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Active monitoring of long-term immunity to SARS-CoV-2 infection and vaccination

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Settore Scientifico Disciplinare MED/09

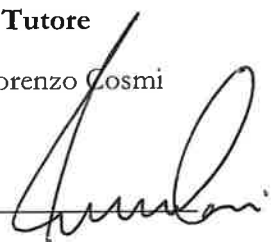
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ABSTRACT

Background: The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has reminded us of the critical importance of monitoring immunity against infectious pathogens, providing essential insights for clinical practice. The transition of the coronavirus disease 2019 (COVID-19) pandemic to an endemic phase depends on the long-term dynamics of adaptive immunity against SARS-CoV-2 elicited by natural infection and/or vaccination as well as the impact of highly transmissible viral variants. However, immunocompromised individuals are particularly vulnerable to infections and characterized by an impaired ability to respond effectively to vaccination. Monitoring the durability of SARS-CoV-2-specific immunity in both immunocompetent and immunocompromised individuals may allow for improvements in global immunization strategies.

Objectives: The aim of this PhD project is to investigate the immune response induced by SARS-CoV-2 infection and anti-SARS-CoV-2 vaccination, focusing on the profiling and long-term kinetics of virus-specific humoral and cell-mediated responses in both immunocompetent and immunocompromised individuals.

Materials and Methods: A total of 209 individuals were enrolled from March 2020 to March 2022 at Careggi University Hospital and Meyer Children's Hospital, Firenze, Italy, including 125 healthcare workers with or without history of SARS-CoV-2 infection who received the original BNT162b2 mRNA vaccine, 14 unvaccinated individuals hospitalized for COVID-19 in March-April 2020 followed up to one year post-hospital discharge, 41 patients with B-cell lymphoma either undergoing or previously treated with rituximab and 21 solid-organ transplant recipients on immunosuppressive therapy who received the original mRNA-1273 vaccine, and 8 people living with HIV who received a fourth original BNT162b2 mRNA vaccine dose. Main clinical data were collected. Humoral (serum levels of anti-Spike IgG and IgM, anti-RBD IgG, Spike-neutralizing antibodies, and anti-Nucleoprotein IgG) and cell-mediated (peripheral blood circulating Spike-specific T and B cells by multiparametric flow cytometry) responses were monitored.

Results: In individuals who recovered from COVID-19, one mRNA vaccine dose was sufficient to reactivate immunological memory. However, naïve individuals, with no prior history of SARS-CoV-2 infection, required two mRNA vaccine doses to generate a robust immune response. SARS-CoV-2-Spike-specific CD4⁺ T and B cell-mediated immunity was still detectable 8 months after the second vaccine dose, while antibody levels significantly declined at 6 months, especially in naïve individuals. Immunological memory induced by vaccination showed similar decay kinetics to that observed following infection. Additionally, a booster dose was necessary for naïve individuals, as it not only restored but also enhanced anti-Spike immunity to levels similar to those observed in vaccinated COVID-19 recovered individuals. In patients with B-cell lymphoma receiving rituximab therapy, anti-SARS-CoV-2 mRNA vaccination induced a weak or absent humoral response, but a strong Spike-specific CD4⁺ T cell response. Bendamustine-containing chemotherapy further impaired the ability to mount a humoral response, even after prolonged discontinuation of treatment, and it was associated with the severity of breakthrough infections in vaccinated patients with B-cell lymphoma. Similarly, solid-organ transplant recipients on immunosuppressive regimens containing antimetabolites exhibited lower humoral and cell-mediated immune responses following vaccination compared to those on regimens without antimetabolites. Finally, in people living with HIV and lower CD4⁺ T cell counts, a fourth vaccine dose transiently boosted Spike-specific adaptive immunity, with cross-reactive CD4⁺ T cell and B cell responses against the Omicron variant.

Conclusions: Active monitoring of long-term immunity against SARS-CoV-2, whether induced by infection and/or vaccination, provides essential information for optimizing immunization strategies. Anti-SARS-CoV-2 mRNA vaccines induce long-term adaptive immunity. Additional vaccine doses boost immunological memory, particularly in naïve and in immunocompromised individuals. Spike-specific cell-mediated immune responses induced by the original anti-SARS-CoV-2 mRNA vaccines are preserved against the Omicron variant.

SUMMARY

1. INTRODUCTION	1
1.1 THE EMERGENCE OF SARS-CoV-2.....	1
1.2 IMMUNITY TO SARS-CoV-2.....	5
1.3 COVID-19 VACCINES: DEVELOPMENT AND CHALLENGES	12
2. OBJECTIVES	19
3. MATERIALS AND METHODS.....	21
3.1 Study population	21
3.2 Evaluation of SARS-CoV-2-specific T cells	25
3.3 Evaluation of SARS-CoV-2-specific B cells	26
3.4 Flow cytometry analysis	27
3.5 Evaluation of SARS-CoV-2-specific humoral response	30
3.6 Statistics	30
3.7 Study approval.....	30
4. RESULTS	31
4.1 LONG-TERM EVALUATION OF ADAPTIVE IMMUNITY TO SARS-CoV-2.....	31
4.2 LONG-TERM EVALUATION OF VACCINE-INDUCED IMMUNITY	32
4.2.1 Rapid decline of Spike-specific humoral response in naïve individuals compared to COVID-19 recovered after vaccination	35
4.2.2 Higher early Spike-specific B cell frequencies in recovered COVID-19 individuals converging with naïve subjects by 8 months after vaccination.....	38
4.2.3 Comparable frequencies of Spike-specific CD4+ T cells in naïve and recovered COVID-19 subjects after vaccination	40
4.2.4 Booster dose reactivates immunological memory to Spike protein more effectively in naïve individuals compared to COVID-19 recovered.....	42
4.3 LONG-TERM EVALUATION OF SARS-CoV-2-INDUCED IMMUNITY.....	44
4.4 EFFECT OF IMMUNOSUPPRESSION ON LONG-TERM VACCINE-INDUCED IMMUNITY.....	47
4.4.1 Higher vaccine-induced Spike-specific humoral response following rituximab discontinuation compared to ongoing therapy	47
4.4.2 Strong vaccine-induced Spike-specific CD4+ T cell response independent of rituximab timing.....	50
4.4.3 Increased severity of breakthrough infections in patients treated with both rituximab and bendamustine.....	51
4.4.4 Impact of chemotherapy type on vaccine-induced humoral response in rituximab-treated patients	53
4.4.5 Long-term vaccine-induced immunity in solid-organ transplant recipients.....	55
4.4.6 Antimetabolite therapy weakens vaccine-induced immunity in solid-organ transplant recipients	56
4.4.7 Transient boost in Spike-specific immune response after fourth vaccine dose in people living with HIV	60
5. DISCUSSION	63
6. CONCLUSIONS	75
7. REFERENCES.....	77

FIGURES

Figure 1 – Visualization of SARS-CoV-2 using Transmission Electron Microscopy (TEM).	1
Figure 2 – Schematic structure of the SARS-CoV-2 virion.	2
Figure 3 – Innate immune pattern-recognition receptors and sensing of SARS-CoV-2.....	5
Figure 4 – Schematic overview of kinetics of immune response in viral infections.	6
Figure 5 – Adaptive immune responses to viral infections.....	6
Figure 6 – The major components of adaptive immunity to SARS-CoV-2.....	7
Figure 7 – Hallmarks of immune response in COVID-19.	8
Figure 8 – Dysregulated type I IFN activity and hyperinflammation.....	9
Figure 9 – Lymphopenia and lymphocytic impairment.....	9
Figure 10 – Dysregulated myeloid response.....	10
Figure 11 – Uncoordinated adaptive immunity to SARS-CoV-2.....	10
Figure 12 – CD4+ T cell responses during and following SARS-CoV-2 infection, stratified by symptomatic versus asymptomatic individuals.	11
Figure 13 – Indicative timeline for COVID-19 vaccines compared to standard vaccines.....	12
Figure 14 – Structure of SARS-CoV-2 Spike protein in the prefusion conformation and mRNA vaccine delivery.	13
Figure 15 – Study Population.....	22
Figure 16 – Time points of analysis for cohort 1 and cohort 2.....	23
Figure 17 – Time points of analysis for cohort 3, cohort 4, and cohort 5.	24
Figure 18 – Schematic overview of alignment of Spike protein and different peptide pools.	26
Figure 19 – Schematic overview of <i>in vitro</i> evaluation of SARS-CoV-2 Spike-specific B and T cells.	27
Figure 20 – Gating strategy for the identification of Spike-specific T cells.	28
Figure 21 – Gating strategy for the identification of Spike-specific B cells.	29
Figure 22 – Composition of the study population.	32
Figure 23 – Long-term evaluation of anti-SARS-CoV-2 antibody levels after two-dose vaccination in 30 individuals with or without history of COVID-19.....	36
Figure 24 – Long-term evaluation of anti-SARS-CoV-2 antibody levels after two-dose vaccination in 125 individuals with or without history of COVID-19.....	37
Figure 25 – Long-term evaluation of Spike-specific B cells after two-dose vaccination in individuals with or without history of COVID-19.....	38
Figure 26 – Long-term evaluation of Spike-specific circulating CD4+ T cells before and after two- dose vaccination in individuals with or without history of COVID-19.....	40
Figure 27 – SARS-CoV-2-specific adaptive immune response before and after the first booster dose in individuals with or without history of COVID-19.....	43
Figure 28 – Evaluation of Spike-specific circulating B and CD4+ T cells before and after the first booster dose in individuals with or without history of COVID-19.	43
Figure 29 – Long-term evaluation of SARS-CoV-2-specific antibody levels and Spike-specific B cells up to 12 months after SARS-CoV-2 infection.	45
Figure 30 – Long-term evaluation of SARS-CoV-2-specific CD4+ T cells up to 12 months after SARS-CoV-2 infection.....	46
Figure 31 – Long-term evaluation of SARS-CoV-2-specific antibody levels in patients with B-cell lymphoma after vaccination and correlation with time from last rituximab treatment.....	50
Figure 32 – Long-term evaluation of Spike-specific CD4+ T cells in patients with B-cell lymphoma after vaccination.....	51
Figure 33 – Evaluation of breakthrough infections in vaccinated B-cell lymphoma patients according to chemotherapy regimen.....	52
Figure 34 – Evaluation of Spike-specific IgG levels and CD4+ T cells following vaccination in B- cell lymphoma patients who underwent chemotherapy regimens with or without bendamustine.....	54
Figure 35 – Evaluation of SARS-CoV-2-specific antibody levels, Spike-specific B cells, and Spike- specific CD4+ T cells in solid-organ transplant recipients following vaccination.	57
Figure 36 – Evaluation of SARS-CoV-2-specific antibody levels, Spike-specific B cells, and Spike- specific CD4+ T cells in solid-organ transplant recipients following boosters.	58
Figure 37 – Evaluation of Spike-specific antibody levels, Spike-specific B cells and Spike-specific CD4+ T cells in people living with HIV following fourth vaccine dose administration.....	61

Figure 38 – Evaluation of CD4+ T cells and CD27+ B cells specific to the Wuhan SARS-CoV-2 Spike and the Omicron (B.1.1.529) BA.1 variant in people living with HIV following fourth vaccine dose.	62
Figure 39 – Schematic overview of long-term SARS-CoV-2 Spike-specific vaccine-induced and natural adaptive immune responses.....	67
Figure 40 - Hybrid immunity with COVID-19 vaccination.	68

TABLES

Table 1 – COVID-19 disease severity classification according to WHO.....	24
Table 2 – List of fluorochrome-conjugated antibodies used for flow cytometric analysis of SARS-CoV-2-specific T cells.....	26
Table 3 – List of fluorochrome-conjugated antibodies used for flow cytometric analysis of SARS-CoV-2 Spike-specific B cells.....	27
Table 4 – Basic demographic and clinical characteristics of 30 healthcare workers who received a two-dose vaccination series of the original BNT162b2 vaccine, followed up to 8 months after the second dose.....	33
Table 5 – Basic demographic and clinical characteristics of 125 healthcare workers who received a two-dose vaccination series of the original BNT162b2 vaccine, followed up to 6 months after the second dose.....	34
Table 6 – Basic demographic and clinical characteristics of 14 healthcare workers who received the first booster dose (original BNT162b2), followed up to one week after booster dose.....	34
Table 7 – Basic demographic and clinical characteristics of 14 patients hospitalized for COVID-19 between March and April 2020 and followed up to 12 months post-hospital discharge.....	44
Table 8 – Basic demographic and clinical characteristics of 41 patients with B-cell lymphoma who received vaccination.....	48
Table 9 – Laboratory data of 41 patients with B cell-lymphoma who received vaccination.....	49
Table 10 – Basic demographic and clinical characteristics of 21 solid-organ transplant recipients and control group who received vaccination.....	55
Table 11 – Breakthrough SARS-CoV-2 infection in 21 solid-organ transplant recipients and control group.....	59
Table 12 – Basic demographic and clinical characteristics of 8 people living with HIV (PLWH) who received vaccination.....	60

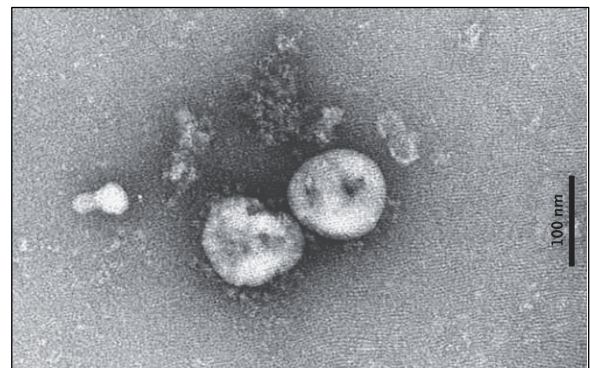
1. INTRODUCTION

1.1 The emergence of SARS-CoV-2

Human coronaviruses (HCoVs) are enveloped single-stranded RNA viruses first isolated in the 1960s from nasal secretion of individuals with common cold [1,2]. Since then additional HCoVs have been discovered, with four - 229E, OC43, NL63, and HKU1 - being prevalent and typically causing common cold and two - severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) - being more pathogenic and capable of causing severe, sometimes fatal, respiratory disease. SARS-CoV is the pathogen responsible for the outbreak of severe respiratory disease that occurred in China between 2002 and 2003, while MERS-CoV has been spreading in the Middle East since 2012 [3,4]. In December 2019, a cluster of patients with severe pneumonia of unknown cause was reported in Wuhan, Hubei province, China [5,6]. Human airway epithelial cells were used to isolate a novel coronavirus, initially named 2019-nCoV and later SARS-CoV-2, which was classified within the clade of *Sarbecovirus*, part of *Orthocoronavirinae* subfamily (*Coronaviridae* family) [5] (Figure 1). Different from both SARS-CoV and MERS-CoV [7,8,9], SARS-CoV-2 was identified as the seventh coronavirus known to infect humans. The illness caused by this virus was named coronavirus disease 2019 (COVID-19) [10].

Figure 1 – Visualization of SARS-CoV-2 using Transmission Electron Microscopy (TEM).

SARS-CoV-2 particles are typically spherical in shape, with a diameter ranging from 60 to 140 nm. The virus particles exhibit distinctive spike-like projections, measuring approximately 9 to 12 nm, resembling a crown, or “corona” in Latin. The term “coronavirus” was originally coined based on the appearance of virions when observed under TEM. Figure from Zhu et al. NEJM 2020 [5].



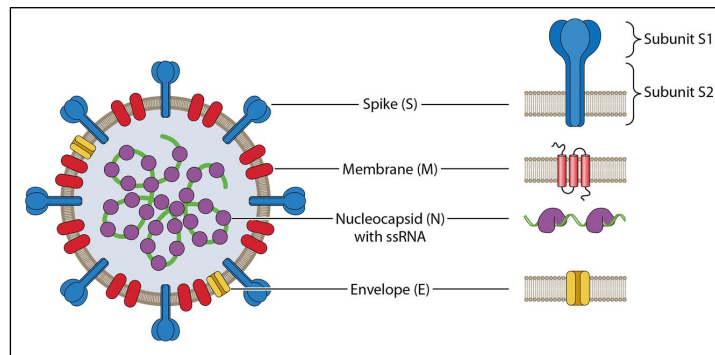
In the following weeks and months, SARS-CoV-2 rapidly spread across the globe, initiating a pandemic [11,12]. As of October 2024, more than 776 million cases of COVID-19 have been reported worldwide, resulting in more than 7 million deaths [13]. In Italy, 26 million cases have been recorded so far, with more than 197000 deaths attributed to the disease [13]. The unique dynamics of the COVID-19 pandemic, both nationally and internationally, have been characterized by periodic surges [14,15]. These surges are primarily driven by the emergence of viral variants with

enhanced transmissibility and immune escape capabilities, as well as by the varying effectiveness of vaccination strategies and the implementation of public health containment measures [16,17,18].

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus, with a genome of approximately 30 kbp. Like other *Betacoronaviruses*, SARS-CoV-2 features four structural proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid protein (N) (Figure 2) [19]. The Spike protein is a large protein that comprises a short C-terminal tail located within the virion, a transmembrane domain that anchors it to the viral envelope, a rod-like S2 domain responsible for mediating membrane fusion, and a large globular S1 domain. Within the S1 domain lies the receptor-binding domain (RBD) which is essential for the virus to attach to and fuse with the host cell membrane. This process occurs following the interaction of the RBD with the angiotensin-converting enzyme 2 (ACE2) receptor expressed by host cells [20].

Figure 2 – Schematic structure of the SARS-CoV-2 virion.

SARS-CoV-2 virion is characterized by four structural proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid protein (N). Figure from Synowiec et al. Clin Microbiol Rev. 2021 [19].



SARS-CoV-2 is primarily transmitted through exposure to infectious respiratory fluids, which occurs mainly via inhalation of fine respiratory droplets and aerosol particles that contain the virus [21]. These droplets and particles deposit on exposed mucous membranes in the mouth, nose, or eye by direct splashes and sprays, or by touching mucous membranes with hands that have been soiled either directly by virus-containing respiratory fluids or indirectly less commonly by touching contaminated surfaces and fomites [22]. The risk of infection diminishes with increased distance from the source and as more time passes after exhalation [23,24].

Coronavirus entry into host cells typically requires two cleavage events in the Spike protein: one at the junction of the S1 and S2 subunits, and a second at the S2' site within the S2 subunit. For SARS-CoV-2, the S1-S2 boundary is cleaved by furin during virus maturation in infected cells, while cleavage at the S2' site happens in the target cell after binding to the ACE2 receptor [25]. Upon binding to ACE2, the S1 subunit undergoes conformational changes that expose the S2' site, allowing protease cleavage to initiate membrane fusion. Depending on the entry route taken by the virus, the S2' site is cleaved by different proteases. In cells expressing transmembrane protease serine 2 (TMPRSS2), cleavage at the S2' site occurs directly at the cell surface. If the target cell expresses insufficient TMPRSS2 or if the virus-ACE2 complex does not encounter TMPRSS2, the virus-ACE2 complex is internalized through clathrin-mediated endocytosis into endolysosomes,

where cathepsins cleave the S2' site [21]. In both entry pathways (surface entry or endosomal internalization), the cleavage of the S2' site exposes the fusion peptide, while the separation of the S1 from S2 triggers significant structural changes in the S2 subunit. These changes, particularly in the region heptad repeat 1, push the fusion peptide toward the target cell membrane, initiating the membrane fusion process that allows viral entry into the host cell. The fusion between viral and cellular membranes creates a fusion pore, allowing the release of viral genome into the host cell cytoplasm [21]. Most part of the SARS-CoV-2 genome (ORF1a and ORF1b) encodes for two large polyproteins, pp1a and pp1b, which via autoproteolytic cleavage are processed into 16 distinct non-structural proteins (NSPs), each with specialized roles in viral replication and transcription [26]. The remaining genome encodes for the structural proteins S, E, M, and N, as well as several open reading frames (ORFs; 3a, 6, 7a, 7b, 8, 9b, and 10) [27].

All viruses, including SARS-CoV-2, change over time. Like most RNA viruses, the evolution of coronaviruses is rapid on timescales of months or years and occurs concomitantly with transmission events and ecological dynamics (including immune responses) [28]. The majority of alterations have little to no effect on viral properties, but certain genetic mutations may affect the functional characteristics of the virus, such as transmissibility, disease severity, immune escape, diagnostic or therapeutic escape [29,30]. After the SARS-CoV-2 outbreak in late 2019, there was an 11-month period of relatively evolutionary stability [31]. However, since late 2020, SARS-CoV-2 evolution has been characterized by the appearance of sets of mutations [31]. These alterations impacted virus properties (i.e., transmissibility and antigenicity), probably in response to the changing immune profile of the human population [31]. The more significant of SARS-CoV-2 variants are grouped in three different ways by the World Health Organization (WHO): variants under monitoring (VUM), variants of interest (VOI) and variants of concern (VOC) [32]. A VUM indicates to public health authorities that a specific variant may warrant prioritized surveillance and evaluation; the primary goal of this classification is to assess whether this variant (or closely related strains) could present an increased risk to global public health compared to other circulating variants. A VOI refers to a SARS-CoV-2 variant with genetic changes that are known to influence its behavior or potential impact on human health, including factors such as increased transmissibility, potential to cause severe disease, or variations that may affect detection or treatment efficacy. A VOC is often designated when it demonstrates a higher rate of spread compared to other circulating variants, indicating a possible emerging risk to global public health. A VOC refers to a SARS-CoV-2 variants of interest that, through a comparative assessment, have been demonstrated to be associated with one or more among: (a) detrimental change in disease severity, (b) substantial impact on the ability of health systems to provide care to patients with COVID-19 or other illnesses, (c) significant decrease in the effectiveness of available vaccines in protecting against severe disease. As of October 2024, there are no VOCs circulating, two VOIs (BA.2.86 and JN.1), and seven VUMs (JN.1.7, KP.2, KP.3, KP.3.1.1, JN.1.18, LB.1, and XEC). The latest VOC circulating has been the Omicron variant, which emerged in late 2021 and subsequently became dominant in mid-2022 [33,34]. The first Omicron sub-lineage, designated as BA.1, carried numerous mutations, particularly

in the Spike protein, leading to high transmissibility, partial evasion of neutralizing antibodies developed against the original wild-type SARS-CoV-2 (Wuhan-Hu-1), and an increased risk of reinfection [35,36,37].

As of October 2024, COVID-19 has resulted in over 776 million confirmed cases worldwide and more than 7 million deaths [13]. The COVID-19 pandemic has not only been a public health crisis but also a socioeconomic catastrophe, triggering widespread economic disruptions, exacerbating inequalities, and challenging healthcare systems around the world [38]. It exposed weaknesses in global and national infrastructures, particularly straining on hospital and healthcare providers causing shortages of critical supplies and disrupting routine healthcare services. Although a relevant proportion of infected subjects (at least one third) never develop symptoms, symptomatic individuals can have a variable disease course [39,40,41]. COVID-19 presents with a broad spectrum of clinical manifestations, including fever, cough, fatigue, anorexia, shortness of breath, myalgias, sore throat, nasal congestion, headache, gastrointestinal manifestations (such as diarrhea, nausea, and vomiting), as well as anosmia and ageusia [42]. In more severe cases, COVID-19 can progress to critical disease, with patients developing acute respiratory distress syndrome (ARDS), hyperinflammation, cardiac dysfunction, neurologic disorders, hypercoagulability, acute kidney injury, autoimmune complications (MDA5-autoimmunity and interstitial pneumonitis contemporaneous with the COVID-19 pandemic), and in children the multisystem inflammatory syndrome (MIS-C) [43,44,45]. COVID-19 is a complex inflammatory disease that can even cause long-term sequelae [46,47,48]. These can be distinguished in residual organ system dysfunction, which is directly related to organ system damage (such as interstitial lung disease, cardiovascular abnormalities, or kidney damage), and a constellation of symptoms that are not fully explained by apparent pathogenic processes and that collectively are termed “long COVID” [49]. Subjects experiencing long COVID often report a range of debilitating symptoms, including extreme fatigue, persistent myalgias, unexplained dyspnea, dysautonomia (temperature dysregulation, tachycardia), sleep disturbances, depression, anxiety, or cognitive impairment [50,51,52].

