third infection¹⁰⁰.

Fc-effector functions are also modified by the presence of new variants. A recent study showed that serum from DG14G-infected patients displayed a smaller decrease in ADP, ADCC, ADCD and ADCT (antibody-dependent cellular trogocytosis) against the Beta and Delta patients than serum from Beta-infected patients. In addition, ADCD was the Fc-effector function most affected, consistent with an epitope-based Fc-dependent response¹⁰¹. The greater cross-reactivity of serum from Beta-infected individuals suggests that ADP, ADCC, ADCD and ADCT are less affected by variants. Mutations affecting the RBD and NTD regions targeted by ADCC were found to have no effect on this function, whereas they did alter neutralization, suggesting that Fc-dependent effector functions may make a greater contribution to decreasing the number of severe cases after infection with a variant than neutralization alone¹⁰¹. As demonstrated for other viruses, such as dengue and MERS, it is important to understand whether these antibodies can facilitate infections (ADE) under certain conditions or contribute to disease severity.

ANTIBODIES IN COVID-19 PATHOPHYSIOLOGY

Severe COVID-19 is characterized by dysregulation of the immune response, with the development of a cytokine storm responsible for rapid disease progression to ARDS and death in some patients. Several studies have reported an association between disease outcomes and dysregulation of the immune system, particularly in terms of the innate response^{102,103}. However, the intrinsic mechanisms of COVID-19 immune pathophysiology are not fully understood.

Neutrophils are the most abundant leukocytes and are often the first to respond to injury and infection¹⁰⁴. They express the Fc alpha receptor (FcαRI/CD89) and can perform various effector functions, including ADP and neutrophil extracellular trap formation (NETosis)^{105,106}. In vitro studies have also shown that serum IgA antibodies are more potent than IgG for stimulating NETosis for many types of viral particles, including the SARS-CoV-2 virus^{107,108} (Fig. 3). These studies also showed that NETosis could be potentiated by monomeric but not secretory IgA from saliva, suggesting that the secretory component may be involved in steric interference with binding to FcαRI¹⁰⁸. These data suggest that NETosis may occur in tissues and in the vasculature, but probably not in the airways. The NETs in serum from patients suffering severe COVID-19 have a lower degradation capacity than those from asymptomatic patients and patients with mild symptoms¹⁰⁹, suggesting that NETs may persist in the tissues for longer periods, resulting in local inflammation. Furthermore, plasmablasts from patients with severe disease, rapidly switch to the IgA2 isotype after stimulation with IL-21 and TGF-β¹¹⁰. This isotype has been shown to be associated with NETosis in patients

with severe COVID-19¹¹¹ or rheumatoid arthritis (RA)¹¹². The IgA1 isotype is the most abundant IgA isotype in the blood of COVID-19 patients. No data are available concerning the levels of the IgA1 and IgA2 isotypes in mucosal secretions. The differences in glycosylation pattern between IgA1 and IgA2 may underlie the greater stimulation of NETosis by IgA2 than by IgA1¹¹². IgA2 is less sialylated than IgA1, and the desialylation of IgA1 has also been reported to increase NETosis and IL-8 production by macrophages in vitro¹¹². It remains unknown whether IgA1 glycosylation patterns are modified in COVID-19 patients, but these data are potentially important, to shed light on the pathological role of IgA isotypes associated with disease severity.

Another pathological effect of the immune response observed in some patients with severe COVID-19 is a small-vessel vasculitis driven by deposits of IgA-C3 immune complexes (ICs)¹¹³. IgA vasculitis, formerly known as Henoch–Schönlein purpura (HSP), has also been reported in children with COVID-19, suggesting that SARS-CoV-2 infection may be a trigger¹¹⁴. SARS-CoV-2 may induce the secretion of cytokines, such as IL-6, affecting the glycosylation machinery and leading to the synthesis of galactose-deficient IgA1, which then forms IgA-ICs in blood¹¹⁵. Complement activation can result in protective effector functions and pathogen clearance. However, during SARS-CoV-2 infection, deposition of the complement components C4b and C3bc, and of the terminal complement complex (TCC) was restricted to anti-RBD IgG antibodies¹¹⁶. These deposits were correlated with both IgG levels and disease severity¹¹⁶. In addition, the IgG response to SARS-CoV-2 RBD after natural infection appears to be based on the IgG1 and IgG3 isotypes, which are the main ligands for C1q-mediated classical complement pathway activation^{40,116}. The aberrant immune responses produced during SARS-CoV-2 infection in patients with severe disease may be explained by structural deficiencies in anti-SARS-CoV-2 specific antibodies (Fig. 3). Lower levels of galactosylation have been observed for all IgG subclasses in patients with severe disease, but not in convalescent patients or patients with mild disease, and the capacity of IgG to bind to FcγRs and C1q is positively correlated with disease severity¹¹⁷. Furthermore, ICs composed of the trimeric spike protein and afucosylated anti-SARS-CoV-2 IgG isolated from COVID-19 patients induce the production of TNF-α, IL-6, CCL3, CCL4, CXCL1, and CXCL10 and robust neutrophil infiltration in the lungs of mice¹¹⁸. In vitro studies have shown that the ICs formed by the SARS-CoV-2 S protein and anti-S IgG antibodies enhance platelet-mediated thrombosis in a FcγRIIA-dependent manner, but only when the Fc domain of the anti-S IgG is modified to match the aberrant glycosylation pattern identified in patients with severe COVID-19¹¹⁹. Low levels of anti-S IgG fucosylation may return to normal within a few weeks of initial infection with SARS-CoV-2, indicating that higher levels of antibody-dependent inflammation occur principally at the time of seroconversion¹²⁰. Nevertheless, early-phase S-specific IgG in the serum of patients with severe COVID-19 induces the production of IL-1β, IL-6, IL-8, and TNF by human alveolar macrophages, which may then breach pulmonary endothelial barrier integrity and induce microvascular thrombosis in vitro¹²⁰. But, can we transpose the observations made during infection to vaccination?

HUMORAL IMMUNE RESPONSE TO VACCINATION

In the face of the pandemic, a number of vaccines were developed very rapidly, with at least three rapidly approved in most countries^{121,122}. More than 11.3 billion doses have been administered worldwide to date¹²³, with demonstrated efficacy against the various VOCs, including the Omicron variant. However, we will need a better understanding of the immunological fingerprint of these vaccines to improve vaccine design, and vaccination policies to increase protection against new variants. After full vaccination with BNT162b2 and CoronaVac, lower serum anti-spike and anti-

RBD IgG concentrations and neutralization capacities have been shown to be associated with being male, older age, immunosuppression, and comorbid conditions such as diabetes, hypertension, heart disease, and autoimmunity^{124–126}. However, after the second dose, a similar protective response is observed in individuals of all ages, highlighting the importance of booster doses for the elderly^{124,127}. Subsequent immunizations, whether natural (through infection) or by vaccination, can modulate the immune response to SARS-CoV-2. Following the second dose of BNT162b2 or mRNA-1273, individuals convalescing from COVID-19 develop higher titers of anti-spike IgG and IgA antibody titers with potent neutralization capacity in serum than individuals who have not had COVID-19^{125,128–130}. In addition, 5 months after the boost, memory antibodies from convalescent vaccinated patients have an improvement in neutralizing activity compared to 1-month post-boost, not been like this for naïve vaccinated individuals¹³⁰. Several studies, including that by Wei et al.¹²⁵, have shown that unvaccinated individuals with prior infections have lower anti-spike IgG titers than vaccinated individuals (with the BNT162b2 or ChadOx1 vaccine)¹³¹, but that they also require lower antibody concentrations to achieve the same level of protection¹²⁵. Indeed, the better and broader response after vaccination in naïve individuals compared to infected patients has the basis in the formation of germinal centers (GCs)^{131–134}. While GCs in vaccinees are well formed and B and Tfh cells are greater stimulated, there is an impairment of GCs in patients with severe disease¹³¹. This suggests that protection depends on the mechanism of antibody generation and that the antibodies generated after infection and vaccination differ in quality.