Therapeutic management of COVID-19 has evolved during the pandemic, with the development and repurpose of antivirals (remdesivir, darunavir/cobicistat, nirmatrelvir/ritonavir, molnupiravir) as well as several monoclonal antibodies (sotrovimab, bebtelovimab, tixagevimab/cilgavimab, casirivimab/imdevimab, bamlanivimab/etesevimab), together with the use of immunosuppressants and immunomodulators (corticosteroids, JAK-inhibitors, tocilizumab), as well as oxygen supplementation and in some cases therapeutic plasma exchange [53,54,55,56]. With the emergence and widespread dominance of the Omicron variant (B.1.1.529) at the end of 2021, only bebtelovimab and sotrovimab retained neutralizing activity against SARS-CoV-2. However, as Omicron continued to evolve into new sub-variants in 2022, these antibodies also lost cross-neutralizing efficacy [57]. As a result, none of the previously authorized monoclonal antibodies currently maintain sufficient activity against the circulating SARS-CoV-2 strains, leading to the cessation of their use for COVID-19 prophylaxis, pre-exposure prophylaxis, and treatment [58].

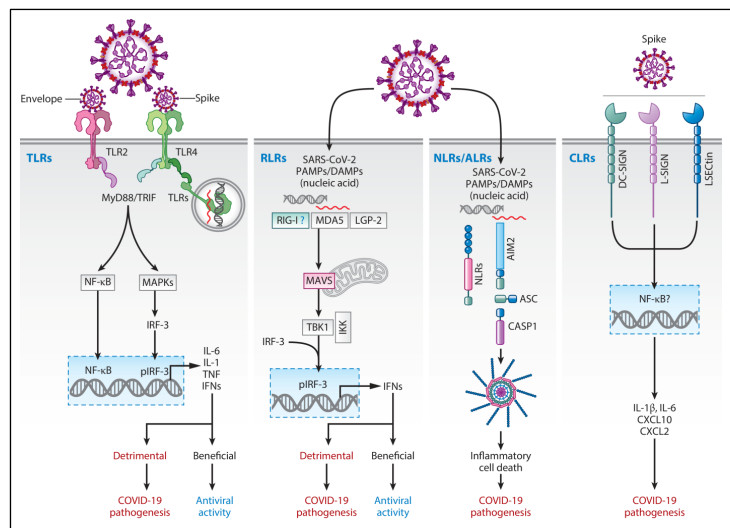
1.2 Immunity to SARS-CoV-2

Innate immunity represents the first line of defense against SARS-CoV-2 invasion and infection, providing a rapid non-specific response that is critical for controlling viral replication and initiating subsequent adaptive immunity [59,60]. Innate immune cells, including macrophages, neutrophils, dendritic cells, and natural killer (NK) cells, are equipped with a range of pattern recognition receptors (PRRs) that recognize pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) during SARS-CoV-2 infection [61]. Upon recognition of viral RNA or other infection-associated molecular signals, PRRs such as Toll-like receptors (TLRs), RIG-I-like receptors, and NOD-like receptors trigger downstream signaling pathways that activate transcription factors, such as NF- κ B and interferon regulatory factors (IRFs) (Figure 3) [62,63,64,65]. This leads to the production of type I and type III interferons (IFNs), along with other pro-inflammatory cytokines and chemokines, which are crucial for orchestrating an antiviral state, recruiting additional immune cells to the site of infection, and enhancing the antigen-presenting function of dendritic cells. SARS-CoV-2 is particularly adept at delaying or evading type I and type III IFN responses. Innate immune dysregulation has a relevant role in the pathophysiology of COVID-19, underscoring the dual role of immunity in combating SARS-CoV-2 infection [66]. Generally, an efficient innate immune response can clear SARS-CoV-2, prevent symptom onset, and prepare the adaptive immune system to establish immunological memory.

Figure 3 – Innate immune pattern-recognition receptors and sensing of SARS-CoV-2.

During SARS-CoV-2 infection, pattern-recognition receptors engage with PAMPs and DAMPs and activate transcriptional programs that drive the expression of inflammatory cytokines, chemokines, and interferons. While these processes are beneficial in clearing the virus, they can also be detrimental and contribute to the pathogenesis of COVID-19.

Figure from Malireddi et al. *Annu Rev Immunol.* 2024 [61].



Activation of adaptive immunity requires approximately one week post-infection, allowing for the selection and expansion of antigen-specific effector cells to contrast the virus effectively (Figure 4) [67]. Adaptive immunity is essential for the control of SARS-CoV-2 infection and the establishment of durable protective immunity. It represents the “second” line of defense against SARS-CoV-2 and involves antigen-specific recognition of viral epitopes [68]. Adaptive immunity

consists of two branches: humoral and cellular immunity (Figure 5). Humoral immune response to SARS-CoV-2 is mediated by antibodies; cellular immunity encompasses virus-specific B cells and helper CD4+ and cytotoxic CD8+ T cells (Figure 6).

Figure 4 – Schematic overview of kinetics of immune response in viral infections.

Figure from Sego et al. PLOS 2020 [69].

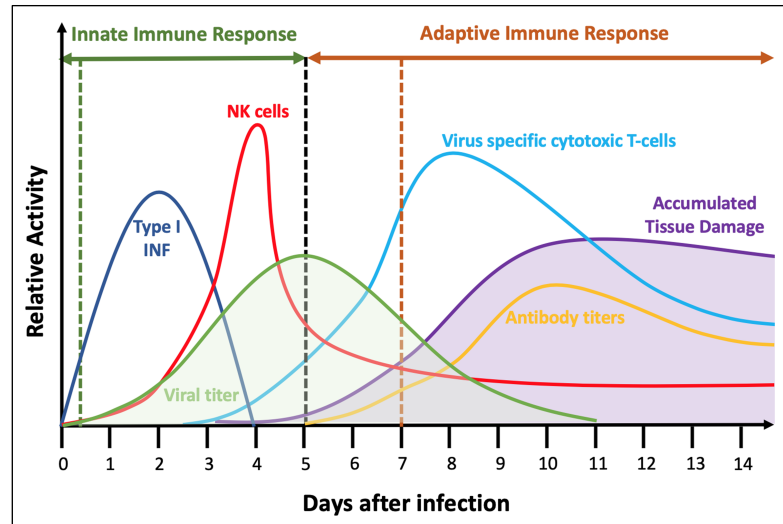
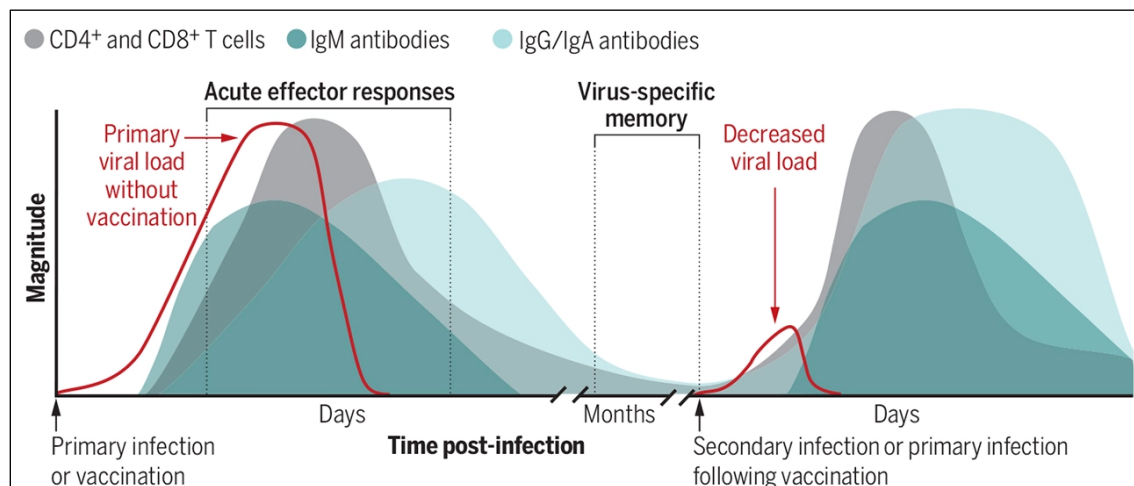


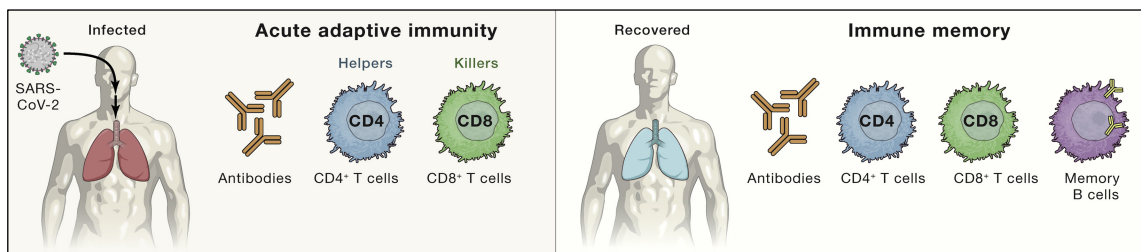
Figure 5 – Adaptive immune responses to viral infections.



Adaptive immune responses control and eliminate viral infections that have evaded innate immune control. Days after infection, virus-specific cytotoxic CD8+ T cells migrate to the site(s) of infection, where they eliminate virally infected cells. Early-responding B cells produce and release virus-specific IgM antibodies; CD4+ T helper cells promote the class-switching of germinal center B cells, thereby enabling virus-specific antibody production from IgM to IgG or IgA. Following the clearance of the virus, as a result of the acute effector responses, a pool of memory IgG/IgA B cells and memory T cells is maintained (virus-specific memory cells). These cells constitute the virus-specific immunological memory and are rapidly reactivated upon reinfection with the same virus (secondary infection). The objective of vaccination is to elicit protective adaptive immune memory without the necessity for a bona fide primary infection. Vaccine-induced immunity can curb viral spread and mitigate severe disease outcomes by facilitating rapid immune activation upon exposure to the virus. Figure from Hope et al. Science 2021 [70].

B cells, essential for humoral immunity, differentiate into plasmablasts and plasma cells that produce antibodies specific to SARS-CoV-2, especially after interacting with follicular helper T cells. The humoral response includes IgA, IgM, and IgG antibodies, each with distinct roles in infection clearance and immune memory. IgG, critical in secondary responses, remains circulating post-infection, providing ongoing immunity. The appearance of neutralizing antibodies, particularly against the RBD of the Spike protein, is fundamental in blocking viral entry into host cells, either neutralizing the virus or facilitating its elimination via other mechanisms, such as the antibody-dependent NK cell activation [71]. T and B cells have specific receptors - T-cell receptor (TCR) and B-cell receptor (BCR), respectively - that recognize foreign antigens and confer critical immunological features such as memory, specificity, self-tolerance, and regulatory functions. T cells, which originate in the bone marrow and mature in the thymus, differentiate into naïve or memory cells depending on antigen exposure. CD4⁺ helper T cells and CD8⁺ cytotoxic T cells (CTLs) perform key roles: CD4⁺ T cells facilitate diverse immune functions, including assisting cytotoxic activity and antibody production, thus cooperating in adaptive immune responses, while CD8⁺ T cells directly eliminate infected cells via perforin and granzymes, a cytotoxic activity central to viral clearance. During acute COVID-19, a decrease in CD8⁺ T cells and expression of senescence markers has been linked to poor outcomes, whereas robust CTL-responses, such as the secretion of IFN- γ , are associated with viral clearance.

Figure 6 – The major components of adaptive immunity to SARS-CoV-2.

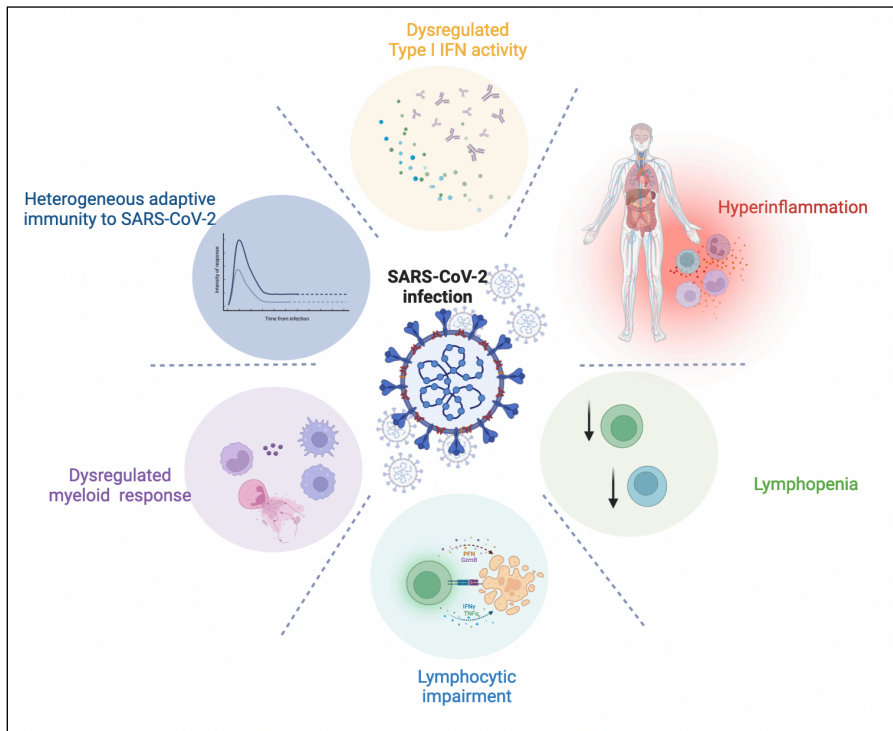


Virus-specific CD4⁺ T cells, CD8⁺ T cells, and antibodies (produced by B cells) constitute the three major components of acute adaptive immunity to SARS-CoV-2 infection. The control of SARS-CoV-2 in individuals with COVID-19 is the result of the combined actions of CD4⁺ T cells, CD8⁺ T cells, and neutralizing antibodies. Immunological memory in COVID-19 recovered individuals consists of memory B cells, antibodies, virus-specific CD4⁺ T cells, and virus-specific CD8⁺ T cells. Virus-specific B and T cells provide long-term immunological memory and undergo rapid expansion following exposure to the antigen. Figure from Sette et al. Cell 2021 [68].

The interplay between innate and adaptive immunity is crucial in COVID-19. A dysregulation of the immune response to SARS-CoV-2 occurs in severe COVID-19 [72]. SARS-CoV-2 can exert profound effects on immune function, leading to dysregulation and exhaustion within immune cell populations. Key abnormalities in both innate and adaptive immunity during COVID-19 can be summarized in six primary hallmarks (Figure 7) [73]. When a rapid, well-coordinated response involving both innate and adaptive immune branches is established early, it facilitates effective viral clearance with minimal symptom progression. Conversely, a delayed and uncoordinated immune response can exacerbate disease severity, inducing a cascade of immune

abnormalities that affect multiple compartments and amplify one another through a self-reinforcing feedback loop.

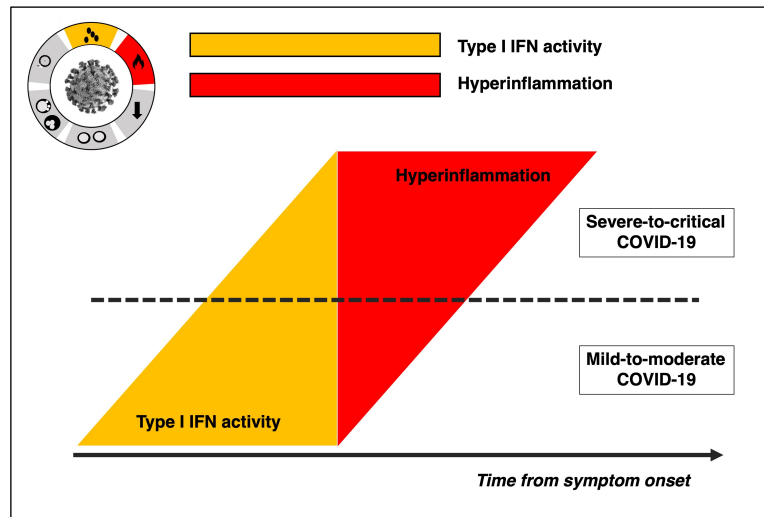
Figure 7 – Hallmarks of immune response in COVID-19.



Effective recovery from viral infections requires a robust host immune response that either eliminates the virus or limits viral replication. The immune responses effector activities are counterbalanced by regulatory mechanisms to prevent excessive inflammation. In COVID-19, the early phase of infection is often marked by dysregulated type I IFN response, which undermines the prompt establishment of a rapid antiviral defense (Figure 8) [74]. This impairment fosters elevated levels of pro-inflammatory cytokines, setting the stage for systemic hyperinflammation – a characteristic feature of severe COVID-19. A dysregulated immune response fails to eliminate the viral spread and contributes to epithelial and endothelial damage, vascular leakage, up to organ failure. SARS-CoV-2 infection exemplifies the importance of an effective immune response and highlights the detrimental effects of immune dysregulation [75]. After an initial response with generally mild to moderate symptoms, some patients show a progression to an excessive and dysregulated inflammatory response (Figure 8). This is a hyperinflammatory response that equals a “cytokine storm”, a potentially life-threatening systemic inflammatory syndrome involving elevated plasma cytokine levels and immune-cell hyperactivation with consequent acute systemic inflammatory symptoms and organ dysfunction [76].

Figure 8 – Dysregulated type I IFN activity and hyperinflammation.

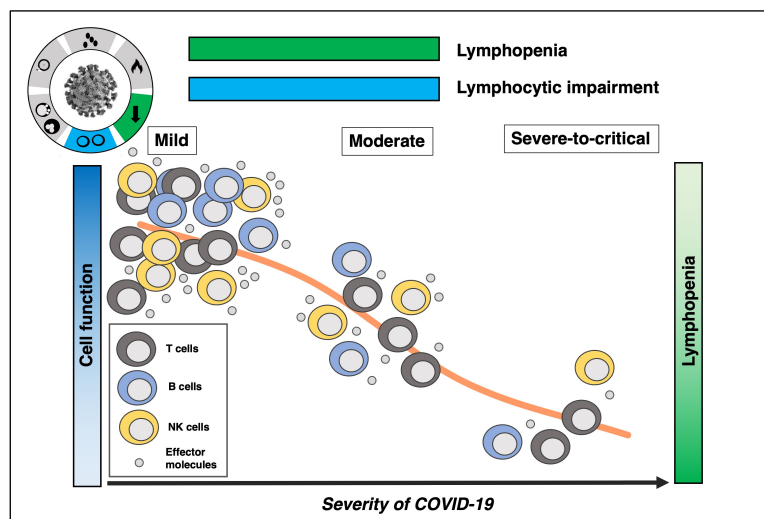
In the early stages of mild-to-moderate COVID-19, an effective type I IFN response limits viral spread, leading to a balanced immune response with controlled cytokine release. However, several mechanisms can impair the early response, including viral evasion, anti-IFN auto-antibodies or genetic mutations in IFN-signaling pathway [77,78,79,80]. Delayed and/or reduced type I IFN activity results in uncontrolled viral replication, prompting compensatory excessive production of pro-inflammatory cytokines. This imbalance underpins the cytokine storm associated with severe COVID-19. Figure from Mazzoni et al. Semin Immunol. 2021 [73].



In addition to cytokine overproduction, acute severe COVID-19 is characterized by substantial lymphocyte depletion (lymphopenia) in both blood and secondary lymphoid organs [81,82]. Lymphocytes are not only affected in terms of absolute numbers, but also functionally impaired (Figure 9) [83]. Functionally, lymphocytes show diminished pro-inflammatory cytokine production and cytotoxic potential. Lymphopenia and impaired lymphocyte functionality correlate strongly with disease severity, and all lymphocyte subsets display an exhausted phenotype [84].

Figure 9 – Lymphopenia and lymphocytic impairment.

COVID-19 progression towards severe illness involves a profound reduction in lymphocytes count across all subsets. Lymphocytes are also profoundly affected in terms of functionality. Figure from Mazzoni et al. Semin Immunol. 2021 [73].



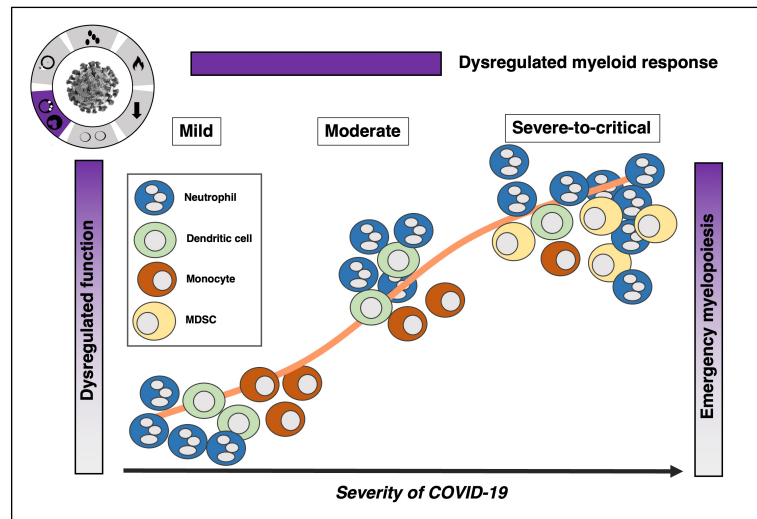
Myeloid cells also exhibit response dysregulation in COVID-19, encompassing both quantitative and qualitative alterations (Figure 10) [85,86]. Neutrophils accumulate and show aberrant NETs formation, enhanced activation marker expression, and PD-L1 expression [87]. Monocytes exhibit

compositional changes with a depletion of non-classical subsets, increased pro-inflammatory cytokine production, and reduced HLA-DR expression [86]. Dendritic cells (DCs) are significantly depleted in severe cases and produce low levels of IFN- α [88]. Additionally, myeloid-derived suppressor cells (MDSCs) emerge, contributing immunosuppressive effects in severe disease [89].

Figure 10 – Dysregulated myeloid response.

Severe COVID-19 is marked by an increase in immature myeloid cells in circulation, reflecting emergency myelopoiesis driven by inflammation.

Figure from Mazzoni et al. *Semin Immunol.* 2021 [73].



The sixth hallmark of COVID-19 is a highly heterogeneous adaptive immune response in terms, which correlates with clinical outcome (Figure 11 and Figure 12) [90]. Asymptomatic individuals demonstrate rapid activation of adaptive immunity with limited SARS-CoV-2-specific antibody and T cell response, while symptomatic individuals often show delayed but more intense adaptive responses.

Figure 11 – Uncoordinated adaptive immunity to SARS-CoV-2.

In asymptomatic individuals, rapid activation of adaptive immunity results in the prompt control of infection, with limited production of virus-specific antibodies and T cells. In these individuals, T cells are more functionally competent, producing higher levels of pro-inflammatory cytokines compared to symptomatic patients. Solid lines indicate the SARS-CoV-2 specific immune response trends in symptomatic and asymptomatic individuals, while dotted lines suggest hypothetical long-term memory responses. Figure from Mazzoni et al. *Semin Immunol.* 2021 [73].

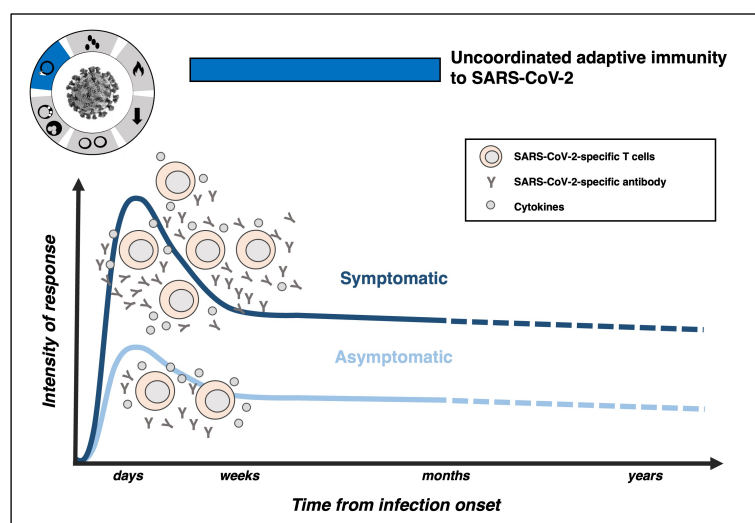
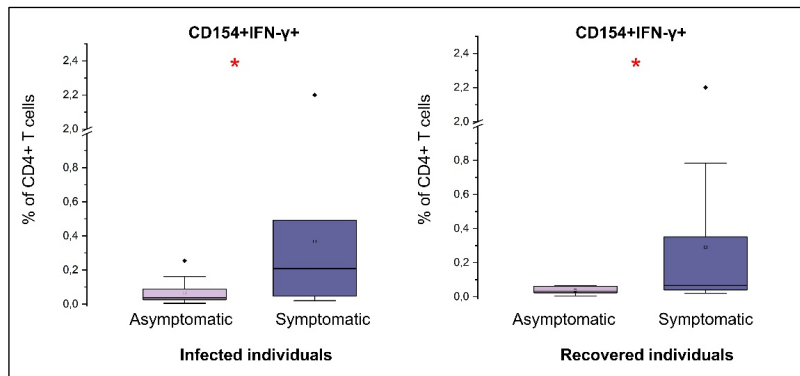


Figure 12 – CD4+ T cell responses during and following SARS-CoV-2 infection, stratified by symptomatic versus asymptomatic individuals.

The frequency of SARS-CoV-2-specific CD154+ CD4+ T cells producing IFN- γ was measured during infection and at 5 months post-infection in asymptomatic (n=10) or symptomatic (n=20) subjects. Symptomatic patients had a significantly higher frequency of Spike-specific CD4+ T cells compared to asymptomatic individuals. This difference is significant even in recovered individuals, indicating a potentially heightened and prolonged immune memory response among those with more severe symptoms (unpaired two-tailed Student's T test; * p <0.05). Data from Mazzoni et al. Clin Transl Immunology. 2021 [74].



Overall, the complex balance and timing of immune responses, particularly the early engagement of adaptive immunity, are pivotal in dictating the course and severity of SARS-CoV-2 infection.

1.3 COVID-19 vaccines: development and challenges

“When SARS-CoV-2 emerged in late 2019 and rapidly spread to all parts of the world, few thought that vaccines could be developed in time to help curb the increasing global disease burden. Yet, several vaccines were approved in record time, with two of the fastest approved and most effective vaccines produced with the new mRNA technology. The concept of using mRNA for vaccination and in vivo delivery of therapeutic proteins was first proposed over 30 years ago, but several hurdles had to be overcome to make this a clinical reality. Early experiments demonstrated that in vitro transcribed mRNA stimulates undesired inflammatory responses and inefficient protein production in cells and tissues. A turning point was the discovery by Karikó and Weissman demonstrating that mRNA produced with modified nucleoside bases evades innate immune recognition and improves protein expression. These findings, combined with the development of efficient systems for in vivo mRNA delivery, stabilization of the SARS-CoV-2 Spike antigen, and unparalleled investments by industry and governments, led to the approval of two highly successful mRNA-based COVID-19 vaccines in late 2020. The discovery by Karikó and Weissman was critical for making the mRNA vaccine platform suitable for clinical use at a time when it was most needed, making this an extraordinary contribution to medicine and paving the way for future mRNA applications.” [91] For their discoveries concerning nucleoside base modifications that enabled the development of effective messenger RNA (mRNA) vaccines against COVID-19, Katalin Karikó and Drew Weissman received the 2023 Nobel Prize in Physiology or Medicine. In fact, the rapid development of highly efficacious vaccines against SARS-CoV-2 at unprecedented speed in the history of vaccinology and the subsequent global vaccination campaign have changed the course of the COVID-19 pandemic [92]. The process of vaccine development is lengthy and expensive [93]. Given the high cost and elevated failure rates, developers often adhere to a linear sequence of steps, incorporating frequent pauses for data analysis and manufacturing-process evaluations (Figure 13A). However, the rapid development of vaccines in a public health emergency necessitates a fast-track process. This involves initiating multiple steps concurrently, without waiting for the confirmation of success from one step before proceeding to the next (Figure 13B).

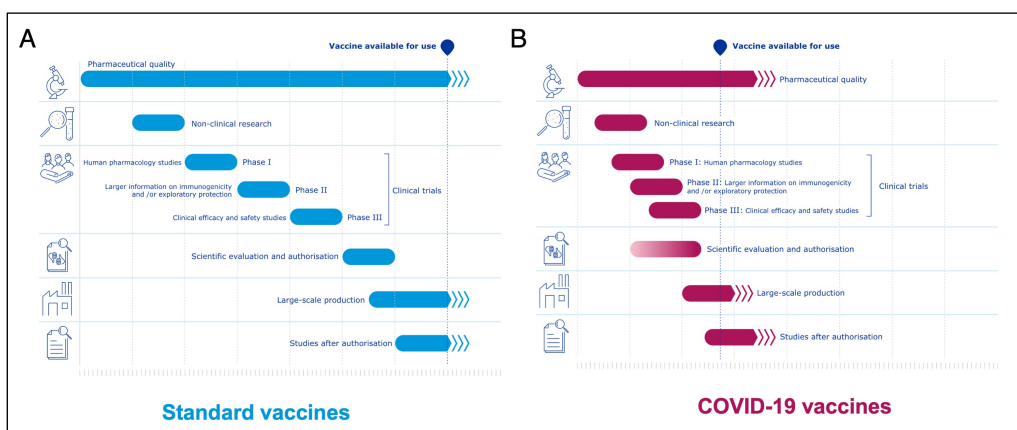


Figure 13 – Indicative timeline for COVID-19 vaccines compared to standard vaccines.

Compared to the standard vaccine development timeline (A), the COVID-19 vaccine development process was significantly expedited (B), resulting in the rapid approval and distribution of vaccines within a year of the onset of COVID-19 pandemic. Figure modified from the European Medical Agency [94].

This approach resulted in the approval of the first COVID-19 mRNA vaccine by the end of 2020. Structure-based vaccine design was used to develop COVID-19 vaccine [95]. A few months after the initial COVID-19 outbreak in China, Wrapp et al. determined a 3.5-Å-resolution cryo-electron microscopy structure of the SARS-CoV-2 Spike trimer in the prefusion conformation [96]. Based on the first published SARS-CoV-2 genome sequence, they expressed ectodomain residues 1 to 1208 of the Spike protein, incorporating two proline mutations within the C-terminal S2 fusion domain to enhance stability (Figure 14A-B) [96,97,98,99]. Before 2005, using mRNA for therapeutic purposes was hampered by low expression and strong inflammatory responses, but breakthrough studies by Karikó, Weissman, and colleagues found that chemically modifying nucleosides in mRNA could significantly reduce immune activation while enhancing protein expression [100,101]. Ionizable lipid nanoparticles are used as carriers protecting the mRNA from degradation by RNases and facilitating its delivery into the cytosol of cells, but also as adjuvant [102]. BNT162b2 and mRNA-1273 are lipid nanoparticle-encapsulated RNA vaccines expressing the prefusion stabilized SARS-CoV-2 full-length Spike protein (Figure 14C) [102,103,104]. They both enter the cell by endocytosis (or direct fusion with the plasma membrane). After endosomal escape into the cytoplasm, the Spike-mRNA is translated at ribosomes associated with the endoplasmic reticulum and newly synthesized Spike is transported into the lumen of this compartment, similar to the process occurring in natural infection. Further transport occurs via the exocytic pathway, leading to expression of Spike at the plasma membrane. The intracellularly synthesized protein is also degraded and enters the major histocompatibility complex (MHC) I and II pathways.

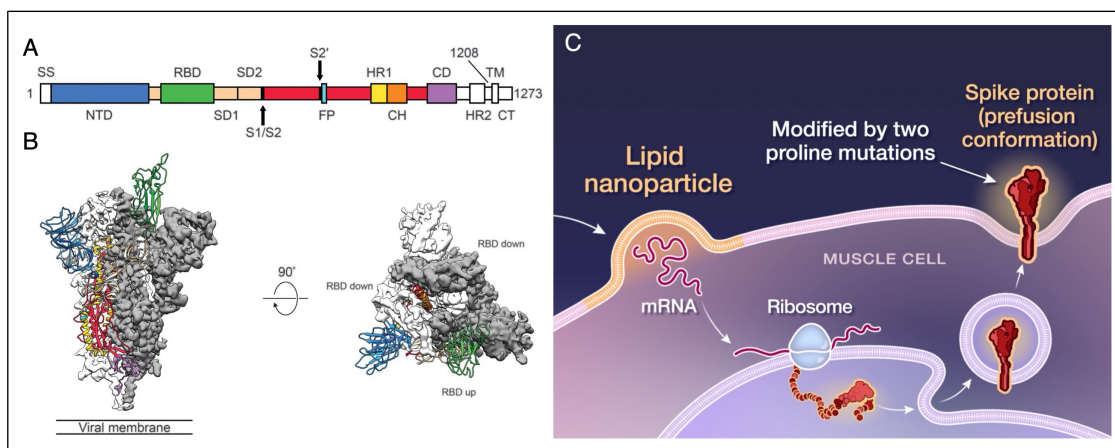


Figure 14 – Structure of SARS-CoV-2 Spike protein in the prefusion conformation and mRNA vaccine delivery.

(A) Schematic of SARS-CoV-2 Spike primary structure colored by domain. Domains that were excluded from the ectodomain expression construct or could not be visualized in the final map are colored white. SS, signal sequence; S2', S2' protease cleavage site; FP, fusion peptide; HR1, heptad repeat 1; CH, central helix; CD, connector domain; HR2, heptad repeat 2; TM, transmembrane domain; CT, cytoplasmic tail. Arrows denote protease cleavage sites at S1/S2 boundary and at S2' site. **(B)** Side and top views of the prefusion structure of the SARS-CoV-2 Spike protein with a single receptor binding domain (RBD) in the up conformation. The two RBD protomers down are shown as cryo-EM density in either white or gray and the RBD protomer up is shown in ribbons colored corresponding to the schematic in panel (A). **(C)** Schematic representation of mRNA vaccine-mediated expression of the Spike protein in transduced cells. Figures modified from Wrapp et al. Science 2020 and NEJM 2020 [96,105].

In addition to mRNA vaccines Pfizer-BioNTech's Comirnaty BNT162b2 and Moderna's Spikevax mRNA-1273, other COVID-19 vaccines involving distinct platforms were developed, such as inactivated virus vaccines, adenovirus vector-based vaccines, and adjuvanted protein subunit vaccines. Vaccination schedules differ based on the vaccine platform and primarily consider factors such as an individual's immune status, age, and history of prior SARS-CoV-2 infection. While the original vaccines targeted the ancestral Spike protein from the Wuhan strain, updated or bivalent vaccines that include protection against viral variants are now recommended [106]. Regarding mRNA vaccination, studies on early recipients highlighted the necessity of administering two spaced doses to individuals without history of previous SARS-CoV-2 infection to establish a robust humoral and adaptive immune response comparable to those observed in previously infected individuals, for whom one single dose is sufficient to reactivate immunological memory [107,108,109].

Vaccination efforts have been monumental in slowing the spread of the virus, particularly in reducing the severity of disease and preventing hospitalizations and deaths. It has been estimated that vaccinations prevented almost 20 million deaths from SARS-CoV-2 infection in 185 countries between December 2020 to December 2021, representing a global reduction of 63% in total deaths during the first year of vaccination [110]. A retrospective surveillance study calculated that COVID-19 vaccination programmes in the WHO European Region were associated with a 59% reduction in the number of deaths among individuals aged 25 years or older between December 2020 to April 2023, accounting for over 1.6 million lives saved [111]. Of the total lives saved 96% were among people aged 60 years or older and 52% among people aged 80 years or older [111]. Remarkably, 60% of all lives were estimated to be saved during the Omicron period [111]. Despite the global vaccination campaign being a historic achievement, it has also underscored significant disparities in access, distribution, and efficacy. Globally, as of end-December 2023, more than 13.6 billion vaccine doses have been administered [112]. Approximately 67% of the global population has completed a full primary series of a COVID-19 vaccine and 32% has received at least one booster dose [112]. Nonetheless, access to vaccination worldwide has remained inequitable [112,113,114]. Countries with higher economic resources have generally achieved better vaccination coverage [115]. In high-income nations, over 90% of the population has been vaccinated, with booster doses being administered regularly, particularly for vulnerable populations. However, in many low-income countries, vaccination rates remain low, with less than 40% of the population fully vaccinated in many regions and limited booster campaigns available [112].

Disparities also extend to individual differences, particularly among high-risk populations, including people with compromised immune status, people with comorbidities and older adults [116,117]. Patients with inborn errors of immunity are at increased risk of severe COVID-19 and their response to COVID-19 vaccination can vary significantly based on the type and severity of their immune deficiency [118,119]. Similarly, individuals with secondary immunodeficiency exhibit impaired vaccine responses due to therapies that suppress or modulate the immune system, such as chemotherapy for malignancies, immunomodulatory treatments for immune-mediated diseases, and

anti-rejection therapies for organ transplantation [120]. These variations in immune response underscore the need for tailored vaccination strategies and close monitoring for high-risk populations to enhance their protection against COVID-19 [121]. As of November 2021, when the PhD project was started, there were limited data on the effect of additional vaccine doses on the reactivation and durability of the immunological memory against SARS-CoV-2 in immunocompromised individuals [122,123]. However, in response to emerging variants and the waning immunity observed over time, booster doses have become a critical component of vaccination strategies in many countries [124]. To evaluate the effect of immunosuppression on vaccine-induced immunity, three main cohorts have been identified in the current study: patients with B-cell lymphoma either undergoing or previously treated with rituximab (RTX), solid-organ transplant recipients on immunosuppressive therapy, and people living with HIV (PLWH).

RTX is widely used in the treatment of various CD20-expressing lymphoid malignancies, including indolent and aggressive forms of B-cell non-Hodgkin lymphoma and it has become the standard-of-care for follicular lymphoma, diffuse large B-cell lymphoma, chronic lymphocytic leukemia, and mantle cell lymphoma [125,126]. RTX is a human/murine chimeric monoclonal antibody that targets CD20 on B lymphocytes, resulting in rapid and prolonged depletion of B cells. Typically, circulating B cells become detectable again approximately 6 to 9 months after the end of treatment, with the emergence of a predominately immature naïve B cell population [127,128]. However, the kinetics of B-cell reconstitution can be affected by the concurrent use of other therapies, such as alkylating agents (e.g. bendamustine, chlorambucil, and cyclophosphamide) and purine analogues (e.g. fludarabine) [129]. In such cases, B-cell aplasia may persist for an even longer duration. In patients with hematological malignancies, anti-CD20 therapy is usually combined with intensive chemotherapy regimens to enhance the antineoplastic effects of RTX. Consequently, this approach also results in greater immunosuppression, compared to RTX regimens for treating patients with immune-mediated diseases [130]. Patients undergoing these highly immunosuppressive treatments experience increased risk of infections and a more severe disease course during SARS-CoV-2 infection [131,132]. Additionally, they exhibit impaired vaccine-induced immunity due to a deficient humoral immune response [133,134]. It has been shown that patients with autoimmune rheumatic diseases treated with RTX have poor humoral immune responses after anti-SARS-CoV-2 vaccinations [135]. However, T-cell responses appear to remain intact [136,137]. While T-cell immunity may help to compensate for ineffective humoral responses in patients with B-cell depletion, antibodies remain essential for effective viral clearance.

Maintenance immunosuppression therapy following transplantation typically consists of a combination of calcineurin inhibitors (i.e. tacrolimus or cyclosporine), mTOR inhibitors (i.e. sirolimus or everolimus), antimetabolites (i.e. mycophenolate mofetil or azathioprine), and corticosteroids (i.e. prednisone) [138]. Tacrolimus (originally designated FK506) and cyclosporine inhibit calcineurin, a calcium- and calmodulin-dependent phosphatase that is critical for T-cell activation, as it phosphorylates a family of transcription factors (NF-AT), which then translocate to the nucleus to upregulate the expression of IL-2, TNF- α , IL-3, IL-4, CD40L, GM-CSF, and IFN- γ

[139]. Sirolimus and its derivative, everolimus, exert their immunosuppressive effect by binding to FKBP12, so that the formed complexes block mammalian target of rapamycin (mTOR), thereby disrupting DNA and protein synthesis and preventing proliferation of T, B, and NK cells [140,141]. Mycophenolate mofetil is a prodrug of mycophenolic acid, an inhibitor of inosine-5'-monophosphate dehydrogenase, which depletes guanine nucleotides, specifically targeting T and B lymphocytes to inhibit their proliferation, thereby suppressing cell-mediated immunity and antibody production [142]. Azathioprine, a purine analog, is metabolized to 6-mercaptopurine and subsequently converted into 6-thiouric acid, 6-methyl-mercaptopurine, and 6-thioguanine; these metabolites are incorporated into replicating DNA and inhibit the de novo pathway of purine synthesis [143,144]. This mechanism is thought to target lymphocytes selectively, as they lack a salvage pathway [143,144]. Glucocorticoids are included in maintenance immunosuppression regimens for their broad immunosuppressive and anti-inflammatory effects; the majority of their actions can be attributed to the transcriptional consequences of glucocorticoid receptor (GR) agonism, altering the transcription of a multitude of genes in leukocytes, resulting in both up- and downregulation [145,146]. Solid-organ transplant recipients have a suboptimal response to vaccination, mainly because they receive these immunosuppressive agents which are essential to maintain graft function and prevent organ rejection [147,148]. These regimens can significantly impair immune responses, leading to increased susceptibility to infections [149,150,151]. Hence, optimization of vaccination strategies for solid-organ transplant recipients is necessary and includes the careful monitoring of vaccine-induced immunological memory.

People living with HIV (PLWH), particularly those with advanced disease, low CD4+ T cell counts, or unsuppressed viral loads, are classified as high-risk population, due to human immunodeficiency virus (HIV) primary targeting of CD4+ T lymphocytes [152,153]. HIV enters host cells via binding to CD4 receptor and chemokine receptor, CCR5 or CXCR4, initiating reverse transcription of its RNA into proviral DNA, which subsequently integrates into the cell genome [154]. This process leads to progressive depletion of CD4+ T lymphocytes and results in an immunodeficient state, culminating in the acquired immunodeficiency syndrome (AIDS), which predisposes individuals to severe opportunistic infections and complications [155]. Studies have shown that PLWH who contract SARS-CoV-2 infection experience higher rates of hospitalization and intensive care unit admissions compared to individuals without HIV [156,157,158]. Immune responses elicited by vaccines, including those for influenza and pneumococcus, are diminished, likely due to CD4+ T cell depletion and associated B cell dysfunction [159]. Vaccination, including anti-SARS-CoV-2 immunization, may transiently elevated HIV viral loads, potentially exposing latent viral reservoirs [160,161,162,163,164]. It has been demonstrated that a third mRNA vaccine dose significantly enhanced humoral immunity in PLWH, regardless of CD4+ T cell counts [165]. In early 2022, some countries introduced additional booster doses for high-risk populations, including PLWH [166,167]. A fourth dose of the original BNT162b2 mRNA vaccine, administered during the Omicron wave, reduced the short-term risk of COVID-19-related outcomes among persons who had received a third dose at least 4 months earlier as well as the short-term risk of

death from all causes in residents of long-term care facilities and in individuals aged 80 years or older [168,169]. However, in generally healthy individuals, following a fourth vaccine dose, the antibody response peaked around 4 weeks, then declined to pre-dose levels by 13 weeks, and stabilized thereafter [170]. Thus, the added immunologic benefit of a fourth vaccine dose appears to wane by 13 weeks post-administration.

2. OBJECTIVES

The goal of this PhD project is to comprehensively investigate the immune response induced by SARS-CoV-2 infection and anti-SARS-CoV-2 vaccination, focusing on the profiling and long-term kinetics of virus-specific T cells and B cells in both immunocompetent and immunocompromised individuals. The long-term monitoring of the adaptive immune response at multiple time points post-vaccination is particularly aimed to characterize the phenotypic and functional properties of T and B cells after booster doses. Additionally, the project aims to assess T cell cross-reactivity to viral variants of concern in vaccinated individuals.

The project is geared towards:

- Investigating the adaptive immune response at multiple time points post-infection and post-vaccination, focusing on the kinetics of Spike-specific T and B cells.
- Characterizing the phenotypic and functional properties of Spike-specific T and B cells, particularly after the administration of booster doses, to assess immune memory and protection longevity.
- Assessing T cell reactivity to viral variants of concern, determining how mutations in SARS-CoV-2 affect immune recognition and functionality in vaccinated individuals.
- Comparing immune responses between immunocompetent and immunocompromised individuals, including those with underlying conditions or on immunosuppressive therapies, to identify key differences that may impact vaccine efficacy.
- Informing strategies for optimizing vaccination protocols, especially for high-risk populations, by providing insights into the quality and durability of immune responses across different cohorts.

3. MATERIALS AND METHODS

3.1 Study population

Participants were enrolled at Careggi University Hospital and Meyer Children's Hospital, Firenze, Italy. The study population included the following cohorts (Figure 15):

- (1) Cohort 1: Healthcare workers who received the two-dose BNT162b2 mRNA vaccine series (administered 21 days apart) between December 2020 and January 2021 and the third dose in October 2021, 9 months after the second vaccine dose; follow-up assessments were conducted at 1 month, 6 months, and 8 months after the second vaccine dose and 1 week after the booster.
- (2) Cohort 2: Patients hospitalized for COVID-19 between March and April 2020 who had not been vaccinated; follow-up evaluations were conducted at 1 month, 6 months, and 12 months post-discharge.
- (3) Cohort 3: Patients with B-cell lymphoma, either currently undergoing or previously treated with chemotherapy, including rituximab (RTX), who received the two-dose mRNA-1273 vaccine series (administered 28 days apart) between April and June 2021 and the third vaccine dose between December 2021 and January 2022; follow-up assessments were conducted up to 1 month post-second vaccine dose and 1 month post-third vaccine dose.
- (4) Cohort 4: Solid-organ transplant recipients on immunosuppressive therapy who received the two-dose mRNA-1273 vaccine series (administered 28 days apart) in March 2021, the third vaccine dose between September and October 2021 and the fourth vaccine dose between April and May 2022; they were evaluated up to 1 month post-second vaccine dose and 1 month post-booster.
- (5) Cohort 5: People living with HIV (PLWH) who received a fourth dose of BNT162b2 mRNA vaccine in March 2022; they were evaluated at 7 days, 1 month and 2 months post-vaccination.