One major unanswered question concerns the cross-protection against new variants conferred by vaccination. One dose of the BNT162b2 mRNA vaccine elicited a better neutralizing response to B.1, Gamma (P.1), and Delta (B.1.617.2) variants in seropositive than in seronegative individuals or non-vaccinated patients hospitalized for COVID-19¹³⁵. The Moderna mRNA-1273 vaccine elicits a SARS-CoV-2-specific antibody response equivalent to that induced by natural infection, targeting wild-type virus and the Alpha, Beta, Gamma, and Delta variants¹²⁹. Individuals with pre-existing immunity vaccinated with mRNA-1273 had higher titers of IgG1 and IgA against all variants (WT, Alpha, Beta, Gamma, and Delta) than fully vaccinated naïve individuals, indicating that hybrid infection/mRNA vaccine-induced immunity triggers cross-reactive antibody responses¹²⁹. Furthermore, individuals infected with the Delta variant after BNT162b2 or mRNA-1273 vaccination were found to have higher anti-RBD IgG titers and higher viral neutralization capacities than vaccinated individuals without prior infection¹³⁶, again suggesting that hybrid immunity triggers a robust anamnestic response, probably contributing to the lower risk of severe disease after vaccination. The vaccination of naïve individuals with three doses, or of previously infected individuals with two vaccine doses as a booster has been shown to enhance the anti-SARS-CoV-2 immune response and, thus, protection as shown by recent studies in which such strategies reduced the loss of neutralization function against various VOC, including Omicron^{97,136–139}. In particular, this enhancement is due to an increase and evolution of RBD-¹³⁷ and NTD-¹⁴⁰ specific memory B cells, notably new clones that developed after the third dose targeting more conserved regions of the RBD¹³⁷. Indeed, Kim et al. demonstrated that 6 months after vaccination, spike-specific memory B cells and long-lived bone marrow plasma cells had increased levels of somatic hypermutation, thus producing anti-spike antibodies with increased affinity and avidity as well as neutralization capacities¹³². Heterologous or multivalent boosting strategies may, therefore, be important for increasing protection against new variants, because the exposure to multiple spike variants expands the breadth of neutralization^{97,136}. We recently demonstrated this in a study in which serum from individuals with heterologous vaccination schedules (ChadOx1/BNT162b2) had

stronger neutralizing activity than serum from individuals with homologous vaccination schedules (BNT162b2/ BNT162b2), regardless of the SARS-CoV-2 variant¹⁴¹. Furthermore, studies in vitro and in vivo with the monoclonal antibody S2P6, isolated from a convalescent patient, demonstrated that targeting highly conserved epitopes in the spike protein (stem helix in this case) is crucial for protection mediated by neutralization and Fc-effector functions¹⁴².

Importantly, mRNA vaccination against COVID generates structurally different antibodies, with effector functions different from those of the antibodies generated in response to natural infection, accounting for the efficacy of SARS-CoV-2 vaccination for reducing the likelihood of severe disease. Vaccination induces more sialylated, fucosylated, and galactosylated antibodies, especially IgG1, than natural infection leading to severe disease^{117,118}. These structural modifications increase the ability of anti-SARS-CoV-2 IgG antibodies to engage in FcγR pathways, which may serve as another mechanism for reducing infection, in addition to their Fab-mediated neutralization activity¹¹⁷. Kaplonek et al. showed that RBD-specific antibody depletion from vaccinated patients had little or no impact on Fc-dependent effector functions, such as ADCC or ADCP, against the Alpha, Beta, Gamma, and Delta variants. Furthermore, plasma from vaccinated individuals displayed high levels of C1q and C3d binding to spike-specific antibodies, or RBD-specific antibodies, unlike convalescent plasma¹⁴³. This difference can be explained by the higher levels of IgG1 and IgG3 elicited by vaccination than by natural infection and by the fact that these isotypes have a higher potency for activating the complement cascade^{86,117}. Therefore, even if the spike mutations found on VOCs decrease vaccine efficacy by reducing neutralizing activity, Fc-dependent effector functions may also contribute to the lower incidence of severe disease after vaccination.

SARS-CoV-2 is a respiratory virus, and the induction of a mucosal immune response is, therefore, crucial to prevent infection. Specific antibody levels are not high in the mucosa, but such antibodies are nevertheless detected. Anti-spike and anti-RBD IgG levels in saliva are almost two orders of magnitude lower than those in serum. Anti-spike and anti-RBD IgA antibodies have also been detected at very low levels in the saliva, these levels peaking after the second dose of BNT162b2 mRNA vaccine¹⁴⁴. Low levels of neutralizing antibodies have also been detected in saliva, nasal swabs, and nasopharyngeal lavages after mRNA vaccination^{69,144}. However, a stronger mucosal response has been measured locally in seropositive than in naïve vaccinated individuals^{69,83,145}, suggesting that hybrid immunity effectively promotes and stimulates the anamnestic response in mucosal tissues. Nevertheless, anti-S1 IgA antibodies with neutralizing capacity were present in the saliva at lower levels for a shorter time after BNT162b2 vaccination than after natural infection, possibly because the route of vaccine administration is different from the natural route of infection^{127,146}. Little is known about the functionality of the mucosal immune response against the various VOCs described to date, but cross-neutralization in the mucosa is expected to be much lower than that against the initial strain, in line with findings for the systemic immune response. Indeed, Garziano et al. reported a fourfold reduction in neutralization titers against the Delta variant in saliva from infected and vaccinated individuals¹⁴⁷.

Antibody-dependent enhancement (ADE) has been reported to facilitate infections with different viruses, including coronaviruses, such as MERS. Some studies have reported that, in subneutralizing concentrations, serum from convalescent COVID-19 patients can enhance SARS-CoV-2 infection in vitro via the FcγR or C1q protein, suggesting that ADE may promote SARS-CoV-2 infection, particularly after vaccination or prior infection^{148–150}. Others have suggested that neutralizing and enhancing antibodies recognize different epitopes^{151–154}. Liu et al. showed that monoclonal

antibodies from COVID-19 patients targeting the NTD region in the spike protein enhanced SARS-CoV-2 infection in vitro through conformational changes induced in the RBD by the antibody binding, facilitating the Spike-ACE2 interaction¹⁵¹. In addition, Lempp et al. demonstrated, using in vitro models, that monoclonal antibodies also directed against the NTD region or against a conserved site at the base of the RBD cannot inhibit viral infection in ACE2 overexpressing cells, however, they can block the lectin-facilitated infection¹⁵⁴. On the contrary, monoclonal antibodies specific to the receptor-binding motif (RBM) effectively block ACE2-dependent infection but do not neutralize lectin-dependent infection promoting cell-to-cell fusion¹⁵⁴. Serum from convalescent, but not vaccinated individuals can mediate FcγR-dependent virus uptake by monocytes in vitro without infectious viral particle production, but pyroptosis may be triggered, increasing inflammation and COVID-19 pathogenesis¹⁵⁵. By contrast, other in vitro studies reported no ADE in convalescent sera, even though at subneutralizing concentrations^{156,157}. It has been suggested that ADE could contribute to SARS-CoV-2 infection in vitro, but preclinical and clinical evidences suggest that this is not the case in vivo. Studies performed in vivo in macaques and mice have shown that antibodies directed against the RBD and NTD do not increase infection rates or disease severity¹⁵³. Thus, even if ADE is sometimes reported in vitro, other Fc effector functions may make a positive contribution in vivo. Furthermore, vaccination/challenge studies in nonhuman primates revealed no increase in disease^{158,159}. In addition, the risk of severe disease is not higher in COVID-19 patients treated with convalescent plasma^{160–162}, or in vaccinees. Moreover, even if new variants, such as Omicron, decrease the efficacy of the neutralizing antibodies elicited by vaccination, no enhancement of disease has yet been reported^{162–164}.

In general, the intramuscular vaccines currently available reinforce systemic immune responses, but only weakly activate existing mucosal responses in previously infected individuals. This may account for the lack of sterilizing immunity to SARS-CoV-2 and subsequent infections even after vaccination. There is, therefore, an urgent need to improve the SARS-CoV-2 vaccine design to target the mucosal immune response more effectively.

CONCLUSIONS AND PERSPECTIVES

Many studies have demonstrated the protective function of the humoral immune response in COVID-19, and after vaccination. Neutralizing antibodies are known to protect against infections, but they are also associated with severe disease in COVID-19. After vaccination, neutralizing antibody titers are correlated with protection against severe disease in most cases, but infections occur due to poor activation of the specific mucosal immune response. Nevertheless, Fc-effector functions, such as ADCC, seem to contribute to protection even in the presence of new variants. However, if SARS-CoV-2 IgG antibodies have an aberrant glycosylation pattern, Fc-mediated protective functions are dysregulated, contributing to disease severity. The jury is still out as to whether the humoral immune system is “guilty” in the case of SARS-CoV-2 infection, but it appears to be a matter of balance, and additional studies are required to shed light on the factors influencing this dual role of antibodies in COVID-19. A full understanding of the humoral immune response to SARS-CoV-2 will be a major asset in the face of possible future coronavirus pandemics.

Despite all the progress made, many questions about the humoral immune response to SARS-CoV-2 remain unanswered. What are the intrinsic factors influencing the uncontrolled humoral immune response in some patients that lead them to develop the severe disease? IgG glycosylation modifications can hamper the immune response in COVID-19, but do IgA antibodies also have aberrant glycosylation patterns in severe disease patients that contribute to the pathophysiology of the disease? Anti-Spike antibodies are the most likely marker of correlates of

protection in SARS-CoV-2 infection. However, the presence of viral variants with different neutralization sensitivities, differences in vaccine formulations, and the fact that antibodies alone cannot fully explain immune protection let a question open: what constitutes an immune correlate of protection for SARS-CoV-2 infection? If we want to get to a sterilizing immunity against SARS-CoV-2, next-generation vaccines should target the mucosal immune response and should include more conservative epitopes that cover a broader spectrum of SARS-CoV-2 variants.

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AUTHOR CONTRIBUTIONS

M.Y.N., T.B., and S.P. analyzed the data. M.Y.N., T.B., and S.P. wrote the paper. All the authors approved the final version of the paper.

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The authors declare no competing interests.

ADDITIONAL INFORMATION

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Annexe 2: New method to detect FcR-dependent functions
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Article

Antiviral Activities of HIV-1-Specific Human Broadly Neutralizing Antibodies Are Isotype-Dependent

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Abstract: Broadly neutralizing antibodies (bNAbs) offer promising opportunities for preventing HIV-1 infection. The protection mechanisms of bNAbs involve the Fc domain, as well as their Fab counterpart. Here, different bNAb isotypes including IgG1, IgA1, IgA2, and IgA122 (IgA2 with the hinge of IgA1) were generated and then produced in CHO cells. Their ability to neutralize pseudovirus and primary HIV-1 isolates were measured, as well as their potential ADCC-like activity using a newly developed assay. In our work, gp41-specific IgA seems to be more efficient than IgG1 in inducing ADCC-like activity, but not in its virus neutralization effect. We show that either gp120-specific IgA or IgG1 isotypes are both efficient in neutralizing different viral strains. In contrast, gp120-specific IgG1 was a better ADCC-like inducer than IgA isotypes. These results provide new insights into the neutralization and ADCC-like activity of different bNAbs that might be taken into consideration when searching for new treatments or antibody-based vaccines.

Keywords: HIV; broadly neutralizing antibodies; gp41; gp120; ADCC; neutralization



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1. Introduction

Immunoprophylaxis with potent bNAbs efficiently protects non-human primates from mucosal transmission even after repeated challenges [1,2]. However, the precise mechanisms of bNAb-mediated viral inhibition in mucosal tissues are currently under-investigated. Constant heavy-chain domains, such as the CH1 domain, modify antibody affinity and specificity, which demonstrates that not only variable regions contribute to antigen binding [3–9]. Increasing evidence shows that non-neutralizing Abs play a significant role in decreasing the viral load, leading to partial and sometimes even total protection. The mechanisms suspected to participate in protection involve the Fc domain of Abs, as well as their Fab counterpart. Consequently, the induced Ab isotype will be determinant for their functions, as well as the quantity and quality of the Fc receptors (FcRs) expressed on immune cells. Fc-mediated inhibitory functions, such as Ab-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), aggregation, and even immune activation, have been proposed [4,5,10–13]. It has been demonstrated that longer hinges are important in the phagocytic activity of IgG1 and IgG3 isotypes, but not for ADCC or neutralization [14]. Recently, Duchemin et al. compared IgA2 and IgG1 isotypes from the 2F5 antibody and showed that the IgA2 isotype induced better ADCC and ADCP effects than the IgG1 isotype [15,16]. Moreover, Jia et al. in 2020 isolated, from chronically infected donors, two bNAbs B cell lineages that class-switched to IgG and IgA. IgA variants reconstituted from these bNAbs had broadly neutralizing activity as well

as plasma IgA fraction [17]. These recent findings highlight the importance of IgA- and IgG-related Fc-effector functions. The RV144 HIV vaccine clinical trial identified plasma IgA responses to HIV Env as correlating to increased HIV acquisition and a decrease in the *in vitro* ADCC activity of vaccine-induced Env-specific IgG [18].