Cohort 1 was divided into two groups: healthcare workers with no prior history of SARS-CoV-2 infection (naïve individuals) and those who had been previously infected by SARS-CoV-2 (COVID-19 recovered) (Figure 15). SARS-CoV-2 infection was confirmed by PCR analysis on nasopharyngeal swabs. Negative history of infection in naïve individuals was based on absence of symptoms, absence of anti-Nucleoprotein IgG and anti-Spike IgG before vaccination, and routine negative monitoring by PCR analysis on nasopharyngeal swabs.

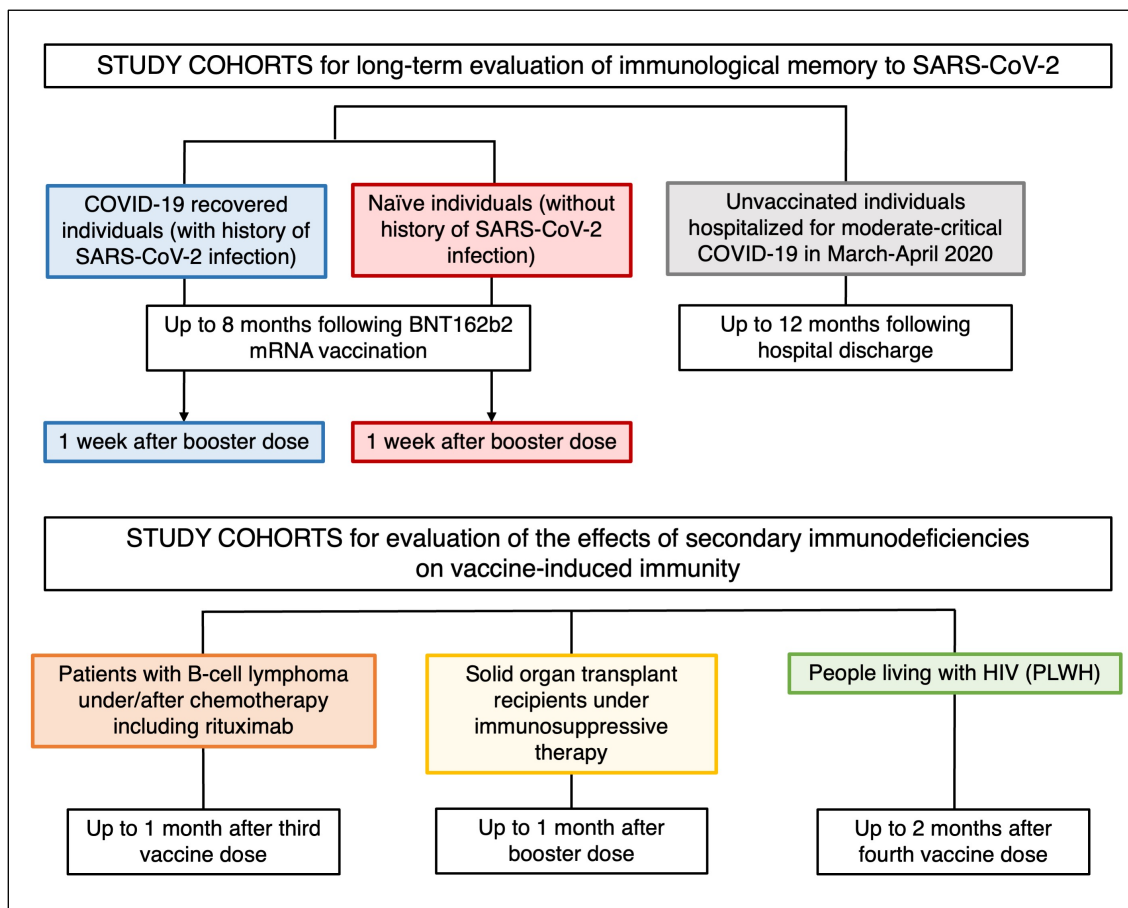


Figure 15 – Study Population.

Study population for long-term evaluation of immunological memory to SARS-CoV-2 included healthcare workers with or without history of SARS-CoV-2 infection followed for up to 8 months after vaccination and one week after booster (cohort 1) and patients hospitalized for COVID-19 during the first wave of the pandemic followed for up to 12 months post-hospital discharge (cohort 2). Study population to evaluate the effects of secondary immunodeficiencies on adaptive immunity induced by anti-SARS-CoV-2 vaccination included patients with B-cell lymphoma vaccinated either after or while under chemotherapy, including rituximab (cohort 3), solid-organ transplant recipients vaccinated while under immunosuppressive therapy (cohort 4), and people living with HIV who were evaluated up to 2 months post-fourth dose (cohort 5).

Following the vaccination schedule approved at the time of study inclusion (December 2020-January 2021), all healthcare workers, regardless of prior SARS-CoV-2 exposure, received two doses of the original BNT162b2 mRNA vaccine, administered 21 days apart. Peripheral blood samples were collected at baseline (before the first vaccine dose), before the second dose, and at 1 month, 6 months, and 8 months post-vaccination (Figure 16A). Additionally, a subcohort of COVID-19 recovered and naïve subjects was evaluated before and one week after receiving the booster dose (Figure 16A). Considering patients hospitalized for COVID-19 between March and April 2020 who had not yet been vaccinated, peripheral blood samples were collected at 1 month, 6 months, and 12 months after hospital discharge (Figure 16B). Demographic (age and gender) and clinical data (COVID-19 severity) on disease course during hospital stay were retrieved from electronic medical records.

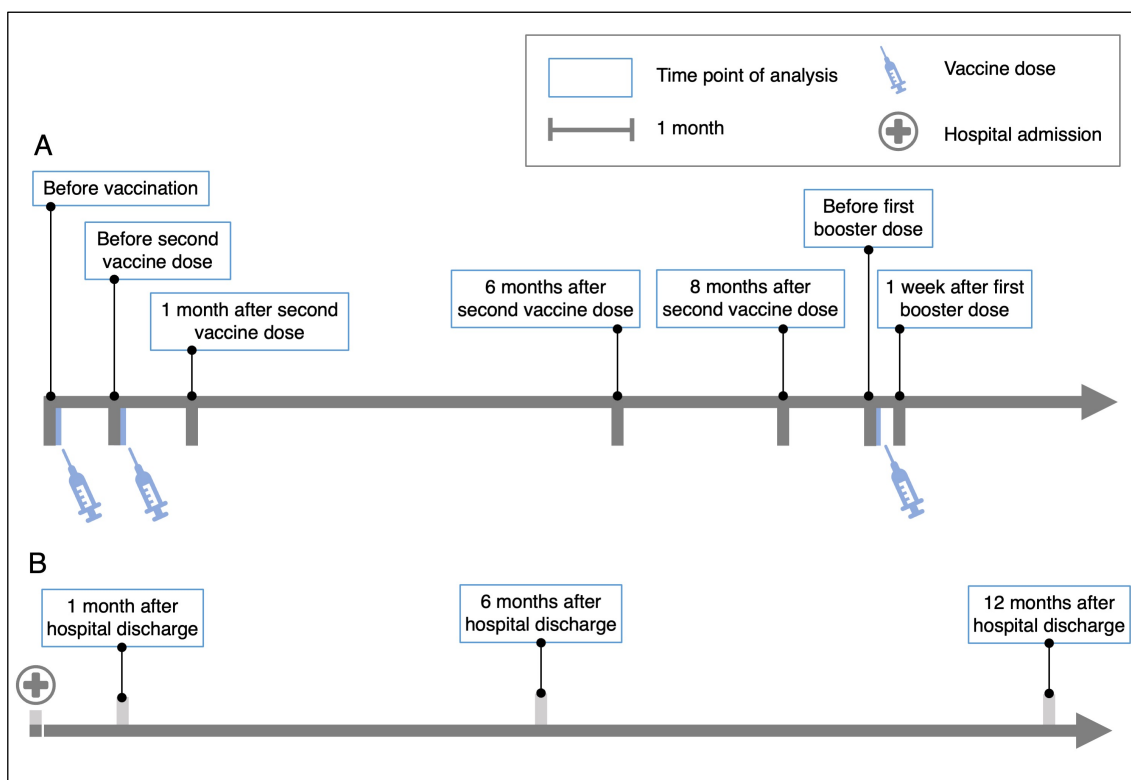


Figure 16 – Time points of analysis for cohort 1 and cohort 2.

Panel A illustrates the time points at which healthcare workers (cohort 1) were analyzed based on their vaccination schedule for the evaluation of long-term vaccine-induced immunity; key points include pre-vaccination (baseline), post-first and post-second dose evaluations, and follow-up assessments at 6 and 8 months post-second dose, as well as one week after the first booster dose. Panel B shows the time points at which patients who were infected with the Wuhan strain between March and April 2020 were evaluated following their hospital discharge (cohort 2). These evaluations were conducted at 1, 6 and 12 months after hospital discharge to assess long-term immunity post-infection.

Cohort 3 was divided in two groups: the first included patients with B-cell lymphoma who received two doses of the original mRNA-1273 vaccine while undergoing RTX therapy, and the second included patients who had completed RTX therapy between 3 and 12 months prior to vaccination. Participants were further stratified based on the chemotherapy regimen received, particularly considering the treatment with bendamustine. Peripheral blood samples were obtained at baseline (before the first vaccine dose), 28 days after (before the second dose), 1 month post-second dose, and 1 month following the third vaccine dose (Figure 17A).

Cohort 4 included three groups: (i) individuals with cystic fibrosis receiving immunosuppressive treatment following lung transplantation, (ii) individuals with cystic fibrosis who did not require lung transplantation, and (iii) individuals under immunosuppressive therapy following kidney transplantation. These study participants were evaluated at baseline (before vaccination), 28 days after (prior to the second dose of the original mRNA-1273 vaccine), and one month after the second dose (Figure 17B). Some lung-transplanted patients with cystic fibrosis were also assessed before and one month after the third or fourth vaccine dose (Figure 17B).

Finally, people living with HIV (cohort 5) who received a fourth vaccine dose in March 2022 were enrolled in the study and sampled at baseline (prior to the administration of the fourth vaccine dose), and subsequently at 7 days, 1 month, and 2 months post-vaccination (Figure 17C)

Demographic and clinical data were collected from electronic medical records.

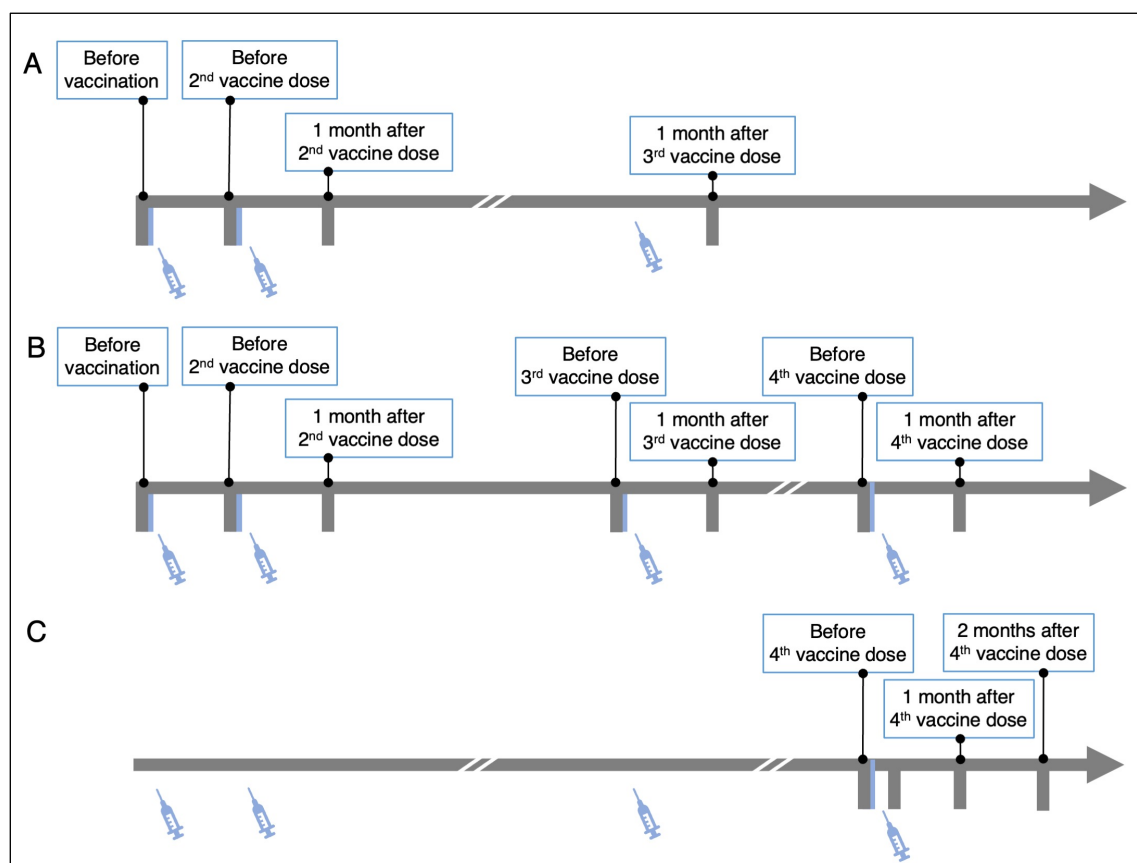


Figure 17 – Time points of analysis for cohort 3, cohort 4, and cohort 5.

Panel A illustrates the time points at which patients with B-cell lymphoma (cohort 3) were analyzed. Panel B shows the time points at which solid-organ transplant recipients (cohort 4) were evaluated. Panel C illustrated the time points at which people living with HIV (cohort 5) were analyzed.

In subjects with COVID-19 or with history of COVID-19, disease severity was defined according to WHO guidelines (Table 1) [171,172].

Table 1 – COVID-19 disease severity classification according to WHO.

Disease		Definition
Mild		Symptomatic patients meeting the case definition for COVID-19 without evidence of viral pneumonia or hypoxia.
Moderate	Pneumonia	Clinical signs of pneumonia (fever, cough, dyspnea, fast breathing) but no signs of severe pneumonia, including $SpO_2 \geq 90\%$ on room air.
Severe	Severe pneumonia	Clinical signs of pneumonia (fever, cough, dyspnea) plus one of the following: respiratory rate > 30 breaths/min; severe respiratory distress; or $SpO_2 < 90\%$ on room air. While the diagnosis can be made on clinical grounds; chest imaging (radiograph, CT scan, ultrasound) may assist in diagnosis and identify or exclude pulmonary complications. Onset: within 1 week of a known clinical insult (i.e., pneumonia) or new or worsening respiratory symptoms.
Critical	Acute respiratory distress syndrome (ARDS)	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 factor present. Oxygenation impairment: Mild ARDS: $200 \text{ mmHg} < PaO_2/FiO_2 \leq 300 \text{ mmHg}$ (with PEEP or CPAP $\geq 5 \text{ cmH}_2\text{O}$) Moderate ARDS: $100 \text{ mmHg} < PaO_2/FiO_2 \leq 200 \text{ mmHg}$ (with PEEP $\geq 5 \text{ cmH}_2\text{O}$) Severe ARDS: $PaO_2/FiO_2 \leq 100 \text{ mmHg}$ (with PEEP $\geq 5 \text{ cmH}_2\text{O}$).

CPAP: continuous positive airway pressure; CT: computed tomography; FiO_2 : fraction of inspired oxygen; PaO_2 : partial pressure arterial oxygen; PEEP: positive end-expiratory pressure; SpO_2 oxygen saturation.

3.2 Evaluation of SARS-CoV-2-specific T cells

Peripheral blood was collected in EDTA tubes (BD Vacutainer® K2E). Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples through density gradient centrifugation using Lymphoprep™. This procedure was followed to isolate mononuclear cells from peripheral blood: (1) blood was added to Lymphoprep™ at 3:2 ratio; (2) the tube was centrifuged at 1500 g for 20 minutes at 4 °C; (3) the ring of PBMCs was carefully collected from the plasma and transferred to a sterile 50 mL conical polypropylene centrifuge tube; (4) the tube was filled with physiological solution; (5) the cells were washed twice with physiological solution by centrifuging at 250 g for 10 minutes to remove any remaining Lymphoprep™. The samples were then cryopreserved in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen. For each individual, longitudinal samples were thawed and analyzed together for T cell and B cell assessments.

For T cell stimulation *in vitro*, 1.5 million PBMCs were cultured in complete RPMI medium supplemented with 5% human AB serum in 96-well flat-bottom plates. Cells were exposed to either medium alone (background, control) or a pool of SARS-CoV-2 Spike peptides (Prot_S1, Prot_S+, and Prot_S) at 0.6 µM/peptide, according to the manufacturer's instructions (PepTivator®, Miltenyi Biotech) (Figure 18). Prot_S covers predicted immunodominant regions of the SARS-CoV-2 Spike protein, Prot_S1 covers the N-terminal S1 domain, Prot_S+ covers part of the C-terminal S2 domain (Figure 18). In patients hospitalized for COVID-19, staphylococcal enterotoxin B (SEB) at 1 µg/ml (Sigma-Aldrich) was used as an additional condition to assess polyclonal cytokine production. PBMCs from patients who recovered from COVID-19 longitudinally assessed up to 12 months following hospital discharge were additionally stimulated with pool of peptides covering the complete sequence of the Membrane protein and the Nucleoprotein of SARS-CoV-2, accordingly to manufacturer's instructions (PepTivator®, Miltenyi Biotech). For evaluation of cross-reactivity against the Omicron variant, cells were stimulated with peptide pools selectively spanning the mutated regions in the Omicron (B.1.1.529) BA.1 variant and harboring the specific mutations (B.1.1.529 BA.1 mutated pool, Miltenyi Biotech). As a control, the same peptide pools but with the original (Wuhan strain) sequence were used (reference pool, Miltenyi Biotech).

After 2-hour incubation at 37°C (98.6°F) and 5% CO₂, Brefeldin A (5 µg/mL) was added, followed by an additional 4-hour incubation. The cells were then fixed and stained using the fluorochrome-conjugated antibodies listed in Table 2. A schematic overview in Figure 19 illustrates how Spike-specific T cells are detected via CD154 (CD40L) expression and production of IFN-γ, TNF-α and/or IL-2.

Table 2 – List of fluorochrome-conjugated antibodies used for flow cytometric analysis of SARS-CoV-2-specific T cells.

Antigen	Fluorochrome	Clone	Company
TNF- α	FITC	6401.1111	BD Biosciences
CD154	PE	TRAP1	BD Biosciences
CD3	PerCP	SK7	BD Biosciences
CD4	PE-Cy7	SK3	Invitrogen
CD8	Super Bright 600	SK1	eBioscience
IL-2	APC	MQ1-17H12	BD Biosciences
IFN- γ	Pacific Blue	B27	BioLegend
L/D	Fixable Viability Stain 780	-	BD Biosciences

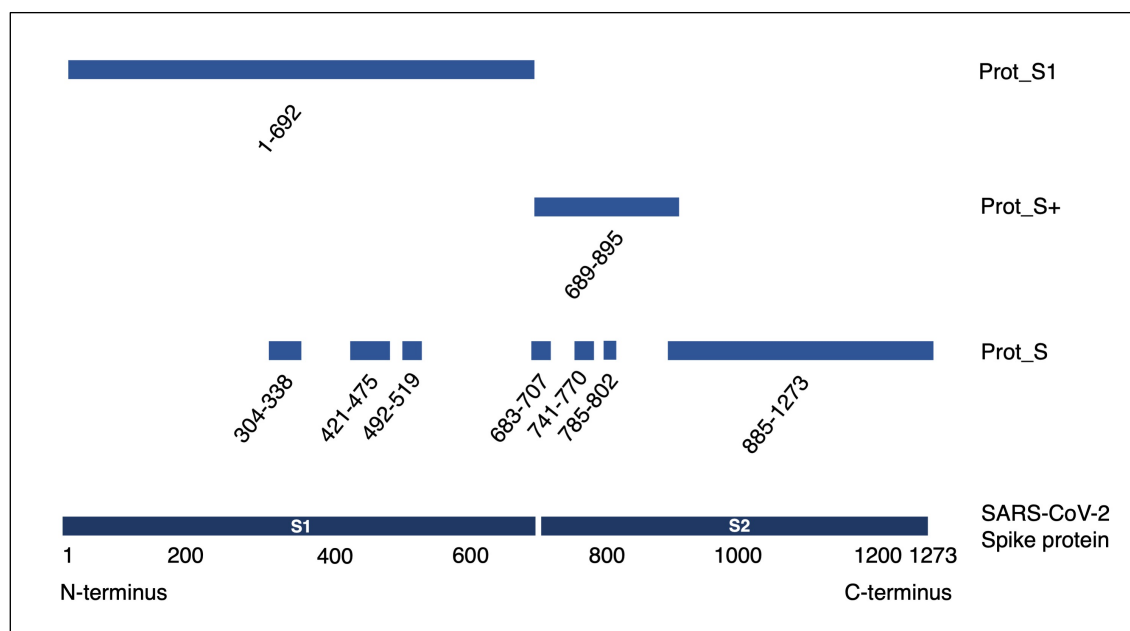


Figure 18 – Schematic overview of alignment of Spike protein and different peptide pools.

The pooled use of the Prot_S1, Prot_S+, and Prot_S peptides allowed for comprehensive coverage of the entire SARS-CoV-2 Spike protein, ensuring that all regions of the protein were represented in the immunological analysis.

3.3 Evaluation of SARS-CoV-2-specific B cells

For *in vitro* evaluation of B cells, 2 million PBMCs were stained for 30 minutes at 4°C using fluorochrome-conjugated antibodies, as detailed in Table 3. After staining, cells were washed with PBS/EDTA buffer (PEB), and incubated for 5 minutes with 7-aminoactinomycin D (7-AAD) to assess viability. Recombinant biotinylated SARS-CoV-2 Wuhan and Omicron Spike proteins (Miltenyi Biotec) were conjugated separately with streptavidin PE (phycoerythrin) and PE-Vio770 for 15 minutes at room temperature. The two conjugates were pooled at a 1:2 ratio and added to the final staining mix (Table 3). The recombinant Spike protein covers the ectodomain, spanning aminoacids V16 to K1211, and includes stabilizing proline substitutions at positions K986P and V987P, along with a mutated Furin cleavage site (RRAR to GSAS substitution at residues 682–685). Additionally, this protein is extended at the C-terminus with a His-tag and an AviTag™. A schematic overview illustrating how the biotinylated Spike protein can be used to detect patient-derived Spike-specific B cells is shown in Figure 19.

Table 3 – List of fluorochrome-conjugated antibodies used for flow cytometric analysis of SARS-CoV-2 Spike-specific B cells.