Here, we conducted a deeper analysis of the isotype impact on the function of bNAbs targeting the gp120 and gp41 regions of HIV-1 Env. The increased interest in understanding the role of ADCC in preventing and controlling HIV-1 infection leads us to assess this function of a panel of antibodies [11]. We studied a panel of Env-specific bNAbs (2F5, B12, PG16, PGT126, PGT128, PGT145, NIH45-46, and 10E8 (Table 1)) for which the ability to mediate non-neutralizing functions is not completely described. IgG1, IgA1, IgA2, and IgA122 (IgA2 with the hinge of IgA1) for the 2F5 bNAb family; IgG1, IgA2, and IgA122 for B12; and IgG1 and IgA122 for the rest of the bNAbs were used to compare their ability to neutralize pseudoviruses and primary HIV-1 isolates, as well as their potential ADCC activity in a new potent gp120- and gp41-targeted assay.

2. Materials and Methods

2.1. Cells

TZM-bl and CHOgp140 cell lines were obtained from the NIH AIDS Reagent Program. HEK 293-gp41MSD (Membrane Spanning Domain) has been previously described [19]. CHO-gp140 and HEK 293-gp41 express gp140 or gp41 in its trimeric form. HEK CD89⁺ and HEK CD16⁺ cell lines were obtained from InvivoGen.

2.2. Construction and Production of Recombinant IgA1, IgA2, IgA122, and IgG

CHO cell lines were transfected with two plasmids: pFUSE-CHIg and pFUSE2-CLIg. These plasmids express the constant regions of the heavy (CH) and light (CL) chains, respectively. Transfection of the CHO cell lines with the recombinant pFUSE-CHIg and pFUSE2-CLIg pair allows the generation of an Ig antibody. Antibodies were purified from the supernatant using the appropriate protein affinity chromatography. IgG1 was purified with protein G, and IgA with peptide M. IgA122 was obtained by replacement of the IgA2 hinge region with the IgA1 hinge region. The purity of recombinant Abs was analyzed on SDS-PAGE in reducing conditions, and the gel was stained with Coomassie Blue.

2.3. Analysis of the Specificity of the Different bNAb Isotypes

Target cells were incubated with broadly neutralizing antibodies (bNAbs) IgA1, IgA2, IgA122, or IgG1 at 5 µg/mL, followed by an anti-human IgGAM (fluorescein isothiocyanate (FITC); Abliance). Antibody binding to target cells was quantified by flow cytometry using a CantoII flow cytometer (BD), and the geometric mean fluorescence intensity (gMFI) was calculated using the software FlowJo, version 10.6.2 (BD).

2.4. HIV-1-Specific Neutralization Assays

Viral primary isolates of clades A (92UG029), B (SF162, 92US660), and D (92UG001) were obtained from the NIH AIDS Reagent Program. Pseudoviruses were produced by co-transfection of the backbone plasmid PSG3Δenv (NIH) and the envelope plasmid of clade B (11035, 11036, and 11307) or clade C (11312) (NIH) in the HEK293 cell line.

2.5. TZMbl/Pseudovirus Assay

Pseudoviruses were incubated with the bNAbs at different concentrations for 2 h, and these mixtures were added to the cells (10,000 cells/well) plated in 96-well plates for 24 h. After 48 h of infection, luciferase activity was used to quantify the infected TZM-bl cells by adding the luciferase substrate, Bright Glo (Promega), and the luminescence was measured (Berthold Technologies ref Tristar LB941). Neutralization activity directly correlates with the reduction in luciferase reporter gene expression after a single round of virus infection in TZMbl cells by the pseudoviruses of panels B and C.

2.6. PBMC/Primary Isolate Assay

Neutralization assay for the primary isolates of clades A (92UG029), B (SF162, 92US660), and D (92UG001) was performed using a pool of PBMCs isolated from healthy donors and stimulated with Phytohemagglutinin (PHA) (2 µg/mL) and recombinant human IL-2 (R&D Systems 202IL) (200 U/mL). A dose of 100 TCID₅₀ of primary isolate virus were incubated for 2 h with the bNAbs at different concentrations, and with these mixtures, PBMCs were infected for 3 h at 37 °C. The amount of p24 in the supernatants was quantified using an ELISA (InfYnity biomarkers). The percentage of neutralization and EC₅₀ were calculated as previously described [20].

2.7. HIV-1 Env-Dependent Antibody-Dependent Cellular Cytotoxicity (ADCC)-like Assay

ADCC-mimicry assay was performed using cells expressing HIV-1 Env (HEKgp41 or CHOSEC/CHOgp140) [21] as target cells. Target cells were first incubated with different bNAb concentrations. Effector cells expressing the Fc receptor (HEK-CD16⁺ or HEK-CD89⁺) were first established by transfection (Invivogen, data not shown). HEK-CD16⁺ or HEK-CD89⁺ cell lines used in the ADCC-mimicry assay have the same expression level of CD16 or CD89 measured by flow cytometry (Invivogen). HEK-CD16⁺ or HEK-CD89⁺ cell lines co-incubated with bNAbs were co-cultured with the target cells at a 1:1 effector/target ratio for 48 h at 37 °C. Alkaline phosphatase was measured in the culture supernatant using Quantiblue (InvivoGen) at 620 nm. Percentage of ADCC was calculated using the formula: $100 \times (\text{OD } 620 \text{ nm with bNAb} - \text{OD } 620 \text{ nm without bNAb}) / \text{OD } 620 \text{ nm with bNAb}$.

2.8. Statistical Analysis

All statistical analyses were performed with InStat software (version 8.01; Graph-Pad Software, La Jolla, CA, USA). The normality was tested for each data set with both Shapiro–Wilk and D’Agostino–Pearson tests. When data could be modelled by a normal distribution, the following tests were performed depending on the comparison: Student’s *t*-test (comparison of two means), one-way ANOVA (multiple mean comparisons) with Bonferroni correction (comparison of all pairs of data sets) or Dunnett’s correction (comparison to a control group). Alternatively, non-parametric tests were performed with the Mann–Whitney test (comparison of two means). In the case of comparisons of two independent variables, a two-way ANOVA test was done. Results are represented as Mean ± SEM. *p* values of less than 0.05 (*), less than 0.01 (**), and less than 0.001 (***) were considered significant. Statistically significant differences between groups are emphasized by bars connecting the relevant columns.

3. Results

3.1. Development of a New Highly Potent Cellular Assay to Measure the ADCC-like Activity

We used a new method able to monitor the potential ADCC-like activity of different mAbs using HEK 293-gp41MSD and CHOgp140 cells as target cells [19,22]. Different HIV-bNAbs from different isotypes (IgA1, IgA2, and IgG1) were generated to compare their functional properties. An antibody IgA122, using an IgA2 Fc with the hinge region of IgA1, was also interestingly included to investigate whether the hinge length can affect the effector function of IgA isotypes. The four isotypes were produced for the bNAb 2F5 and IgG1, IgA2, and IgA122 for B12, while for the rest, we constructed IgG1 and IgA122. The antibodies were generated by recombinant DNA technology, produced in CHO cells, and purified by affinity chromatography according to their isotype (protein G for IgG, peptide M for IgA), obtaining from 2–5 mg of each antibody with more than 98% purity. We could identify the presence of the heavy (H) and light (L) chains at the expected sizes, which proves the integrity of each antibody (Figure 1).

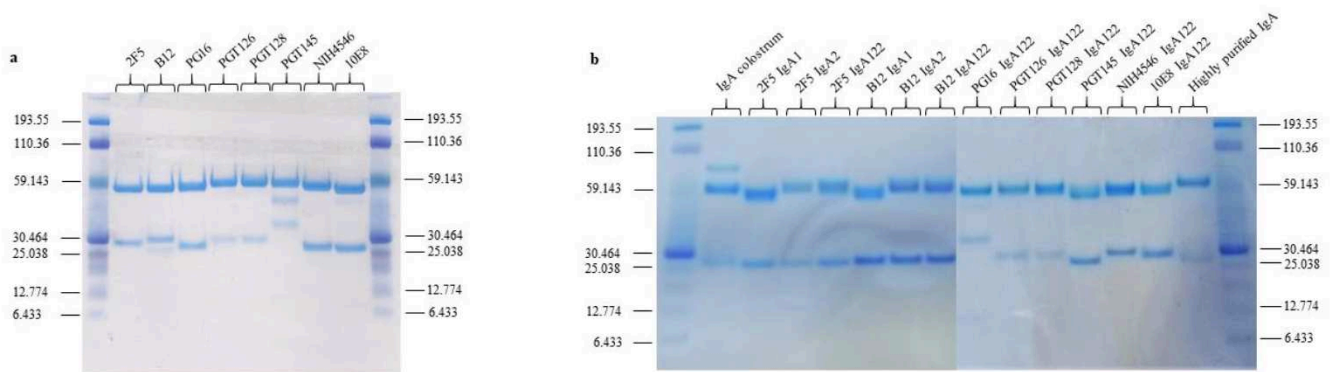


Figure 1. Characterization of the different bNAb isotypes: (a) Separation of bNAbs IgG and (b) IgA isotypes on SDS-PAGE (Bis-Tris 4–12%). An amount of 10 µg of the corresponding antibody was loaded on the gel. The first and last lanes of each gel correspond to the molecular weight marker. Two IgA controls were used: colostrum IgA and a highly purified IgA. Migration was performed at 150 V for 1 h.

The binding capacity of the bNAbs to their respective antigens was first tested by flow cytometry on the two target cell lines. IgG1 recognized its epitopes on gp41 and the CD4 binding site in the gp120 protein (MFI = 407–6784) better than the IgA isotypes (MFI = 152–1548). The V2 and V3 epitopes were similarly unrecognizable by all isotypes (Table 1). All tested antibodies exhibited an ADCC-like dose effect ranging from 0.75 µg/mL to 100 µg/mL (Figure 2). For most of the antibodies, the ADCC-like maximal activity effect was clearly achieved at the maximum tested concentration.

Table 1. Efficacy of binding of the different isotypes on Env-expressing cells by flow cytometry. Percentage of positivity of cells with 5 µg/mL of antibodies (n = 3).

Target Protein	Epitope *	Antibody	Isotype	MFI	% of Positivity
gp41 (HEK-gp41)	MPER	2F5	IgG1	407	93.5%
			IgA1	185	55.0%
			IgA2	152	45.5%
			IgA122	202	64.5%
		10E8	IgG1	228	41.6%
			IgA122	153	8.76%
gp140 (CHOSEC)	CD4 binding site	B12	IgG1	1566	99.6%
			IgA1	886	98.4%
			IgA2	586	73.5%
			IgA122	878	92.3%
	NIH4546	IgG1	6784	100%	
		IgA122	1548	99.4%	
V2 glycan	PG16	IgG1	439	0.049%	
		IgA122	422	0.23%	
	PGT145	IgG1	623	41.4%	
		IgA122	471	4.34%	
	V3 glycan	PGT126	IgG1	638	47.6%
		IgA122	540	27.4%	
PGT128	IgG1	476	13.2%		
	IgA122	442	1.62%		

* Source: NIH AIDS Reagent Program; <https://www.hiv.lanl.gov/> (accessed on 9 December 2021).

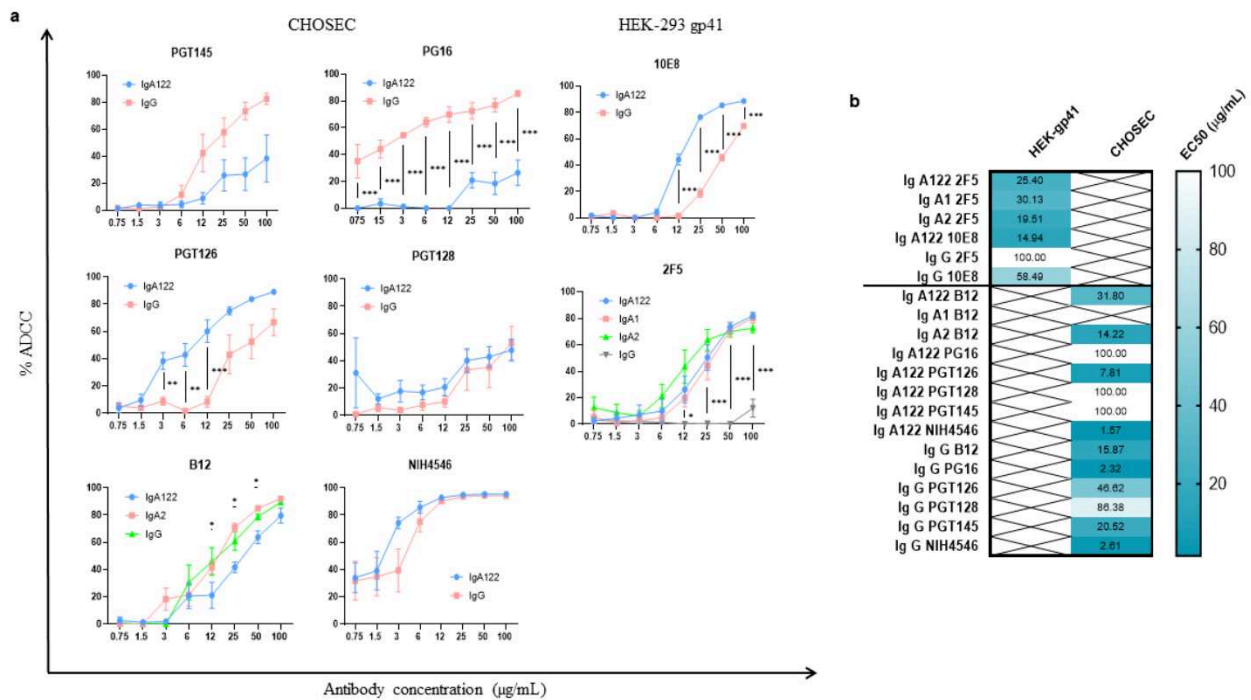


Figure 2. ADCC-like activities of bNAbs depend on their isotypes and their specificity: (a) %ADCC for each evaluated concentration. Each point represents the mean and SEM of triplicates from two different experiments. Student’s t-test was used for antibodies PGT145, PG16, 10E8, PGT126, PGT128, and NIH4546. A 2-way ANOVA with Tukey’s multiple comparisons was used for the antibodies 2F5 and B12. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (b) Heatmap of EC50 (µg/mL) values obtained for ADCC. Numbers indicate the geometric mean values of results from triplicates of at least two independent experiments (n = 6).