Antigen	Fluorochrome	Clone	Company
CD19	APC-Vio770	LT19	Miltenyi Biotech
CD27	VioBright FITC	M-T271	Miltenyi Biotech
IgA	VioGreen	IS11-8E10	Miltenyi Biotech
IgG	VioBlue	IS11-3B2.2.3	Miltenyi Biotech
CD14	PerCP	TÜK4	Miltenyi Biotech
IgM	APC	PJ2-22H3	Miltenyi Biotech
CD3	PerCP	BW264/56	Miltenyi Biotech
7-AAD	-	-	Miltenyi Biotech
Spike	PE	-	Miltenyi Biotech
Spike	PE-Vio770	-	Miltenyi Biotech

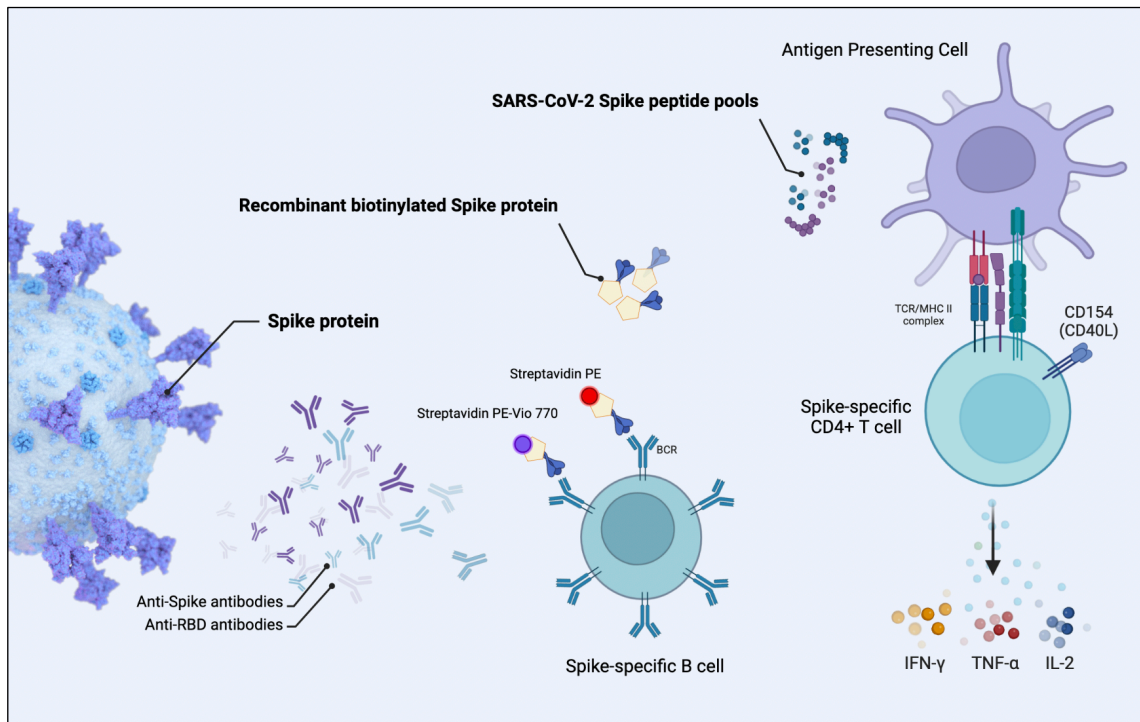


Figure 19 – Schematic overview of *in vitro* evaluation of SARS-CoV-2 Spike-specific B and T cells.

For the detection of Spike-specific B cells, biotinylated Spike protein conjugated with streptavidin PE and PE-Vio770 was used. For the analysis of Spike-specific T cells, PBMCs were stimulated with SARS-CoV-2 peptide pools. Spike-specific T cells were identified by CD154 expression and cytokine production (IFN- γ , TNF- α , IL-2).

3.4 Flow cytometry analysis

Samples were acquired on a BD LSR II flow cytometer (BD Biosciences) with FACSDiva Software and analyzed by FlowJo Software. All flow cytometric analyses were performed following published guidelines [173,174]. Gating strategies used to identify Spike-specific lymphocytes are shown in Figure 20 for T cells and in Figure 21 for B cells.

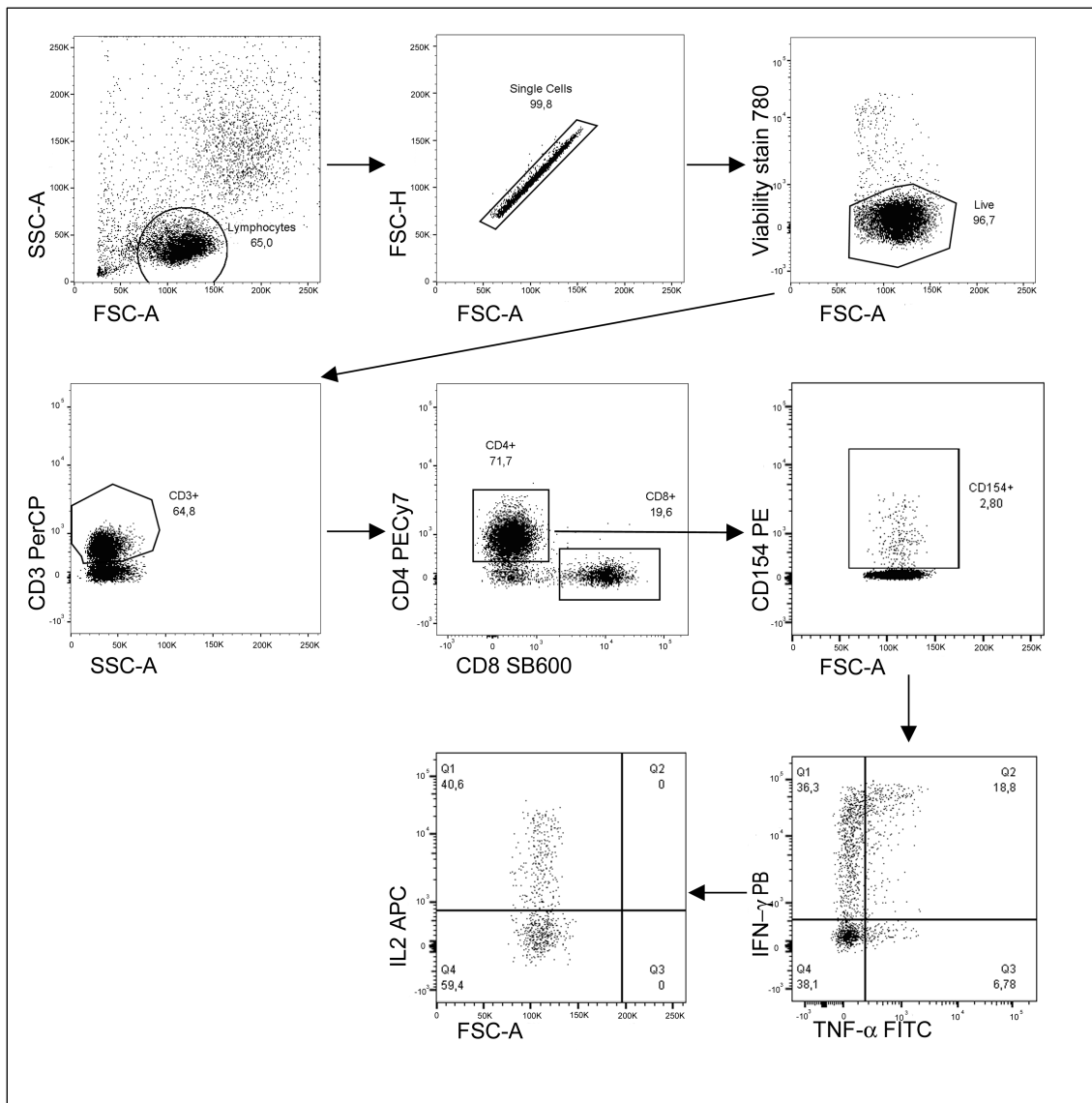


Figure 20 – Gating strategy for the identification of Spike-specific T cells.

Lymphocytes are gated based on physical parameters (FSC-SSC). Doublets are removed using FSC-H and FSC-A parameters. Dead cells are excluded using viability stain 780. T cells are identified by their expression of CD3. CD4+ T cells are further examined and among these, cells expressing CD154 are identified. CD4+CD154+ T cells are then evaluated for IFN- γ , TNF- α , or IL-2 expression.

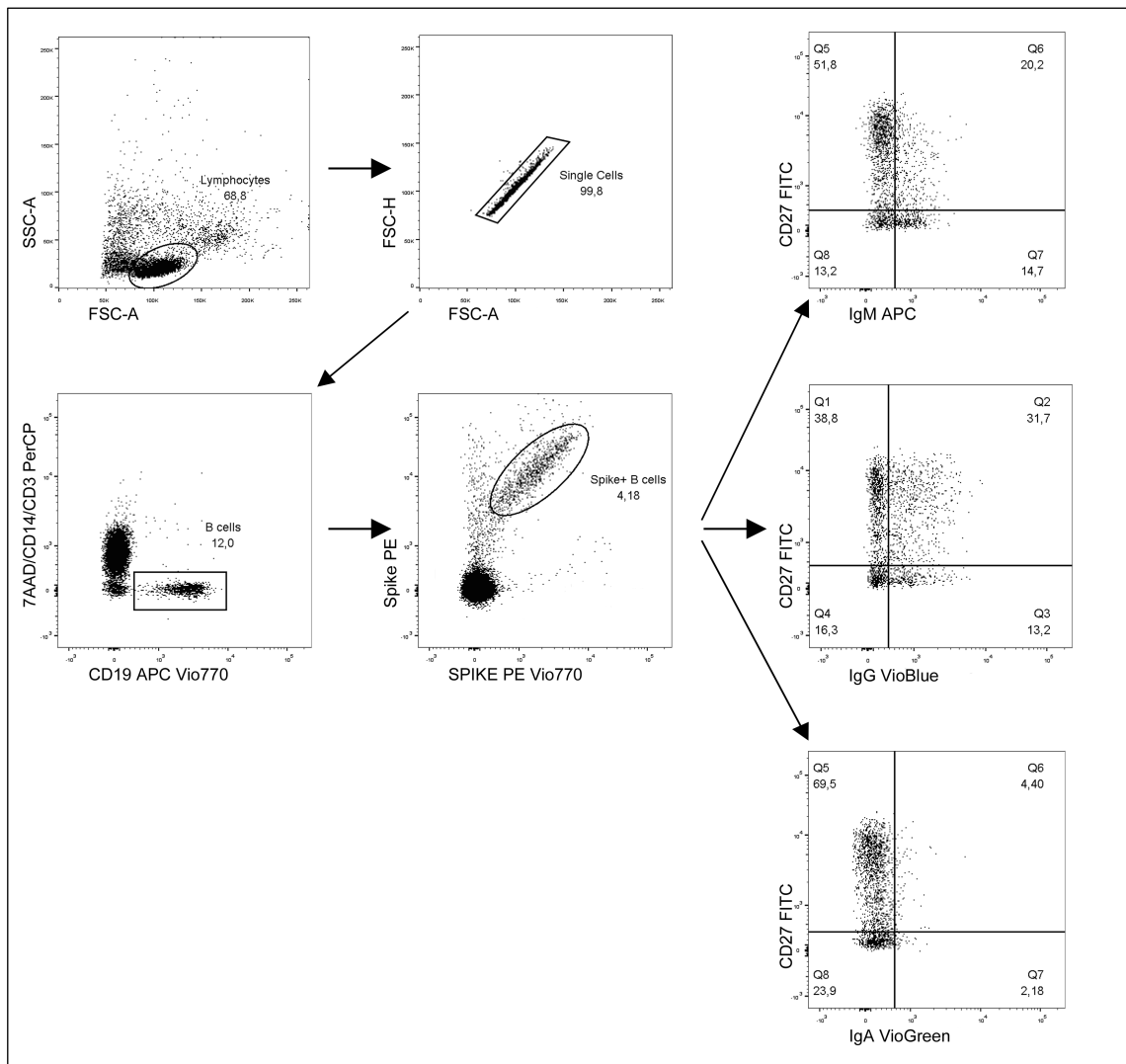


Figure 21 - Gating strategy for the identification of Spike-specific B cells.

Lymphocytes are gated based on physical parameters (FSC-SSC). Doublets are removed using FSC-H and FSC-A parameters. PerCP is used as a dump channel for the exclusion of dead cells (7-AAD), CD3+ cells and CD14+ cells. B cells are identified as CD19+ cells. B cells binding PE- and PE Vio770-conjugated Spike protein are identified as Spike-specific B cells. Among Spike-specific B cells, CD27 expression associated to IgG, IgM and IgA was evaluated.

3.5 Evaluation of SARS-CoV-2-specific humoral response

Serum was collected from serum separator tubes (BD Vacutainer® SST™ II Advance), frozen and stored at -20°C (4°F). Evaluation of anti-Spike protein (in trimeric form) IgG (Diasorin), IgM (Abbot), and IgA (Euroimmun), anti-RBD IgG (Abbott), anti-Nucleoprotein IgG (Abbott), and neutralizing antibodies that block binding of Spike protein with the ACE2 receptor (Dia.Pro Diagnostic Bioprobes) was performed following manufacturers' instructions. Neutralizing antibodies were evaluated via ACE2-RBD neutralization assay (Dia.Pro Diagnostic Bioprobes) which is an enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative determination of inhibition activity of RBD-ACE2 binding induced by antibodies to SARS-CoV-2. The antibody reactivity of each specimen is expressed in AU/ml or by the ratio between optical density and cut-off value (index).

3.6 Statistics

Unpaired samples were compared using the Mann-Whitney U test, while Wilcoxon's signed-rank test was used to compare different time points of analysis within each study group. In all cases, P values ≤ 0.05 were considered statistically significant. Data analysis was performed using OriginPro, Version 95E (OriginLab Corporation, Northampton, MA, USA).

3.7 Study approval

The study was approved by the Careggi University Hospital Ethics Committee [protocol CEAVC 16859 – *COVID-19 - Caratterizzazione fenotipica e funzionale delle cellule del sistema immunitario in pazienti con infezione da SARS-CoV-2* (COVID-19 – Phenotypic and functional characterization of immune cells in patients with SARS-CoV-2 infection), protocol CEAVC 19466 – *COVAC - Caratterizzazione della risposta immunitaria in soggetti sottoposti a vaccinazione per SARS-CoV-2* (COVAC – Characterization of the immune response in subjects vaccinated against SARS-CoV-2), and protocol CEAVC 23847_BIO – *CROSSVAC - Persistenza ed intensità della risposta immunitaria adattativa indotta dal vaccino anti-SARS-CoV-2, compresa la valutazione della cross-reattività alle varianti del virus, in bambini sani ed in pazienti pediatrici e adulti immunocompromessi: implicazione per la strategia di vaccinazione* (Persistence and magnitude of adaptive immune response to anti-SARS-CoV-2 vaccine, including assessment of cross reaction to virus variants, in healthy children and in immunocompromised paediatric and adult patients: implication for vaccination strategy)] and by the Spallanzani Hospital Ethics Committee (protocol SCIG N483 - em. sost. n.1-13.01.2023). Written informed consent was obtained from recruited individuals.

4. RESULTS

4.1 Long-term evaluation of adaptive immunity to SARS-CoV-2

A total of 209 subjects were recruited from March 2020 to March 2022 at Careggi University Hospital and Meyer Children's Hospital, Firenze, Italy and divided into 5 cohorts. Cohort 1 included 30 healthcare workers who received the original BNT162b2 mRNA vaccine (two doses, 21 days apart) between December 2020 and January 2021. In addition, 95 healthcare workers vaccinated in the same period were considered to expand serological evaluation. Of these, 14 healthcare workers who received the third vaccine dose in October 2021 were followed at one week post-booster dose. In total, cohort 1 included 125 subjects which were divided into those with (COVID-19 recovered group) or without (naïve group) history of SARS-CoV-2 infection. 14 patients hospitalized with COVID-19 between March and April 2020, who had not yet been vaccinated, were followed for up to 12 months post-discharge (cohort 2). Cohort 1 and 2 were enrolled to evaluate long-term immunological memory induced either by vaccination or infection. To evaluate long-term vaccine-induced immunity in immunocompromised individuals, the study included 41 patients with B-cell lymphoma followed up to 1 month post-third dose (cohort 3), 21 solid-organ transplant recipients followed-up to 1 month post-third or fourth dose (cohort 4), and 8 people living with HIV (PLWH) followed up to 2 months post-fourth dose. Patients with B-cell lymphoma who were either currently receiving or had previously completed chemotherapy, including rituximab (RTX), received the original mRNA-1273 vaccine (two doses, 28 days apart) between April and June 2021, followed by a third dose between December 2021 and January 2022. Solid-organ transplant recipients (13 lung transplant recipients with cystic fibrosis and 8 kidney transplant recipients) on immunosuppressive therapy received the original mRNA-1273 vaccine (two doses, 28 days apart) in March 2021, followed by a third dose between September and October 2021, and a fourth dose between April and May 2022. PLWH received a fourth dose of the original BNT162b2 mRNA vaccine in March 2022. None of the immunosuppressed individuals had prior PCR-confirmed SARS-CoV-2 infection that was further excluded based on negative history, and IgG antibodies against nucleoprotein.

The composition of the cohorts is summarized in Figure 15 and Figure 22. Main clinical features were collected for each group. A schematic overview of the analysis time points is shown for cohort 1 and 2 in Figure 16, while for cohorts 3, 4, and 5 in Figure 17. The evaluation of anti-SARS-CoV-2 antibody levels and flow cytometric analysis of SARS-CoV-2-specific B and T cells was performed.

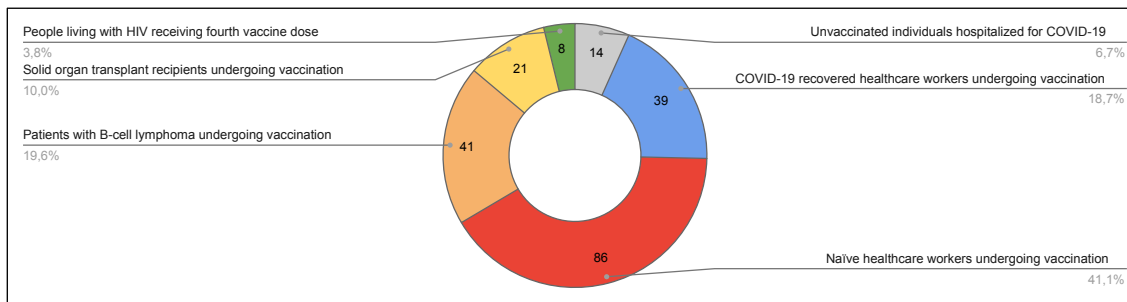


Figure 22 – Composition of the study population.

A total of 209 individuals were recruited in the study.

4.2 Long-term evaluation of vaccine-induced immunity

The assessment of the kinetics of vaccine-induced immunity in both naïve and COVID-19-recovered individuals is crucial for understanding the durability of immunological memory and the impact of hybrid immunity. The vaccinated study population was prospectively followed and monitored to evaluate long-term adaptive immune responses elicited by the vaccine. 30 healthcare workers undergoing the original BNT162b2 mRNA vaccine between December 2020 and January 2021 received a two-dose series (21 days apart) regardless of previous history of infection. Among them, 15 individuals had a previous history of symptomatic SARS-CoV-2 infection (COVID-19 recovered group) and 15 had no history of infection (naïve group). Both groups included 7 (47%) males and 8 (53%) females (Table 4). Recovered individuals were older than naïve subjects, had had COVID-19 on average 8 months before vaccination with mild disease in the majority of cases (67%) (Table 4). In addition, 95 individuals (71 naïve and 24 COVID-19 recovered) were enrolled to expand serological data on a larger cohort. Clinical characteristics of 125 healthcare workers vaccinated with BNT162b2 are reported in Table 5. Recovered individuals had been infected 8 ± 3 months before vaccination and were mainly asymptomatic or had mild disease (79.5%).

In addition, 14 healthcare workers were evaluated before the original BNT162b2 third vaccine dose and 1 week after. All individuals received the booster dose 9 months after the second vaccine dose, in October 2021. 7 (50%) of them had a previous history of SARS-CoV-2 infection and 7 (50%) had a negative history for SARS-CoV-2 infection (Table 6).

Table 4 – Basic demographic and clinical characteristics of 30 healthcare workers who received a two-dose vaccination series of the original BNT162b2 vaccine, followed up to 8 months after the second dose.

Subject (ID)	Age at first vaccine dose (years)	Gender	COVID-19 severity	Time from COVID-19 diagnosis* to first vaccine dose (days)	Comorbidities
COVID-19 recovered individuals					
R1	46	M	mild	214	H
R2	35	F	mild	58	none
R3	51	F	severe	294	H, DYS
R4	52	F	mild	286	none
R5	60	M	moderate	302	CKD
R6	65	M	mild	312	none
R7	53	F	mild	289	migraine
R8	37	M	moderate	297	none
R9	54	F	mild	293	DYS
R10	29	F	mild	289	none
R11	39	M	mild	72	none
R12	61	M	critical	288	H
R13	64	M	critical	292	none
R14	51	F	mild	222	H, DYS
R15	44	F	mild	306	none
Mean±SD	49±11	-	-	254±82	-
Naïve individuals					
NR1	63	M	na	na	none
NR2	41	F	na	na	DYS, DM
NR3	32	M	na	na	none
NR4	44	F	na	na	none
NR5	35	F	na	na	none
NR6	61	F	na	na	none
NR7	41	M	na	na	none
NR8	37	F	na	na	none
NR9	32	M	na	na	none
NR10	28	M	na	na	none
NR11	34	M	na	na	none
NR12	29	F	na	na	none
NR13	46	F	na	na	thyroid disorder
NR14	24	F	na	na	none
NR15	53	M	na	na	none
Mean±SD	40±12	-	-	-	-

CKD: chronic kidney disease; DM: type 2 diabetes mellitus; DYS: dyslipidemia; F: female; H: hypertension; M: male; na: not applicable

*First positive test on nasopharyngeal swab

Table 5 – Basic demographic and clinical characteristics of 125 healthcare workers who received a two-dose vaccination series of the original BNT162b2 vaccine, followed up to 6 months after the second dose.

	COVID-19 recovered individuals	Naïve individuals
No. subjects	39	86
Age (years)		
Mean±SD	50.4±11.3	45.5±14.3
Gender		
Male, n (%)	13 (33.3)	39 (45.3)
Female, n (%)	26 (66.7)	47 (54.6)
COVID-19 severity		
Asymptomatic/mild, n (%)	31 (79.5)	na
Moderate, n (%)	2 (5.1)	na
Severe, n (%)	2 (5.1)	na
Critical, n (%)	4 (10.3)	na
Time from COVID-19 diagnosis* to first vaccine dose (days)		
Mean±SD	230.4±93.6	na

na: not applicable

*First positive test on nasopharyngeal swab

Table 6 – Basic demographic and clinical characteristics of 14 healthcare workers who received the first booster dose (original BNT162b2), followed up to one week after booster dose.

Subject (ID)	Age (years)	Gender	COVID-19 severity
COVID-19 recovered individuals			
RB1	52	F	severe
RB2	53	F	mild
RB3	65	M	critical
RB4	37	M	moderate
RB5	55	F	mild
RB6	62	M	critical
RB7	54	F	mild
Mean±SD	54±9	-	-
Naïve individuals			
NB1	45	F	na
NB2	56	F	na
NB3	36	M	na
NB4	36	F	na
NB5	29	M	na
NB6	31	M	na
NB7	42	M	na
Mean±SD	39±9	-	-

F: female; M: male; na: not applicable.