3.2. ADCC-like Activities of bNAbs Depend on Their Isotypes and Their Specificity

3.2.1. Higher ADCC-like Activity of gp41-Specific IgA Isotypes

For 2F5, the ADCC-like activities of IgA122 (EC50 = 25.40 µg/mL), IgA1 (EC50 = 30.13 µg/mL), and IgA2 (EC50 = 19.51 µg/mL) were statistically different ($p < 0.0001$) from IgG1 ADCC (EC50 = 100 µg/mL) (Figure 2). However, no significant differences were observed between the IgA isotypes. For 10E8, the IgA122 isotype also had more potent ADCC-like activity (EC50 = 14.94 µg/mL) than the IgG1 isotype (EC50 = 58.49 µg/mL) ($p < 0.000001$) (Figure 2). The 2F5 and 10E8 antibodies have their epitopes in the MPER region of gp41. However, 2F5 recognizes a beta sheet epitope and 10E8 an α-helix epitope [23]. Nevertheless, our results suggest that, in the case of 2F5 and 10E8, the IgA isotypes are better ADCC inducers than IgG1 against gp41, independently of the nature of the epitope.

3.2.2. Heterogenous gp120-Specific ADCC-like Activity of IgG1 and IgA Isotypes

Measurements of the ADCC-like activity of gp120-specific bNAbs were tested on CHOgp140 target cells (Figure 2). When comparing the NIH4546 and B12 families, which have their epitopes in the CD4 binding site, we detected that, in the case of NIH4546, IgG1 induced 50% ADCC with a higher antibody concentration (EC50 = 2.61 µg/mL) than IgA122 (EC50 = 1.569 µg/mL) ($p = 0.00095$). In the case of B12, IgA122 needed more concentration to achieve 50% ADCC (EC50 = 31.80 µg/mL) than IgA2 (EC50 = 14.22 µg/mL) and IgG1 (EC50 = 15.87 µg/mL), and the differences were statistically significant in both cases ($p < 0.0001$). However, the EC50 concentrations of the IgG1 and IgA2 isotypes on CHOSEC were not statistically different ($p = 0.48$). For PGT128, no significant differences between 50% ADCC-like activities mediated by the IgA122 and IgG1 isotypes were observed (IgA122 EC50 = 100 µg/mL; IgG1 EC50 = 86.38 µg/mL; $p = 0.558$). Interestingly, for PGT126-specific isotypes, we observed a significant decrease in IgA122 EC50 concentrations compared to

IgG1 (IgA122 EC50 = 7.81 µg/mL; IgG1 EC50 = 46.62 µg/mL; $p < 0.000001$). In contrast, for PGT145, IgG1 had a significantly higher ADCC activity than IgA122 with less antibody concentration (IgA122 EC50 = 100 µg/mL; IgG1 EC50 = 20.52 µg/mL; $p = 0.0004$). For PG16 isotypes, the IgG1 EC50 antibody concentration was significantly smaller than IgA122 (IgA122 EC50 = 100 µg/mL; IgG1 EC50 = 2.32 µg/mL; $p = 0.00003$). The IgA and IgG1 isotypes induced a heterogeneous ADCC effect when facing cells expressing the gp120 glycoprotein. Of note, for V2-specific antibodies, IgG1 induces more ADCC, probably due to the better accessibility of IgG1 than IgA in the V2 glycan shield structure [24,25].

3.3. Development of a New Highly Potent Cellular Assay to Measure the ADCC-like Activity

3.3.1. Gp41-Specific IgG1 Has More Potent Neutralizing Activity

When comparing the role of the bNAbs' Fc portion in their virus neutralization ability, any statistically significant difference between bNAbs was measured. However, for gp41-specific antibody families as previously described, we confirmed the superiority of 10E8 (Figure 3), implying that the slightly greater access of the 10E8 bNAb to the MPER epitope on the cell surface than that of 2F5 may play a critical role in antibody neutralization, as was already shown by Huang et al. in 2012 [26].

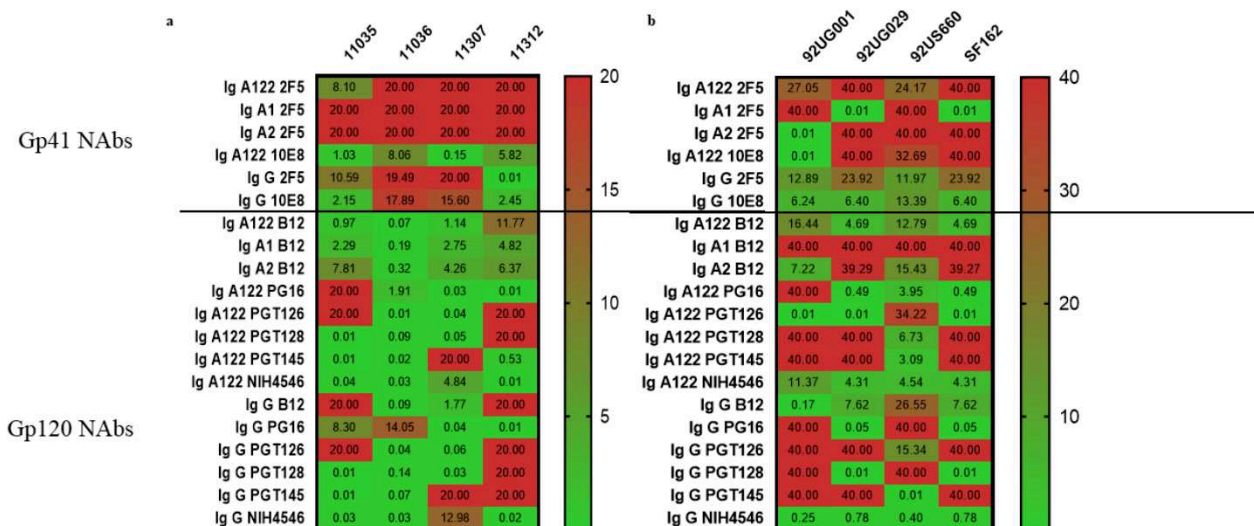


Figure 3. Neutralizing activity of bNAbs depend also on their isotypes and their specificity. Heatmap of EC50 (µg/mL) values obtained for neutralization for all tested antibodies using: (a) T2M-bl cells and pseudovirus or (b) PBMCs isolated from healthy donors and primary isolates. Numbers indicate the geometric mean values of results from duplicates of at least two independent experiments (n = 4). A 2-way ANOVA with Tukey's multiple comparisons was used to compare EC50s from all the antibodies per virus.

When we compared isotypes, IgG1 from the 10E8 and 2F5 bNAbs better neutralized six out of eight viral strains with an EC50 < 7 µg/mL, while the IgA isotypes neutralized one or two viral strains (92UG001 strain for IgA2 2F5 antibody and 92UG029 and SF162 strains for IgA1 2F5; EC50 < 0.02 µg/mL). Only IgA122 from 10E8 neutralized the four pseudoviruses tested (EC50 < 8.06 µg/mL), suggesting that, for this specific family, the hinge length increases the neutralization ability in that case.