4.2.1 Rapid decline of Spike-specific humoral response in naïve individuals compared to COVID-19 recovered after vaccination

Following the administration of the first vaccine dose, serum levels of anti-Spike IgM increased in naïve and COVID-19 recovered individuals, showing interindividual variability among COVID-19 recovered subjects (Figure 23A). In both groups, anti-Spike IgM levels began to decline starting from one month after completing the two-dose vaccination series; by 6 and 8 months post-vaccination, the levels had decreased below the cut-off limit (Figure 23A). Anti-Spike IgA and anti-Spike IgG levels peaked in naïve individuals one month after receiving the second vaccine dose (Figure 23B-C). In individuals who had previously recovered from COVID-19, these antibodies reached their highest level already after the first dose and remained stable up to one month later. However, a slight decline was observed at month 6 and 8 (Figure 23B-C). Anti-Spike IgAs and IgGs decreased at month 6 in naïve individuals (Figure 23B-C). Nonetheless, at month 8 anti-Spike IgA and IgG levels were significantly higher than those before vaccination in both groups. Anti-RBD IgGs increased up to month 1, dropped significantly at month 6, and then remained at higher levels than baseline at month 8 in both groups (Figure 23D). The kinetics of anti-RBD IgGs overlapped with that of anti-S IgGs in naïve individuals (Figure 23C-D). At all time points of analysis, COVID-19 recovered individuals displayed significantly higher levels of anti-Spike IgAs and IgGs as well as anti-RBD IgGs than naïve individuals (Figure 23B-D). Neutralizing antibodies increased in naïve individuals up to one month post-second dose and decreased over month 8 (Figure 23E). Neutralizing antibodies at month 8 were significantly higher than baseline in naïve individuals, whereas they were comparable to baseline levels in COVID-19 recovered subjects. It is important to note that anti-Spike IgG and IgA, anti-RBD IgG and neutralizing antibodies were significantly higher in COVID-19 recovered vaccinated individuals at all time points of analysis compared to the naïve group. COVID-19 recovered individuals displayed significantly higher levels of anti-Nucleoprotein IgG pre-vaccination than naïve individuals, confirming prior SARS-CoV-2 infection (Figure 23F). In COVID-19 recovered subjects, anti-N IgGs decreased over time, reaching values below the cut-off limit at months 6 and 8 (Figure 23F). In naïve individuals anti-N IgGs were below the cut-off value throughout the course of the analysis (Figure 23F).

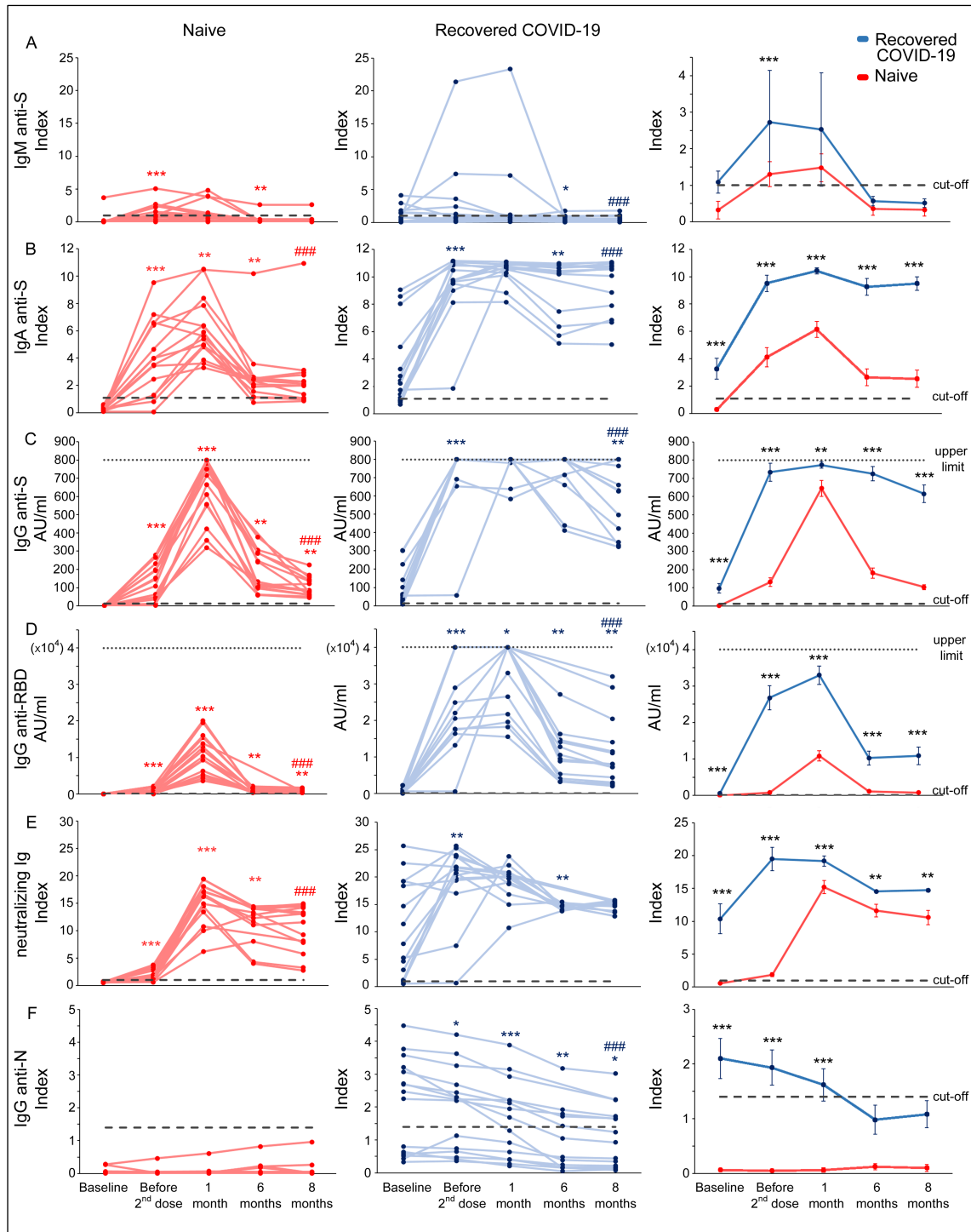


Figure 23 – Long-term evaluation of anti-SARS-CoV-2 antibody levels after two-dose vaccination in 30 individuals with or without history of COVID-19.

Evaluation of (A) anti-Spike IgM, (B) anti-Spike IgA, (C) anti-Spike IgG (D) anti-RBD IgG, (E) Spike-neutralizing Ig, and (F) anti-Nucleoprotein IgG levels in naïve individuals and COVID-19 recovered individuals before vaccination, before the second vaccine dose, at 1 month, 6 months, and 8 months after the second original BNT162b2 mRNA vaccine dose. On the left panels, individual data from 15 naïve subjects are shown (red lines); on the central panels, individual data from 15 COVID-19 recovered subjects are shown (blue lines); on the right panels, mean values $\pm$ SEM of both groups are shown. Dashed lines represent the cut-off values, dotted lines denote the upper detection limits. Red and blue asterisks (left and central panels) refer to paired statistics within each study group compared with the previous time point in the kinetics calculated with Wilcoxon's signed-rank test. Red and blue hashtags (left and central panels) denote paired statistics within each study group between baseline and 8 months, calculated with Wilcoxon's signed-rank test. Black asterisks (right panels) denote unpaired statistics between naïve individuals and COVID-19 recovered individuals at each time point calculated with Mann-Whitney U test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; red and blue hashtags ### $P < 0.001$. Error bars indicate SEM.

Serological evaluation was extended to an additional 95 healthcare workers (71 naïve and 24 COVID-19 recovered) who received the two-dose BNT162b2 mRNA vaccine series. In total, 86 naïve individuals and 39 COVID-19 recovered individuals were prospectively followed for up to 6 months after complete vaccination, and humoral responses were assessed. Main demographic and clinical characteristics are reported above and summarized in Table 5.

In COVID-19 recovered individuals, anti-Spike IgG, anti-RBD IgG and neutralizing antibodies were significantly higher than in the naïve group at all time points of the analysis (Figure 24A-C). Antibody levels increased after the first vaccine dose and gradually declined over 6 months post-second dose (Figure 24A-C). In contrast, in naïve individuals, anti-Spike IgG, anti-RBD IgG, and neutralizing antibodies peaked 1 month after vaccination and rapidly declined by month 6, though levels did not return to baseline. The antibody kinetics in both groups mirrored those observed in the smaller cohort described previously (Figure 23C-E).

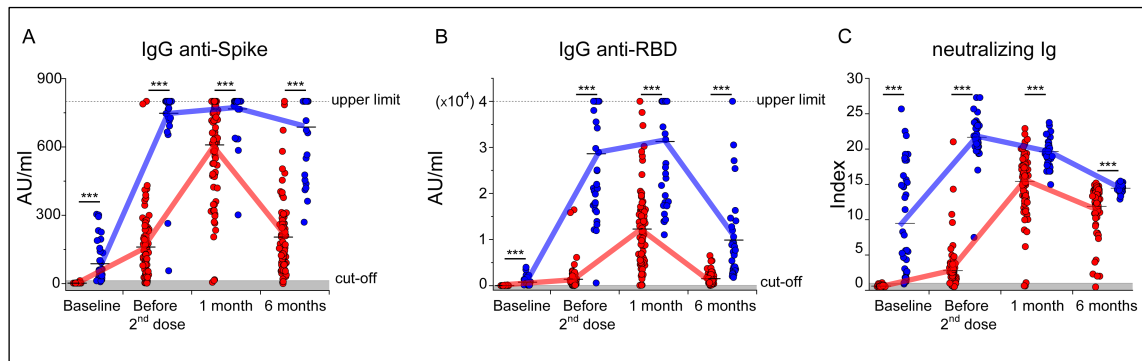


Figure 24 – Long-term evaluation of anti-SARS-CoV-2 antibody levels after two-dose vaccination in 125 individuals with or without history of COVID-19.

Evaluation of (A) anti-Spike IgG, (B) anti-RBD IgG, and (C) neutralizing antibodies levels at baseline, before second vaccine dose, 1 month and 6 months after second vaccine dose in 86 naïve (red dots) and 39 COVID-19 recovered individuals (blue dots). Continuous colored lines show the kinetics. Black asterisks represent unpaired statistics between naïve and COVID-19 recovered individuals at each time point calculated with Mann-Whitney U test. *** $P < 0.001$.

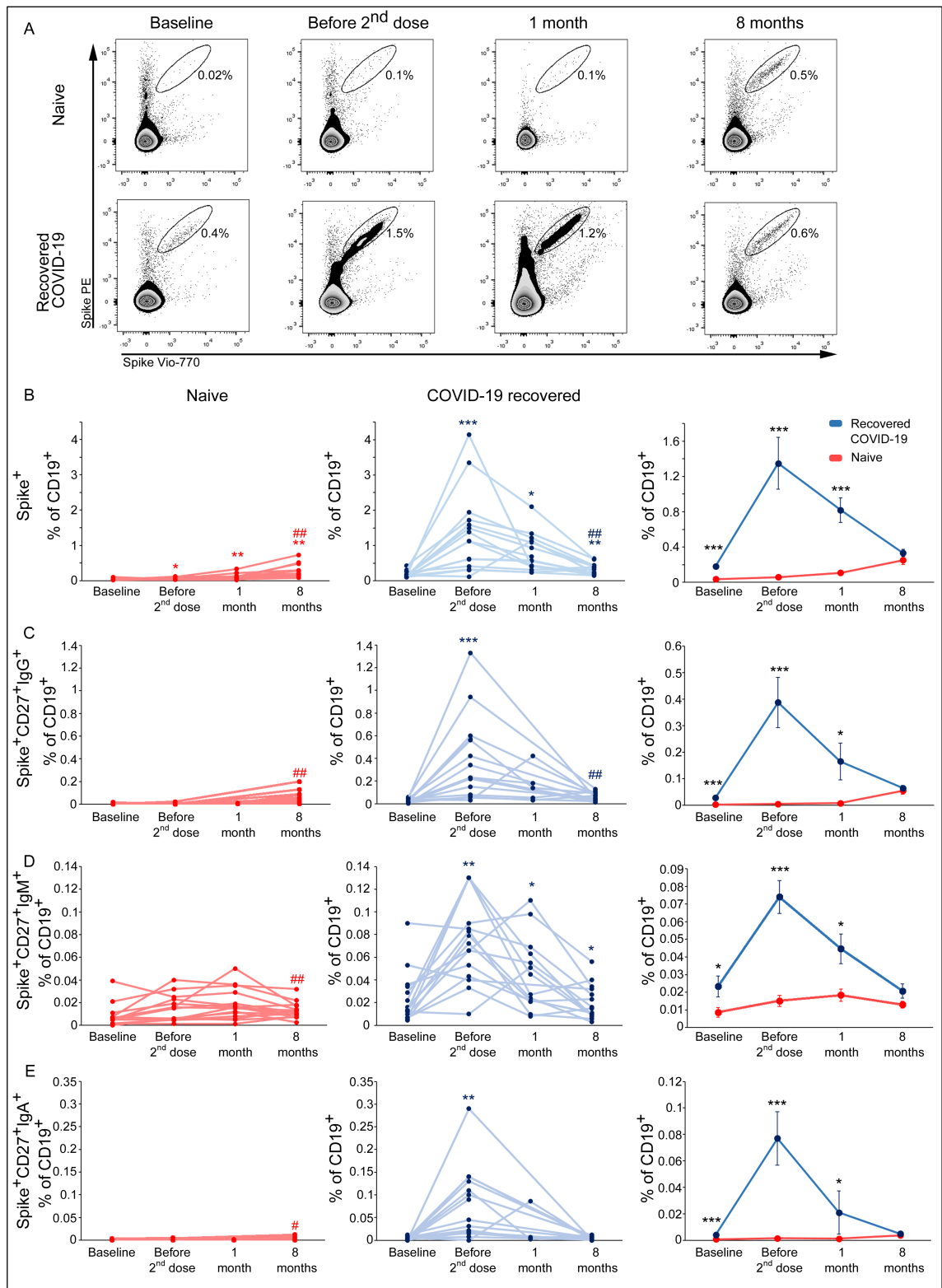
4.2.2 Higher early Spike-specific B cell frequencies in recovered COVID-19

individuals converging with naïve subjects by 8 months after vaccination

Spike-specific B cells were detected up to 8 months after complete vaccination both in naïve and in COVID-19 recovered individuals (Figure 25A). In the latter group, Spike-specific B cells were present before vaccination and significantly increased from baseline to 1 month post-vaccination. By 8 months, their frequencies were comparable to those in naïve subjects (Figure 25A-B). Naïve individuals exhibited a gradual and significant increase in Spike-specific B cells up to 8 months following the second vaccine dose (Figure 25B). COVID-19 recovered individuals displayed different kinetics (Figure 25B). In this group, the frequency of Spike-specific B cells peaked three weeks after first vaccine dose, then progressively declined (Figure 25B). Compared to COVID-19 recovered individuals, naïve subjects showed significantly lower frequencies of circulating Spike-specific B cells from baseline to month 1, but by month 8, frequencies in both groups were similar (Figure 25B). Both naïve and COVID-19 recovered individuals had significantly higher levels of Spike-specific B cells at month 8 compared to pre-vaccination levels (Figure 25B). Spike-specific CD27+ B cells expressing IgG, IgM, or IgA isotype showed kinetics comparable to total Spike-specific B cells (Figure 25C-E). Frequencies of Spike-specific CD27+IgG+, CD27+IgM+ or CD27+IgA+ were significantly higher in recovered individuals compared to naïve subjects from baseline to 1 month, but were similar at 8 months post-vaccination (Figure 25C-E). Thus, by 8 months post-vaccination, the circulating pool of Spike-specific memory B cells had the same frequency in enrolled individuals regardless of previous SARS-CoV-2 infection.

Figure 25 – Long-term evaluation of Spike-specific B cells after two-dose vaccination in individuals with or without history of COVID-19. ►

(A) Representative flow cytometry plots of Spike-specific B cells in 1 naïve individual (top panels) and 1 COVID-19 recovered individual (bottom panels) before vaccination, before the second vaccine dose, at 1 month, 6 months, and 8 months after the second vaccine dose. Longitudinal evaluation of frequencies of **(B)** total, **(C)** CD27+IgG+, **(D)** CD27+IgM+ and **(E)** CD27+IgA+ Spike-specific B cells in naïve individuals (red lines, left panels) and COVID-19 recovered individuals (blue lines, central panels). Cumulative data are represented (right panels) as mean $\pm$ SEM from 15 naïve individuals and 15 COVID-19 recovered individuals. Red and blue asterisks (left and central panels) denote paired statistics within each study group compared with the previous time point in the kinetics, calculated with Wilcoxon's signed-rank test. Red and blue hashtags (left and central panels) denote paired statistics within each study group between baseline and 8 months, calculated with Wilcoxon's signed-rank test. Black asterisks (right panels) represent unpaired statistics between naïve and COVID-19 recovered individuals at each time point, calculated with Mann-Whitney test. * $P < 0.05$; # $P < 0.05$; ** $P < 0.01$; ## $P < 0.01$; *** $P < 0.001$. Error bars indicate SEM.

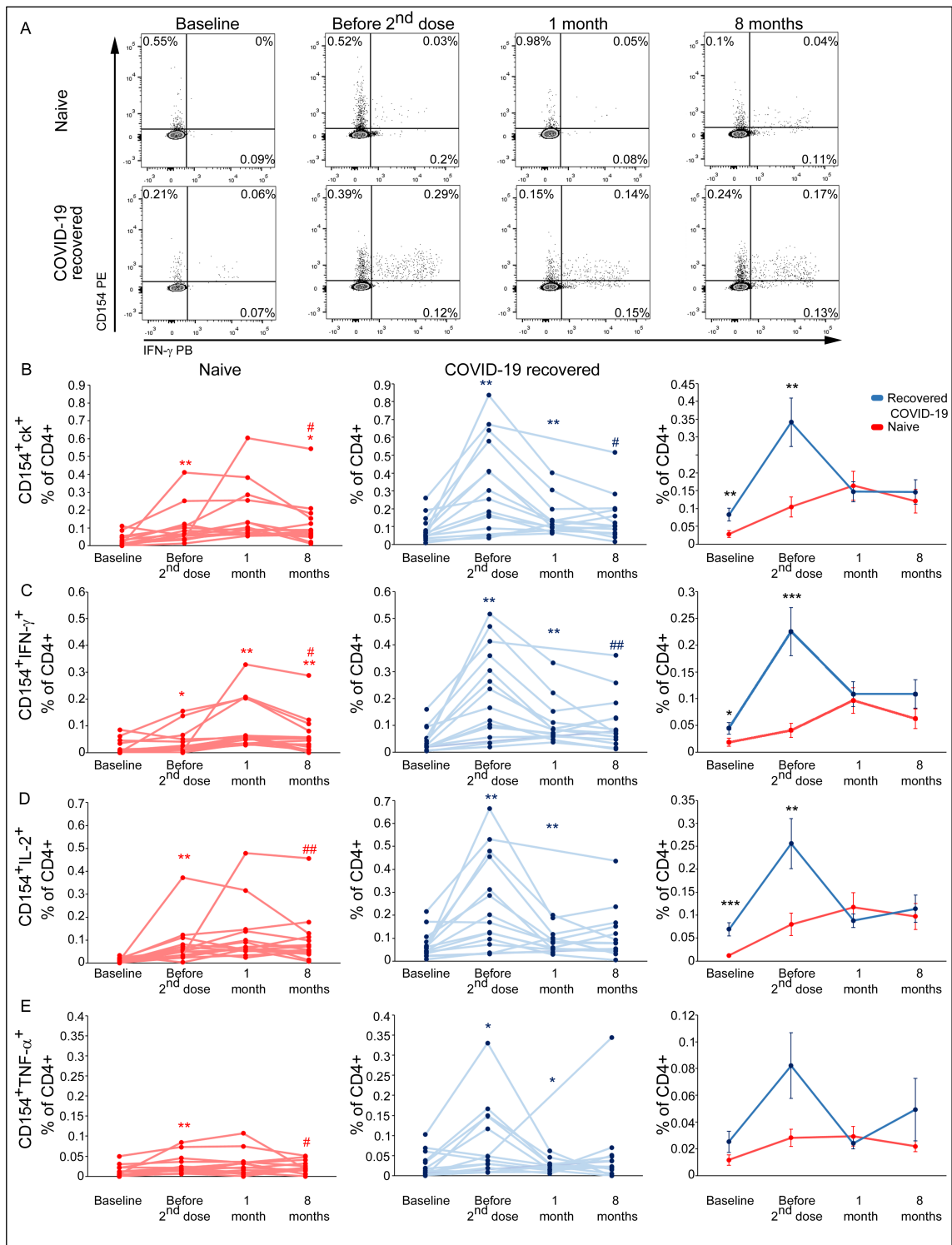


4.2.3 Comparable frequencies of Spike-specific CD4⁺ T cells in naïve and recovered COVID-19 subjects after vaccination

Spike-specific CD4⁺ T cells were detected up to 8 months after two-dose vaccination both in naïve and COVID-19 recovered individuals (Figure 26A). Total Spike-specific CD4⁺ T cells, defined by CD154 expression and the production of at least one cytokine among IL-2, IFN- γ , and TNF- α (CD154+ck+) showed a gradual increase up to one month after the second vaccine dose in naïve individuals (Figure 26B). This was followed by a mild reduction at month 8 (Figure 26B). In COVID-19 recovered individuals, Spike-specific CD154+ck+ CD4⁺ T cells significantly increased three weeks after the first vaccine dose, then declined at month 1 and resulted unchanged at month 8 (Figure 26B). As for antigen-specific B cells, frequency of Spike-specific CD4⁺ T cells was significantly higher by month 8 compared to baseline in both groups (Figure 26B). Total Spike-reactive CD4⁺ T cells were significantly more frequent in COVID-19 recovered individuals than in naïve subjects from baseline to three weeks after first vaccine dose, being comparable at month 1 and 8 (Figure 26B). The kinetics were similar whether focusing on Spike-specific CD4⁺ T cells producing IFN- γ (Figure 26C), IL-2 (Figure 26D), or TNF- α (Figure 26E). Thus, at 1 month and 8 months post-vaccination the circulating pool of Spike-specific CD4⁺ T cells has the same frequency in enrolled individuals regardless of previous SARS-CoV-2 infection

Figure 26 – Long-term evaluation of Spike-specific circulating CD4⁺ T cells before and after two-dose vaccination in individuals with or without history of COVID-19. ►

(A) Representative flow cytometric plots of Spike-specific CD4⁺CD154⁺IFN- γ ⁺ T cells in 1 naïve individual (top panels) and 1 COVID-19 recovered individual (bottom panels) before vaccination, before the second vaccine dose, and 1 month and 8 months after the second vaccine dose. Longitudinal evaluation of frequencies of **(B)** Spike-specific T cells expressing CD154 and producing at least 1 cytokine among IL-2, IFN- γ , and TNF- α , **(C)** CD154⁺IFN- γ ⁺, **(D)** CD154⁺IL-2⁺, and **(E)** CD154⁺TNF- α ⁺ Spike-specific CD4⁺ T cells in naïve individuals (red lines, left panels) and COVID-19 recovered individuals (blue lines, central panels). Cumulative data are represented (right) as mean $\pm$ SEM from 15 naïve individuals and 15 COVID-19 recovered individuals. In B–E, data are shown after subtraction of the background, unstimulated condition. Red and blue asterisks (left and central panels) refer to paired statistics within each study group compared with the previous time point in the kinetics, calculated with Wilcoxon's signed-rank test. Red and blue hashtags (left and central panels) refer to paired statistics within each study group between baseline and 8 months, calculated with Wilcoxon's signed-rank test. Black asterisks (right panels) represent unpaired statistics between naïve and COVID-19 recovered individuals at each time point, calculated with Mann-Whitney test. * $P < 0.05$; # $P < 0.05$; ** $P < 0.01$; ## $P < 0.01$; *** $P < 0.001$. Error bars indicate SEM.



4.2.4 Booster dose reactivates immunological memory to Spike protein more effectively in naïve individuals compared to COVID-19 recovered

A total of 14 healthcare workers (7 naïve and 7 COVID-19 recovered) were evaluated before and 1 week after the administration of the first booster dose. All participants received BNT162b2 mRNA vaccine and had completed a two-dose series of the same mRNA vaccine 9 months earlier (homologous booster dose). Main demographic and clinical characteristics are described above and summarized in Table 6. Anti-Spike and anti-RBD IgG levels significantly increased in naïve individuals after the booster dose (Figure 27A-B), while no significant change was observed in serum titers of neutralizing antibodies (Figure 27C). In COVID-19 recovered individuals, anti-Spike IgGs were similar before and after the booster dose (Figure 27A). Anti-RBD IgGs showed an increasing trend (Figure 27B). Remarkably, a significant reduction of neutralizing antibodies was observed one week after the booster dose in COVID-19 recovered individuals, although the levels were still above the cut-off limit (Figure 27C). Although anti-Spike IgG, anti-RBD IgG, and neutralizing Ig levels were significantly lower in naïve than in COVID-19 recovered individuals before the booster dose, the titers were similar one week after vaccination (Figure 27A-C). The frequency of total Spike-specific B cells increased in naïve individuals but not in COVID-19 recovered after the booster administration (Figure 27D) and it was comparable between the two groups one week later (Figure 27D, Figure 28A). Spike-specific CD27+IgG+, CD27+IgM+, CD27+IgA+ B cells showed an increasing trend after the booster dose (Figure 28). The frequency of CD154+cytokine+ Spike-specific CD4+ T cells increased only in naïve individuals (Figure 27E, Figure 28E). No significant effect was observed in COVID-19 recovered individuals (Figure 27E, Figure 28E). Similarly, CD154+IFN- γ +, CD154+IL-2+, and CD154+TNF- α + Spike-specific CD4+ T cells increased after booster dose only in naïve individuals (Figure 28F-H). Thus, the booster dose reactivates humoral and cellular immunity to Spike protein more efficiently in naïve than in COVID-19 recovered individuals.

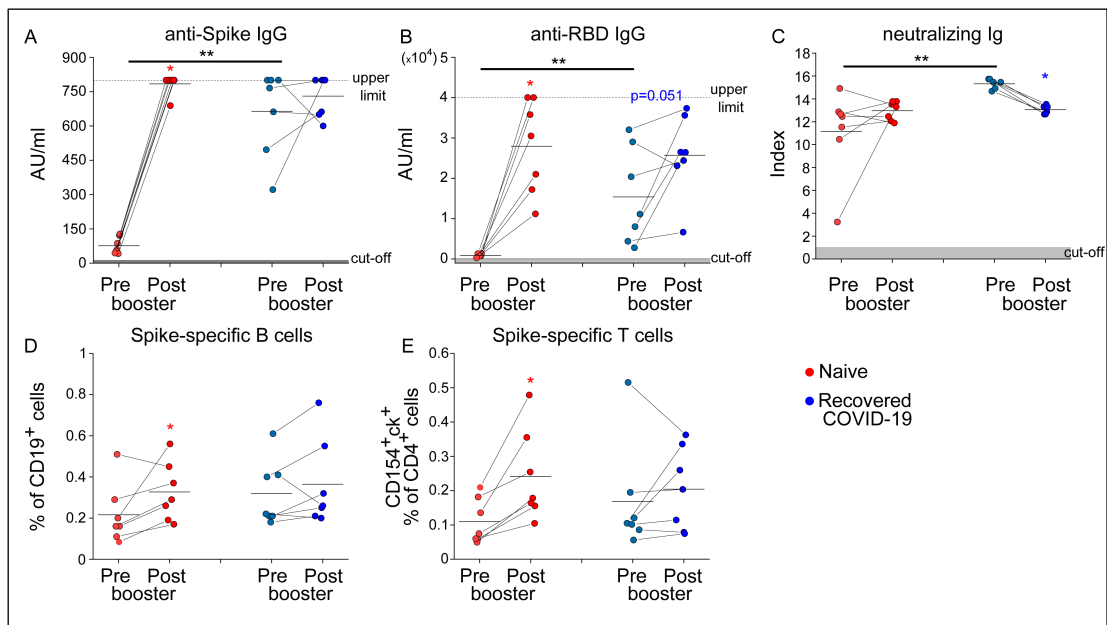


Figure 27 – SARS-CoV-2-specific adaptive immune response before and after the first booster dose in individuals with or without history of COVID-19.

Levels of **(A)** anti-spike IgG, **(B)** anti-RBD IgG, and **(C)** neutralizing Ig in 7 naïve individuals (red) and 7 individuals who have recovered from COVID-19 (blue) before (pre) and 1 week after (post) the administration of the first booster dose. Levels of **(D)** Spike-specific B cells and **(E)** Spike-specific CD4+ T cells (defined as expressing CD154+ and producing at least one cytokine among IFN- γ , IL-2, and TNF- α cells) in 7 naïve individuals (red) and 7 individuals who have recovered from COVID-19 (blue) before and 1 week after vaccine booster administration. Red and blue asterisks denote paired statistics within each study group, calculated with Wilcoxon's signed-rank test. Black asterisks represent unpaired statistics between naïve individuals and individuals who have recovered from COVID-19, calculated with Mann-Whitney U test. * $P < 0.05$; ** $P < 0.01$.

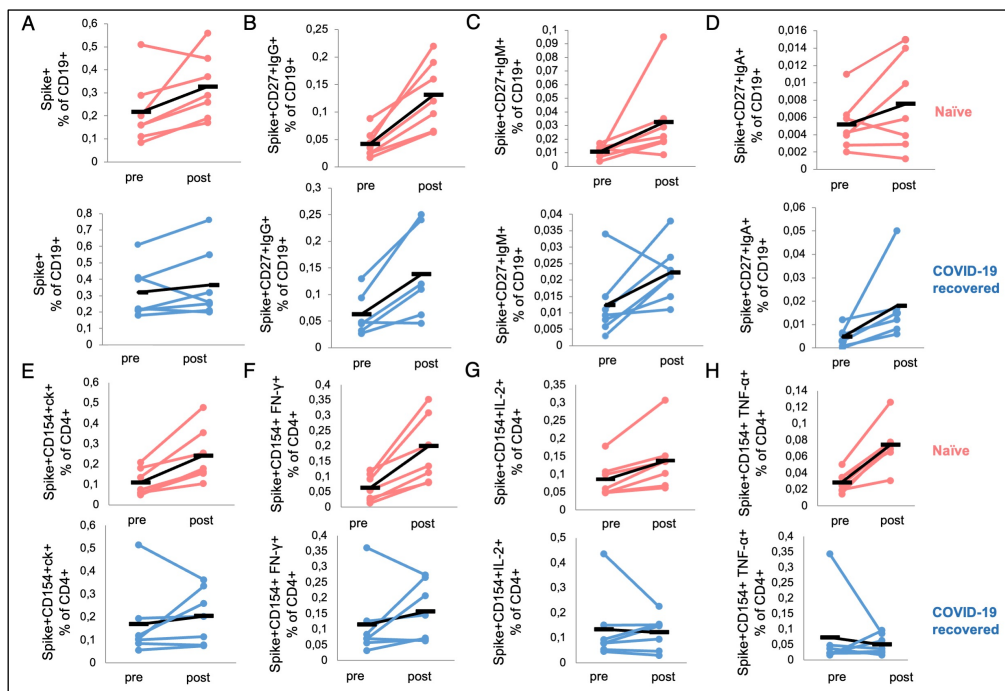


Figure 28 – Evaluation of Spike-specific circulating B and CD4+ T cells before and after the first booster dose in individuals with or without history of COVID-19.

Longitudinal evaluation of **(A)** total, **(B)** CD27+IgG+, **(C)** CD27+IgM+ and **(D)** CD27+IgA+ Spike-specific B cells in naïve individuals (red lines) and COVID-19 recovered individuals (blue lines) before (pre) and 1 week after (post) the administration of the first booster dose. Longitudinal evaluation of **(E)** Spike-specific T cells expressing CD154 and producing at least 1 cytokine among IL-2, IFN- γ , and TNF- α , **(F)** CD154+IFN- γ +, **(G)** CD154+IL-2+, and **(H)** CD154+TNF- α + Spike-specific CD4+ T cells in naïve individuals (red lines) and COVID-19 recovered individuals (blue lines) before (pre) and 1 week after (post) the administration of the first booster dose. Black lines represent mean values.

4.3 Long-term evaluation of SARS-CoV-2-induced immunity

A total of 14 patients hospitalized for COVID-19 between March and April 2020 were followed up to one year after infection to compare the durability of natural immunity to vaccine-induced immunity. These subjects were still not vaccinated at the last follow-up date. They had moderate (43%), severe (28.5%) or critical (28.5%) COVID-19 (Table 7). Mean age at disease onset was 61 ± 8 years (Table 7). Males were 57% and females 43% of cases (Table 7).

Table 7 – Basic demographic and clinical characteristics of 14 patients hospitalized for COVID-19 between March and April 2020 and followed up to 12 months post-hospital discharge.

Subject (ID)	Age (years)	Gender	COVID-19 severity	Comorbidities
U1	56	M	moderate	none
U2	61	M	moderate	H, obesity
U3	58	M	severe	DM, obesity
U4	75	F	severe	H
U5	48	M	moderate	H
U6	62	M	severe	CKD, H, DM
U7	58	M	critical	none
U8	73	M	critical	H, DYS
U9	73	F	severe	H, COPD, GCA
U10	56	F	critical	Thyroid disorder
U11	56	F	moderate	Anxiety disorder
U12	54	F	moderate	none
U13	57	F	moderate	DM
U14	69	M	critical	H
Mean$\pm$SD	61$\pm$8	-	-	-

CKD: chronic kidney disease; COPD: chronic obstructive pulmonary disease; DM: type 2 diabetes mellitus; DYS: dyslipidemia; F: female; GCA: giant cell arteritis; H: hypertension; M: male.

Levels of anti-Nucleoprotein IgG, anti-Spike IgM and IgG, and anti-RBD IgG significantly declined at month 6 and 12 after hospital discharge (Figure 29A–D), but Spike-neutralizing antibodies were stable over time (Figure 29E). In 69% of cases, anti-N IgGs were below the cut-off limit (1.4 Index) at month 12 (Figure 29A). In all individuals, anti-Spike IgGs were still detectable at month 12 with a mean value of 109.8 ± 73.3 AU/mL (Figure 29C). Spike-specific circulating B cells were present up to month 12 (Figure 29F–G). Their frequency was unchanged over time (Figure 29F). Spike-specific CD27+IgM+ B cells significantly declined, while the frequencies of CD27+IgG+ and CD27+IgA+ Spike-specific B cells remained stable over months after SARS-CoV-2 infection (Figure 29H–J). SARS-CoV-2-reactive CD4+ T cells, identified by CD154 expression and the production of at least one cytokine (IL-2, IFN- γ , or TNF- α) upon in vitro stimulation with Spike or a combination of Spike, Membrane, and Nucleoprotein peptide pools, were detectable up to 12 months (Figure 30A–B and F–G). Their frequencies resulted stable over time (Figure 30A,F). Similar kinetics were observed when evaluating the production of specific cytokines (IFN- γ , IL-2, or TNF- α), in response to Spike or to the combination of Spike, Membrane, and Nucleoprotein (Figure 30C–E and H–J). These results indicate that the humoral response significantly declines one year after SARS-CoV-2 infection, while the cellular response remains stable.

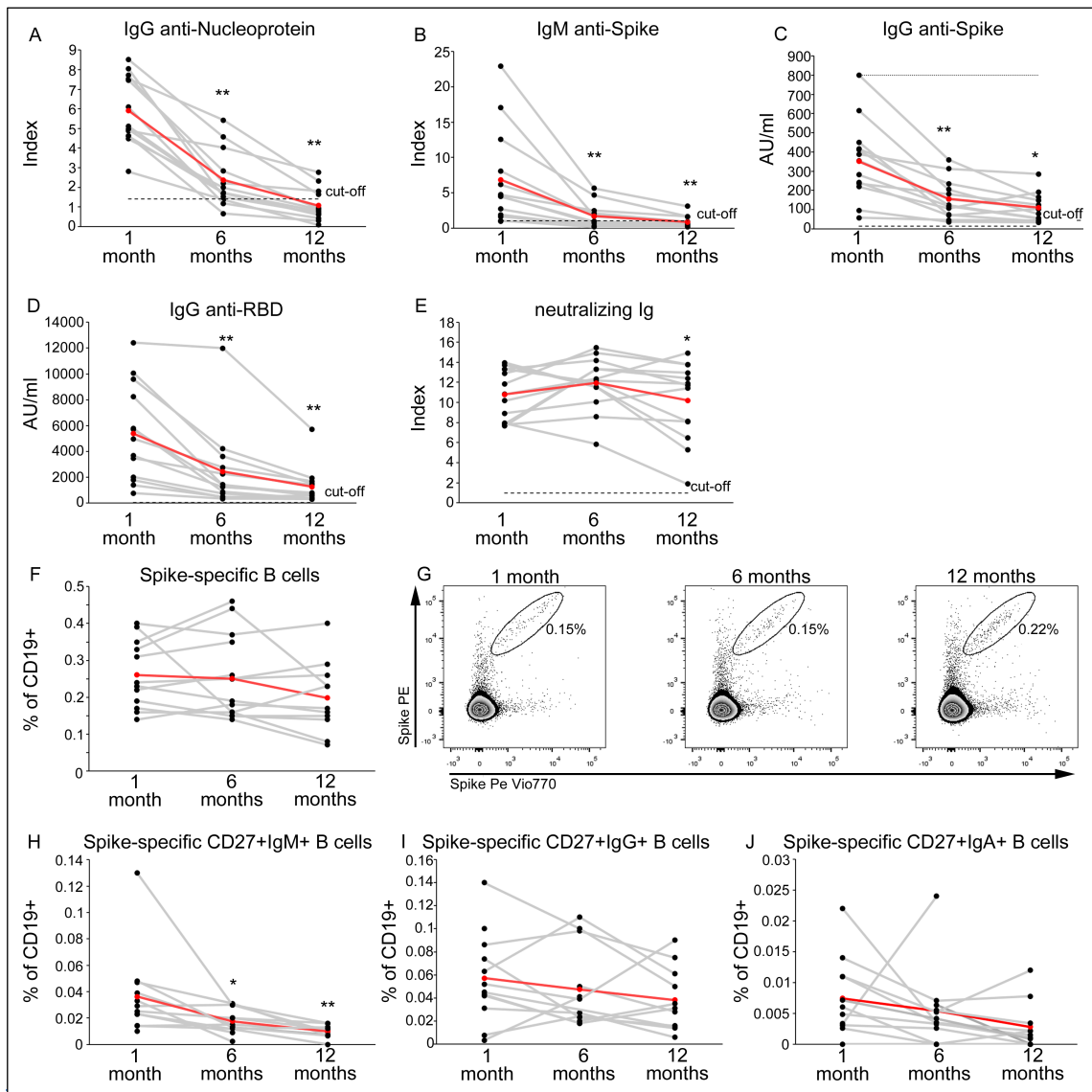


Figure 29 – Long-term evaluation of SARS-CoV-2-specific antibody levels and Spike-specific B cells up to 12 months after SARS-CoV-2 infection.

Levels of **(A)** anti-Nucleoprotein IgG, **(B)** anti-Spike IgM, **(C)** anti-Spike IgG, **(D)** anti-RBD IgG, and **(E)** neutralizing antibodies in 13 COVID-19 recovered unvaccinated individuals evaluated at 1, 6, and 12 months following hospital discharge. Frequencies of **(F)** total, **(H)** CD27+IgM+, **(I)** CD27+IgG+, **(J)** CD27+IgA+ Spike-specific B cells evaluated at 1, 6, and 12 months following hospital discharge in 14 COVID-19 recovered unvaccinated individuals. Representative FACS plots of total Spike-specific B cells at each time point are shown in panel **G**. Red lines represent mean values. Asterisks refer to paired statistics at each time point compared with the previous, calculated with Wilcoxon's signed-rank test. * $P < 0.05$; ** $P < 0.01$.

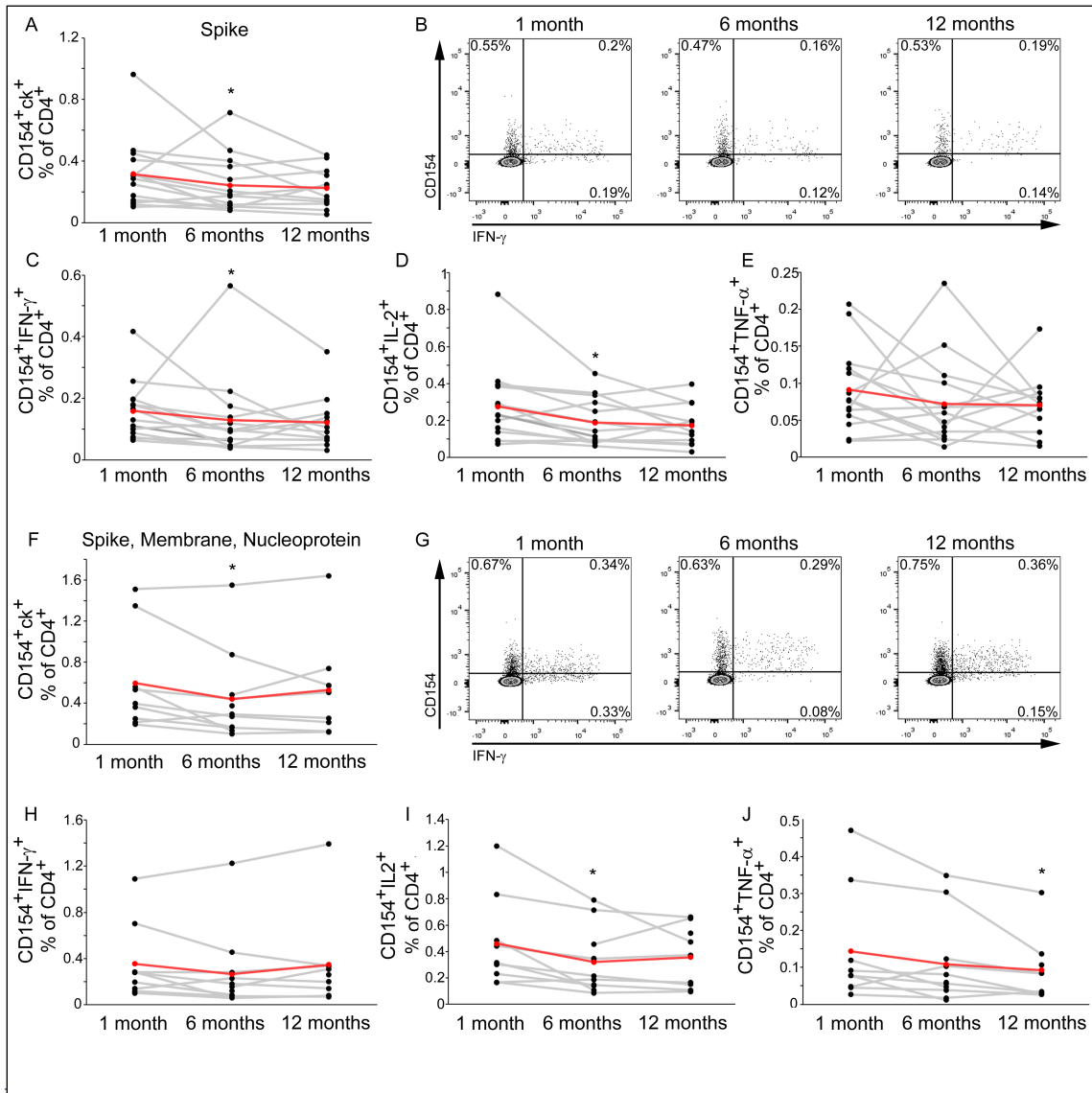


Figure 30 – Long-term evaluation of SARS-CoV-2-specific CD4⁺ T cells up to 12 months after SARS-CoV-2 infection.

Frequencies of **(A)** Spike-reactive CD4⁺ T cells expressing CD154 and producing at least one cytokine among IL-2, IFN-γ, and TNF-α, **(B-C)** CD154+IFN-γ⁺, **(D)** CD154+IL-2⁺, and **(E)** CD154+TNF-α⁺ evaluated at 1, 6, and 12 months following hospital discharge in 14 COVID-19 recovered unvaccinated individuals. Representative FACS plots of CD154+IFN-γ⁺ Spike-specific CD4⁺ T cells at each time point are shown in panel **B**. Frequencies of **(F)** CD154+CD4⁺ T cells reactive to Spike, Membrane, and Nucleoprotein producing at least one cytokine among IL-2, IFN-γ and TNF-α, **(H)** CD154+IFN-γ⁺, **(I)** CD154+IL-2⁺, and **(J)** CD154+TNF-α⁺ evaluated at 1, 6, and 12 months following hospital discharge in 14 COVID-19 recovered unvaccinated individuals. Representative FACS plots of CD154+IFN-γ⁺CD4⁺ T cells reactive to Spike, Membrane, and Nucleoprotein at each time point are shown in panel **G**. Red lines represent mean values. Asterisks denote paired statistics at each time point compared with the previous, calculated with Wilcoxon's signed-rank test. **P* < 0.05.

4.4 Effect of immunosuppression on long-term vaccine-induced immunity

4.4.1 Higher vaccine-induced Spike-specific humoral response following rituximab discontinuation compared to ongoing therapy

A total of 41 patients with B-cell lymphoma were included in the study (Figure 22). Of these, 25 were undergoing rituximab (RTX) therapy, while 16 had completed RTX treatment. In the latter group, the median time from the last therapy cycle to the first vaccine dose was 174 days. The median age for patients receiving ongoing treatment was 63 years (range 37–80) with a male-to-female ratio of 0.9, while for the post-treatment group, the median age was 69 years (range 46–80) with a male-to-female ratio of 3. The most common diagnoses were follicular lymphoma (31.7%) and diffuse large B-cell lymphoma (31.7%), followed by mantle cell lymphoma (14.6%) and nodal marginal zone lymphoma (4.9%). Among the patients undergoing therapy, only one received RTX alone, while the other 24 were treated with RTX in combination with chemotherapy. Specifically, 13 patients received RTX with bendamustine, and 11 patients were treated with RTX plus other chemotherapy regimens, including R-CHOP (rituximab, cyclophosphamide, doxorubicin, prednisone, and vincristine), R-COMP (rituximab, cyclophosphamide, non-pegylated liposomal doxorubicin, prednisone, and vincristine), among others (Table 8). In the post-treatment group, two patients were treated with RTX alone, eight received RTX with bendamustine, and six were treated with RTX alongside other chemotherapy regimens (Table 8). Patients received the first mRNA-1273 vaccine dose in March 2021, followed by a second dose 28 days later. A third dose was administered between December 2021 and January 2022. Peripheral blood samples were collected at baseline (time 0, before the first dose), 28 days after (T1, prior to the second dose), 1 month after the second dose (T2), and 1 month following the third dose (T3) (Figure 17). Additional laboratory data for the recruited patients at baseline are provided in Table 9 showing no differences in hemoglobin levels, white-cell count, lymphocyte count and platelet count between patients with B-cell lymphoma currently undergoing treatment and those who have completed treatment.

Table 8 – Basic demographic and clinical characteristics of 41 patients with B-cell lymphoma who received vaccination.