3.3.2. Gp120-Specific IgG1 and IgAs Have Similar Neutralization Effect

As for the gp41-specific antibodies, we did not find any statistically significant differences between the neutralization effects of any of the gp120-specific tested bNAbs. Nevertheless, we saw that only B12 IgA122 and NIH4546 IGA122 and IgG1 neutralized all viral strains with an EC50 < 13 µg/mL. For the remaining tested bNAbs, the IgG1 and IgA isotypes similarly neutralized all viral strains, even though the pseudoviruses were

better neutralized than the primary isolates (Figure 3). The IgA122 isotype tends to better neutralize all viruses than the respective IgG1 isotype. That is the case for PG16 family, where the IgA122 isotype neutralizes six out of eight viruses with an EC₅₀ < 4 µg/mL, and the IgG1 isotype also neutralizes six out of eight viruses, but with a higher antibody concentration (EC₅₀ < 14 µg/mL). The B12 family is also an example of that because, even if the IgA1 and IgA2 isotypes were exclusively better neutralizers of pseudoviruses and IgG1 had a broader spectrum of neutralization, IgA122 required less antibody concentration to have the same effect (EC₅₀ < 16 µg/mL) against all tested viruses.

3.4. Correlation between ADCC-like Activity and Virus Neutralization

Each of the HIV-1 monoclonal antibodies was tested for neutralization by two methods with different viruses (clades A, B, and D) or pseudoviruses (clades B and C). ADCC-like activity was also measured and correlated with antibody binding to target cells (Figure 4a). For gp41, the ADCC-like EC₅₀ (Spearman's $r = 0.6$) tended to positively correlate with antibody binding to target cells; however, the correlation was not significant ($p > 0.4$) (Figure 4a). Neither did the ADCC-like EC₅₀ significantly correlate with neutral activity for any of the eight viral strains evaluated (Spearman's $r = 0.20; 0.03; 0.34; -0.58; 0.52; -0.70; -0.67; -0.70; p > 0.1$) (Figure 4b). However, there is a clear tendency to a better ADCC-like effect for the IgA isotypes when more potent neutralization is achieved, even though this effect depends on the viral strain used for the neutralization test. On the other hand, the IgG isotype tends to be better for neutralization and less efficient for ADCC, compared to IgA isotypes. For gp120-specific IgA and IgG, the ADCC-like activity inversely correlated with binding to target cells (Spearman's $r = -0.82; -0.09$, respectively) (Figure 4a). Both isotypes seem to be equally efficient for neutralization; nevertheless, IgG tends to generate a better ADCC response than the IgA isotypes. A positive correlation between the ADCC and neutralization of the 92UG001 viral strain was observed when comparing the IgA isotypes (Spearman's $r = 0.88, p = 0.0179$) (Figure 4b).

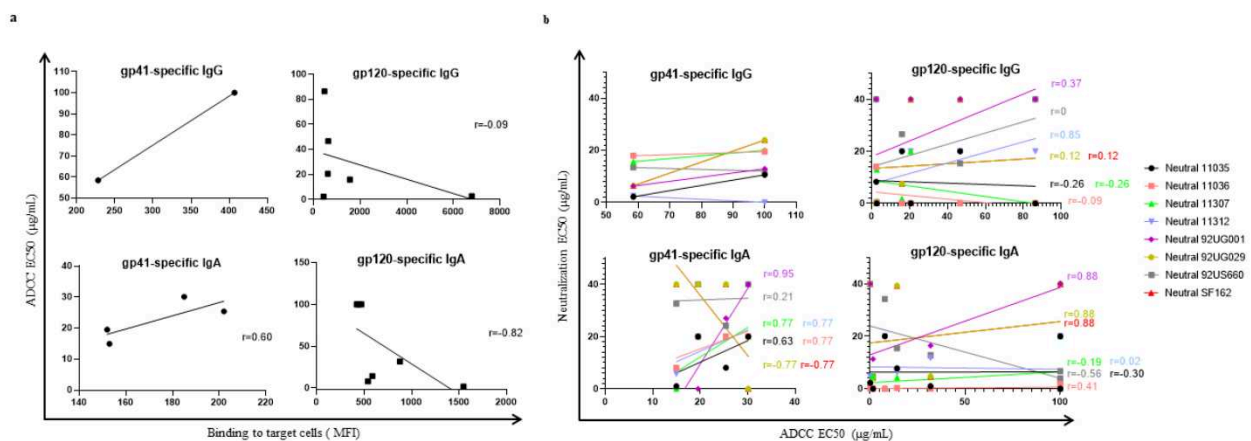


Figure 4. Correlation between: (a) MFI and ADCC-like EC₅₀, (b) ADCC-like EC₅₀ and Neutralization EC₅₀. Each point represents the mean value of triplicates from two different experiments ($n = 6$). Non-parametric Spearman correlation was calculated for each case.

4. Discussion

Here, we compare the impact of different isotypes of bNAbs on their antiviral functions. The evaluation of the potential ADCC of different isotypes from different bNAbs will provide information concerning the contribution of the Fc region of gp41-specific and gp120-specific antibodies in the protection against HIV-1. For that reason, the IgG and IgA isotypes from eight bNAb families were constructed and produced in CHO. The quality of these antibodies was evaluated by their specificity and binding capacity to CHOgp140 or HEK-gp41 target cells; however, IgA was less efficient in binding gp41- and gp140-expressing cells compared to the IgG isotypes. We then tested their potential ADCC-like

activity and their ability to neutralize primary isolates and pseudoviruses. ADCC-like activity was measured using a new sensitive assay designed to measure phosphatase alkaline, from which the expression was enhanced by the binding between FcR-expressing cells and the complex Ab-target cell. Ab antiviral efficiency is highly dependent not only on the Fab–antigen interactions that block viral entry but also on interaction of the Ab Fc domain with its cognate FcR expressed on the innate effector cells [27]. Contrary to other ADCC tests using PBMCs or NKs as effector cells [15,16,28], the assay is simpler and more interesting to gain an idea of the potency of the Fc-dependent antiviral effects that bNAbs can trigger beyond ADCC. However, further studies are needed to compare our assay with a more physiologic assay using PBMCs or NK cells. Our test uses antibody concentrations higher than the ones used by Duchemin et al. in 2020 [16]. Nevertheless, with this new method for evaluating ADCC effect based on cells expressing gp41 or gp120, all bNAbs tested were able to induce an ADCC response without the need to work with HIV-1-infected cells.

Our study reveals that, in the context of gp41, despite the inverse correlation between the ADCC-like EC50 and the effective binding of IgG1 to target cells, IgA is more efficient than IgG1 in inducing ADCC, but not for its neutralization effects in the context of the virus. In accordance with this, the IgG1 isotypes from 2F5 and 10E8 are described to have less or no ADCC effect against HIV-1-infected cells [29], probably due to a low affinity of these Abs for Env protein in the context of HIV-1-infected cells, which can be explained by their specificity to a gp41 epitope that is transiently exposed during fusion and phospholipids [30–33]. Moreover, Tudor et al. and Duchemin et al. showed that the IgA2 isotype from the 2F5 bNAb induced more ADCC and ADCP than its corresponding IgG1 [4,16]. For gp120 specificity, the IgA or IgG1 isotypes are efficient in neutralizing the different viral strains. In contrast, IgG1 was a better ADCC inducer than the corresponding IgA isotypes. The differences found between the Fc effector functions depending on the target protein could be explained by the different structural conformations between the Env glycoprotein on the infected cells and on the virus, making the epitopes variably exposed to the Ab. In accordance with this, Benjamin von Bredow et al. in 2019 showed for the PGT145 Ab that antibody binding affinity can differentiate sensitivity to ADCC from neutralization [34].

Duchemin et al. demonstrated that, for the gp41-specific 2F5 bNAb, the IgA isotype cooperates with the corresponding IgG isotype and also with IgG from 10E8 to increase HIV-1-infected cell lysis by ADCC [15]. However, Tomaras et al. addresses the interference of gp120-specific IgA in the correspondent IgG-mediated ADCC, most likely due to the higher affinity of IgA than IgG for gp120 [35]. In this last study, they used effector cells that did not express the Fc α RI and were unable to lyse target cells mediated by IgA isotypes. Duchemin et al. proposed that one possible explanation of the differences between their study and Tomaras et al. is that the capacity of the gp120 epitopes targeted by the RV144 vaccine-induced Abs in mediating ADCC differs from that of the gp41 epitopes. The results of our study support their conclusions, suggesting that the epitopes targeted by the Ab is crucial for isotype cooperation in ADCC.

Khamassi et al. in 2020 showed that the CH1 α domain of FabA has strong antibody specificity and functional activities, notably, an efficient neutralization of HIV-1-infected CD4+T lymphocytes, which is not true for FabG [36]. The same group saw a gain in affinity and antiviral activity of bNAbs when they performed isotype switching from IgG to IgA [4,36]. Their studies add another layer of complexity to the structure–function relationship of bNAbs, and our results are in accordance with this, as we detected differences between IgA and IgG isotypes' antiviral functions for some families. On the other hand, the production of Ab IgA2 containing the hinge of IgA1 showed that there is no gain in the Fc effector functions tested in this study for the bNAb 2F5. These findings contrast with Richardson et al. in 2019 [10] showing that the hinge region and Fc portion of a bNAb influenced the neutralization and Fc-relative functions. However, for the B12 family, the isotype IgA122 better neutralized pseudoviruses and primary isolated viruses

than the other IgA B12 isotypes. At the same time, IgA2 was a better neutralizer than IgA1 for the same family. It seems that, for the B12 antibody, a strategy of recombinant antibodies with the Fc part corresponding to IgA2 and the IgA1 hinge could be a good approach for immunotherapies against HIV. In addition, Scheepers et al. showed that IgA1 switching from IgG1 improved the neutralization activity of CAP88-CH06 lineage antibodies, and the suggested mechanism was associated to the structure and glycosylation of the IgA1 hinge region [37]. In accordance with the greater exposure of the Env proteins of lab-adapted viruses to antibodies [38–41], pseudoviruses were generally more sensitive to neutralization in terms of the magnitude of responses; notwithstanding, some antibodies were more efficient in neutralizing the primary viruses than the pseudoviruses.

This study has limitations. The test used here to measure ADCC-like activity does not reflect the physiological ADCC that occurs in the body, as there are no effector cells, such as PBMCs or NKs. However, it offers an idea of the potency of the Fc-dependent antiviral effects that bNAbs can trigger beyond ADCC. Nevertheless, further studies are needed to compare our assay with a more physiologic assay using PBMCs or NK cells.

Overall, our results reveal a variety of responses according to the nature of the neutralized virus and the Env-specific ADCC-related effect for the bNAbs tested. Concerning the ADCC-like effect of the gp41-specific tested antibodies, the IgA isotypes seem to be better than IgG1; the contrary seems to be true for the gp120-specific tested antibodies. We consider that the isotype selection should be based on the target epitope and the desired Fc-related effect. These results provide new insights into the neutralization and ADCC effects of different bNAbs that might be taken into consideration when searching for new treatments or antibody-based vaccines.

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Resumé

Mon travail de thèse s'inscrit dans les premières années de la pandémie de COVID-19. Malgré les connaissances accumulées, il reste beaucoup à étudier sur l'infection du SARS-CoV-2 et la réponse immunitaire. Mon objectif est de caractériser la réponse immunitaire humorale contre le virus SARS-CoV-2, d'identifier des marqueurs prédictifs de la gravité de la maladie et d'étudier la réponse immunitaire après vaccination. Dans notre étude, nous avons démontré que les patients atteints de COVID-19 sévère présentaient des niveaux plus bas de sIFNAR1 et des niveaux plus élevés de sIFNAR2 dans le sérum, comparés aux patients asymptomatiques. Ces protéines pourraient être des indicateurs de la gravité de la maladie. Nous avons également constaté que chez les patients les plus gravement atteints, la réponse IgA sérique était plus forte et précoce que chez les patients moins graves ou asymptomatiques. Ces résultats suggèrent un rôle potentiel dans la physiopathologie de la maladie. De plus, notre travail montre que la vaccination hétérologue est plus immunogénique et protectrice contre différents variants du SARS-CoV-2 qu'une vaccination homologue. Cependant, l'incapacité des vaccins à ARN messager (mRNA) à créer une réponse mucoale est une lacune qu'il faudrait combler avec de nouveaux vaccins, ciblant la voie mucoale pour la vaccination. Néanmoins, une première immunisation ne sensibilise pas à une amplification de la maladie dépendante d'anticorps (ADE). L'ensemble des données produites dans ce travail de thèse contribue à éclaircir certains aspects du rôle de la réponse humorale dans la physiopathologie de la COVID-19, ainsi que la réponse immunitaire développée après vaccination. Ce travail ouvre la voie à de nouvelles questions et pistes de recherche pour mieux comprendre la réponse immunitaire dans la COVID-19.

Abstract

My PhD thesis is marked by the early years of the COVID-19 pandemic. Despite accumulated knowledge, there is still much to study regarding SARS-CoV-2 infection and the immune response. My objective is to characterize the humoral immune response against the SARS-CoV-2 virus, identify predictive markers of disease severity, and study the immune response after vaccination. In our study, we demonstrated that severe COVID-19 patients had lower levels of sIFNAR1 and higher levels of sIFNAR2 in the serum, compared to asymptomatic patients. These proteins could serve as predictive markers of disease severity. Additionally, we observed that in the most severely affected patients, the serum IgA response was stronger and more rapid than in less severe or asymptomatic patients, suggesting a potential role in the disease's pathophysiology. Furthermore, our work indicates that heterologous vaccination is more immunogenic and protective against different SARS-CoV-2 variants than homologous vaccination. However, the inability of mRNA vaccines to elicit a mucosal response is a gap that should be addressed with new vaccines targeting mucosal pathways. Nevertheless, initial immunization does not sensitize individuals to antibody-dependent enhancement (ADE) of disease. The data generated in this thesis contribute to elucidating certain aspects of the role of the humoral response in the pathophysiology of COVID-19, as well as the immune response developed after vaccination. This work opens the door to new questions and research topics for a better understanding of the immune response in COVID-19.