Subject (ID)	Age (years)	Gender	Disease	Treatment protocol	Time between rituximab therapy and first vaccine dose (days)	Time from latest bendamustine administration to first vaccine dose (days)	SARS-CoV-2 infection after vaccination; WHO severity
Ongoing rituximab therapy (TP-Ongoing)							
ON1B	80	M	FL	R-BENDA; RTX	NA	383	Yes; 4
ON2	43	M	GZL	DA-EPOCH-R	NA	NA	No
ON3B	70	F	LL	R-BENDA	10	10	No
ON4	79	F	PBCL	RTX	NA	NA	Yes; 1
ON5	73	M	FL	R-CHOP; RTX	12	NA	No
ON6B	75	M	FL	R-BENDA; RTX	NA	309	Yes; 1
ON7B	75	F	FL	BENDA; R-CVP; RTX	NA	521	Yes; 2
ON8	47	F	DLBCL	R-CHOP	NA	NA	Yes; 2
ON9B	75	F	FL	R-BENDA; RTX	NA	478	No
ON10B	72	M	FL	R-BENDA; RTX	NA	712	No
ON11B	53	F	FL	R-BENDA; RTX	NA	444	Yes; 2
ON12B	56	F	FL	R-BENDA; RTX	NA	505	Yes; 1
ON13	63	F	DLBCL	R-COMP; MTX; R-DHAOx	NA	NA	No
ON14	52	M	DLBCL	R-CHOP; R-DHAOx; RTX	NA	NA	Yes; 1
ON15B	65	F	FL	R-CHOP; R-BENDA; RTX	NA	675	Yes; 3
ON16B	71	F	FL	R-BENDA; RTX	NA	504	No
ON17B	53	F	FL	R-BENDA; RTX	NA	496	Yes; 3
ON18B	52	M	DLBCL	R-CHOP; R-DHAOx; PIX; BENDA	21	NA	Yes; 4
ON19B	61	M	FL	R-BENDA; RTX	NA	530	Yes; 4
ON20	46	M	DLBCL	R-CHOP	34	NA	No
ON21	54	M	DLBCL	R-CHOP*	33**	NA	Yes; 1
ON22	67	M	DLBCL	R-CHOP*	24**	NA	Yes; 1
ON23	74	M	DLBCL	R-COMP*	14**	NA	Yes; 1
ON24	59	F	DLBCL	R-CHOP*	10**	NA	Yes; 1
ON25	37	F	PMBCL	R-CHOP*	7**	NA	Yes; 2
Mean±SD	62,1±12,3	-	-	-	-	-	-
Median (IQR)	63 (20)	-	-	-	-	-	-
M/F ratio	-	0,9	-	-	-	-	-
Post-rituximab therapy (Post-TP)							
POST1B	56	F	SMZL	R-BENDA; RTX	163	285	Yes; 1
POST2	63	M	FL	RTX	141	NA	Yes; 1
POST3B	73	M	LPL	R-BENDA	409	409	No
POST4	70	M	DLBCL	R-CHOP; RTX	98	NA	No
POST5B	69	M	MCL	R-BAC	77	NA	No
POST6	73	M	DLBCL	R-CHOP	284	NA	No
POST7B	74	M	MCL	R-BENDA	311	311	No
POST8	68	M	DLBCL	R-COMP	223	NA	No
POST9B	72	M	MCL	R-FND; RTX; R-BENDA	120	120	No
POST10	64	M	MCL	R-CHOP; R-DHAOx	322	NA	No
POST11B	80	M	MCL	R-BENDA	588	588	No
POST12B	46	F	NMZL	R-BENDA	71	71	No
POST13	68	F	NMZL	RTX	185	NA	No
POST14B	69	F	MCL	RTX; R-BENDA; RTX	125	619	Yes; 4
POST15	57	M	BL	R-COMP; MTX	186	NA	No
POST16	78	M	DLBCL	R-COMP	100	NA	Yes; 1
Mean±SD	67,5±8,7	-	-	-	212,7±140,5	-	-
Median (IQR)	69 (9,3)	-	-	-	174 (175,8)	-	-
M/F ratio	-	3	-	-	-	-	-

BCL: B-cell lymphoma; BL: Burkitt lymphoma; DA-EPOCH-R: dose-adjusted etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin, rituximab; DLBCL: diffuse large B cell lymphoma; F: female; FL: follicular lymphoma; GZL: gray zone lymphoma; LL: lymphocytic lymphoma; LPL: lymphoplasmacytic lymphoma; M: male; MCL: mantle cell lymphoma; MTX: methotrexate; NA: not applicable; NMZL: nodal marginal zone lymphoma; PBCL: peripheral B cell lymphoma; PIX: pixantrone; PMBCL: primary mediastinal large B-cell lymphoma; R-BAC: rituximab, bendamustine, cytarabine; R-CHOP: rituximab, cyclophosphamide, doxorubicin, prednisone, vincristine; R-COMP: rituximab, cyclophosphamide, non-pegylated liposomal doxorubicin, prednisone, vincristine; R-CVP: rituximab, cyclophosphamide, vincristine, prednisolone; R-DHAOx: rituximab, dexamethasone, cytarabine, oxaliplatin; R-FND: rituximab, fludarabine, mitoxantrone, dexamethasone; SMZL: splenic marginal zone lymphoma; * treatment protocol followed after first vaccine dose administration; ** time from first vaccine dose administration to the start of rituximab therapy.

Table 9 – Laboratory data of 41 patients with B cell-lymphoma who received vaccination.

Subject (ID)	Hemoglobin (g/dl)	White-cell count (per μ l)	Lymphocyte count (cells/ μ L)	Platelet count (cells/ μ L)
Ongoing rituximab therapy (TP-Ongoing)				
ON1B	14,5	4500	1900	168000
ON2	11,9	8610	870	406000
ON3B	12,9	4790	680	170000
ON4	13,1	4000	1910	164000
ON5	13,4	2950	580	197000
ON6B	14	3210	580	185000
ON7B	11,1	2600	890	174000
ON8	11,2	3060	770	254000
ON9B	12,6	6240	1450	214000
ON10B	11,8	1830	na	179000
ON11B	13,2	6300	1000	300000
ON12B	14,3	5300	na	174000
ON13	10,7	3640	na	323000
ON14	16,4	6720	2040	278000
ON15B	12,4	4540	990	236000
ON16B	13	11600	1430	266000
ON17B	13,1	2600	520	247000
ON18B	9,5	4710	300	66000
ON19B	15,5	5900	na	150000
ON20	15,5	4890	1590	156000
ON21	10,5	3480	1140	325000
ON22	12,8	6700	660	267000
ON23	12,1	3660	220	287000
ON24	9,2	2130	860	171000
ON25	10,1	6150	na	325000
Mean $\pm$ SD	12,6 $\pm$ 1,9	4800 $\pm$ 2200	1000 $\pm$ 500	227300 $\pm$ 75800
Median (IQR)	12,8 (2,2)	4500 (2900)	900 (800)	214000 (107000)
Post-rituximab therapy (Post-TP)				
POST1B	13,8	6020	na	259000
POST2	15,7	5170	1190	230000
POST3B	14,9	7290	730	287000
POST4	13,7	5150	1340	298000
POST5B	7,9	11500	2970	295000
POST6	14	12900	na	218000
POST7B	14	5050	1590	142000
POST8	15,1	9400	na	229000
POST9B	15	4870	1270	238000
POST10	10,2	3600	720	95000
POST11B	16,7	2740	360	153000
POST12B	13	2940	680	166000
POST13	13,3	3100	700	110000
POST14B	12,5	2200	580	201000
POST15	13,3	2890	960	124000
POST16	11,8	4810	1400	244000
Mean $\pm$ SD	13,4 $\pm$ 2,1	5600 $\pm$ 3200	1100 $\pm$ 700	205600 $\pm$ 66500
Median (IQR)	13,8 (3,9)	5000 (3400)	1000 (700)	223500 (100300)
p-value	0.15272	0.63836	0.9124	0.34722

na: not available; the p-values indicate the statistical significance of the difference between the two groups for each variable.

At baseline, no anti-Spike or anti-RBD IgG antibodies were detected in any of the patients (Figure 31A-B). Due to the depletion of CD20+ B cells, SARS-CoV-2-specific IgGs were undetectable in almost all patients vaccinated while receiving RTX treatment (TP-Ongoing) (A-B). Interestingly, within the TP-Ongoing group, two patients after the first dose and three patients 1 month post-second dose demonstrated low but detectable levels of anti-RBD and anti-Spike IgGs (Figure 31A-B). Notably, two of these patients (ON18 and ON22) started RTX therapy shortly after receiving the first vaccine dose (24 and 21 days, respectively) but completed their vaccination course while still undergoing RTX treatment (Table 8). These patients had detectable CD19+ cells at baseline (8.1% and 4.2% of CD19+ B lymphocytes, respectively), but none were present at following time-points of analysis. In contrast, post-TP patients, who completed the vaccination series at least 3 months after discontinuing RTX/chemotherapy, exhibited significantly higher anti-Spike IgG levels after the second and third vaccine dose and anti-RBD IgG levels after the third vaccine dose (Figure 31A-B). However, anti-Spike and anti-RBD IgG levels in patients treated with RTX remained lower

than those in healthy individuals (Figure 23 and Figure 24). Anti-Nucleoprotein IgG levels remained below the cut-off limit at all evaluation time points (Figure 31C). No correlation was found between the time elapsed since the last RTX treatment and the levels of anti-Spike or anti-RBD IgGs measured one month post-second or post-third vaccine dose (Figure 31D-E).

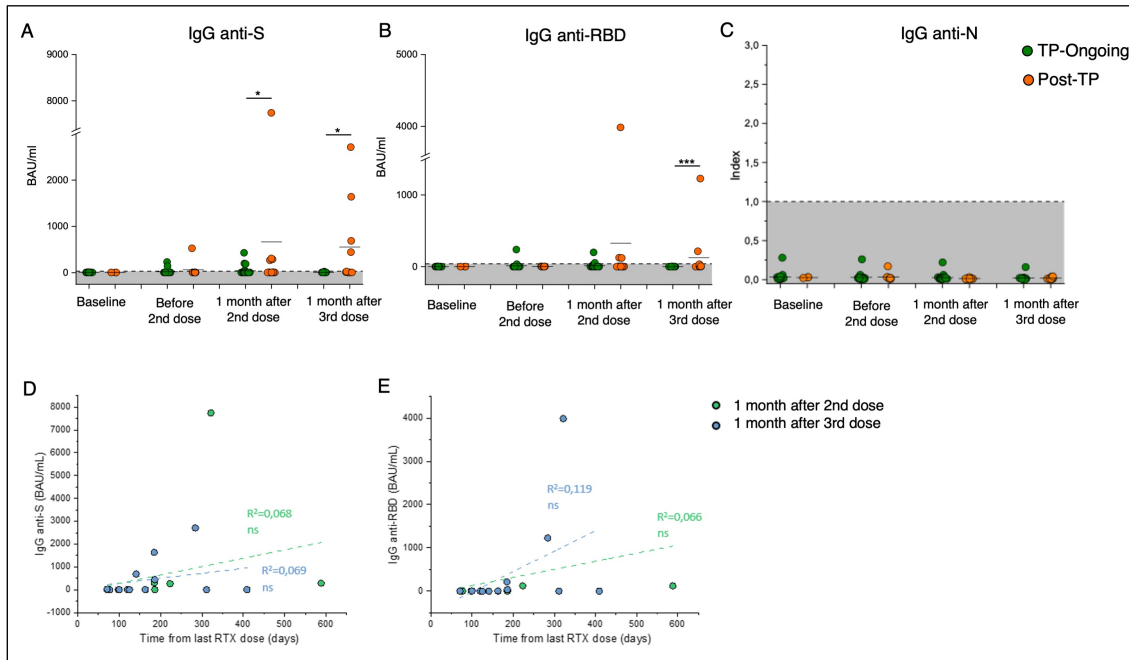


Figure 31 – Long-term evaluation of SARS-CoV-2-specific antibody levels in patients with B-cell lymphoma after vaccination and correlation with time from last rituximab treatment.

Evaluation of **(A)** anti-Spike IgG, **(B)** anti-RBD IgG, and **(C)** anti-Nucleoprotein IgG in therapy-ongoing group (green dots) at baseline (n=24), before second vaccine dose (n=23), 1 month after second dose (n=23) and 1 month after third dose (n=10) and in post-therapy (orange dots) group at baseline (n=3), before second vaccine dose (n=9), 1 month after second dose (n=13) and 1 month after third dose (n=12). Black lines in panels A–C represent mean values. Black asterisks denote unpaired statistics between TP-Ongoing and Post-TP patients at each time point, calculated with Mann-Whitney U test; * $P < 0.05$, *** $P < 0.001$. Correlation between **(D)** anti-Spike IgG or **(E)** anti-RBD IgG levels and the time from last rituximab dose (days) in the post-therapy group patients evaluated at 1 month after second vaccine dose (green dots) and 1 month after third vaccine dose (blue dots). Correlations were calculated using Pearson's correlation coefficients.

4.4.2 Strong vaccine-induced Spike-specific CD4⁺ T cell response independent of rituximab timing

The majority of patients with B-cell lymphoma (92.7%) enrolled in this study received intensive chemotherapy combined with RTX, therefore the reactivation capacity of CD4⁺ T cells was assessed through in vitro stimulation with a peptide pool representing the full-length Spike protein of the Wuhan strain. High levels of Spike-specific CD4⁺ T cells were demonstrated in both TP-ongoing and post-TP patients at all time points after receiving vaccination, up to 1 month post-third vaccine dose (Figure 32A-B). Notably, the frequency of Spike-specific CD4⁺ T cells showed no significant differences between the two groups and it was comparable to that seen in healthy individuals (Figure 32A).

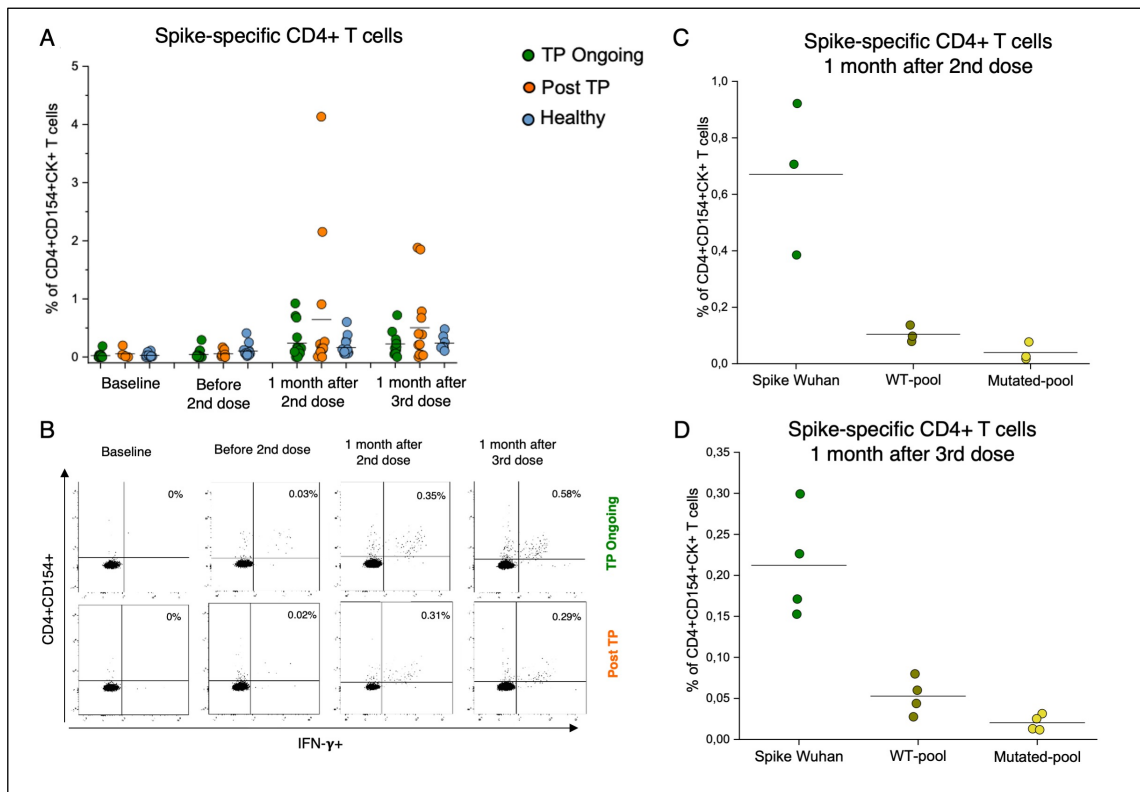


Figure 32 – Long-term evaluation of Spike-specific CD4+ T cells in patients with B-cell lymphoma after vaccination.

(A) Frequency of CD4+ T cells reactive to Spike protein, defined by expression of CD154 and at least one cytokine among IFN- γ , IL-2, and TNF- α (CD4+CD154+ck+) in therapy-ongoing patients (green dots) at baseline (n=14), before second vaccine dose (n=15), one month after second dose (n=15), and one month after third vaccine dose (n=10); in post-therapy patients (orange dots) at baseline (n=3), before second vaccine dose (n=7), one month after second dose (n=13), and one month after third vaccine dose (n=12); and in healthy individuals (light blue dots, n=14). **(B)** Representative flow cytometric plots of Spike-specific CD154+CD4+IFN- γ + T cells, at each time point of evaluation, in one therapy-ongoing patient (upper panels) and in one post-therapy patient (lower panels). **(C)** Frequency of CD4+ T cells specific for Wuhan Spike (wild type), Omicron variant reference or mutated pool in therapy-ongoing patients evaluated one month post-second vaccine dose (n=3). **(D)** Frequency of CD4+ T cells specific for Wuhan Spike (wild type), Omicron variant reference or mutated pool in therapy-ongoing patients evaluated one month post-third vaccine dose (n=4). The “Wuhan Spike” column denotes cells stimulated with peptide pools (Prot_S1, Prot_S+, and Prot_S) that cover the entire sequence of the Spike protein. The “mutated-pool” column corresponds to cells stimulated with peptide pools targeting the mutated regions specific to the Omicron variant. The “WT-pool” column refers to cells stimulated with the same peptide pools as those in the “mutated-pool”, but using the original Wuhan strain sequence.

In a subset of patients of the TP-ongoing group, CD4+ T-cell response to the Omicron variant was assessed one month after the second and after the third vaccine dose (three and four patients, respectively) (Figure 32C-D). The overall CD4+ T-cell response was preserved against the Omicron variant after vaccination in patients undergoing immunosuppressive treatment for B-cell lymphoma (Figure 32C-D).

4.4.3 Increased severity of breakthrough infections in patients treated with both rituximab and bendamustine

The incidence and severity of breakthrough infections during a 1-year follow-up after vaccination was prospectively assessed in both the TP-ongoing and post-TP group. Among the recruited

patients, 21 (51%) individuals experienced breakthrough SARS-CoV-2 infections between 3 and 12 months after the third vaccine dose, with varying severity, according to the WHO COVID-19 severity index (Table 1). Notably, 6 (28.6%) of these patients had a more severe disease course (Figure 33A). In the TP-Ongoing group, 68% of patients developed COVID-19, which was mild (8/17) or moderate (4/17). Only three patients experienced critical disease and two had severe disease. In contrast, in the post-TP group, only 25% of patients developed COVID-19, with the majority (3/4) experiencing mild illness. Importantly, all six patients with a higher WHO COVID-19 severity index (scores of 3 or 4) were treated with RTX + bendamustine (Table 8). When comparing infected patients based on their treatment regimen, patients who received both RTX and bendamustine had a significantly higher WHO COVID-19 severity index (mean = 2.6) than those treated with RTX + other chemotherapy regimens (mean = 1.36) (Figure 33B), although no differences in the overall number of breakthrough infections between the two groups was observed. Upon analyzing the characteristics and the type of therapy received by infected patients, patients with severe disease tended to cluster together (Figure 33C).

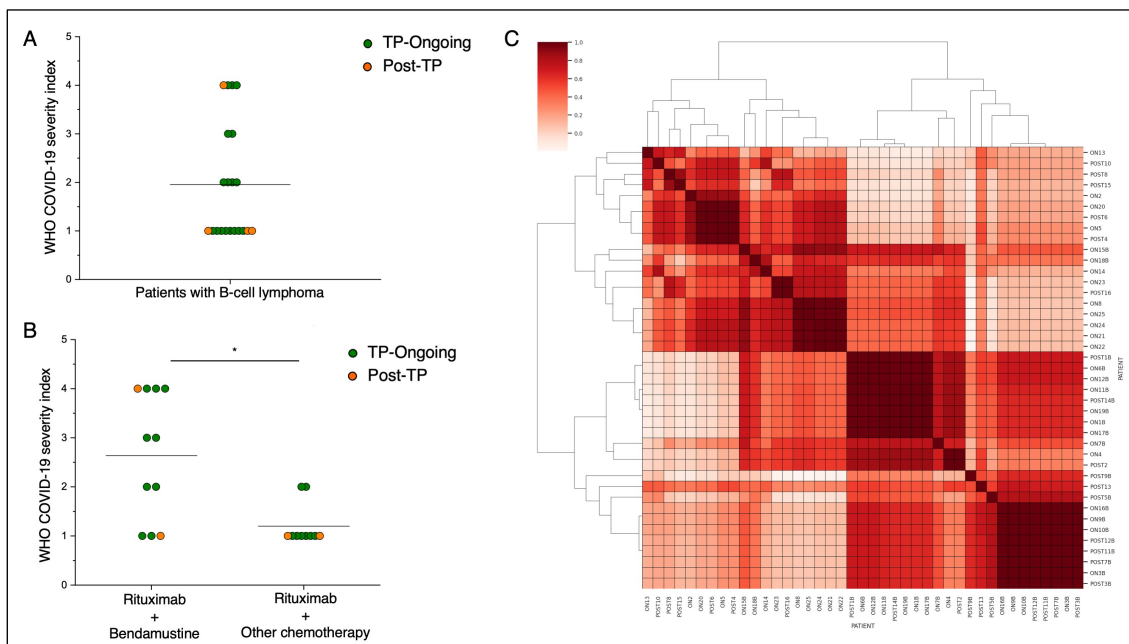


Figure 33 – Evaluation of breakthrough infections in vaccinated B-cell lymphoma patients according to chemotherapy regimen.

(A) WHO COVID-19 severity index of 21 patients with B-cell lymphoma with breakthrough infection, divided in those receiving treatment at the time of vaccination (TP-ongoing, green dots, n=17) and those who had discontinued treatment at the time of vaccination (post-TP, orange dots, n=4). **(B)** Comparison of WHO COVID-19 severity index between 11 patients treated with rituximab + bendamustine and 10 patients treated with rituximab + other chemotherapy regimen. Mean values are shown as black lines. Black asterisks denote unpaired statistics between patients receiving different chemotherapy regimens, calculated with Mann-Whitney U test; * $P < 0.05$ (C) Identification of clusters of B-cells lymphoma patients showing similarities based on type of therapy received and SARS-CoV-2 infection severity. Heatmap and hierarchical clustering of the total cohort of patients with B-cell lymphoma (n=41) based on different treatment received in addition to rituximab, total number of rituximab cycles, breakthrough SARS-CoV-2 infection and WHO COVID-19 severity index. For each patient the color code illustrates the average level of similarities between patients.

4.4.4 Impact of chemotherapy type on vaccine-induced humoral response in rituximab-treated patients

Given the increased risk of developing a more severe disease course following SARS-CoV-2 infection in patients treated with RTX and bendamustine, the groups were further subdivided based on the chemotherapy regimen administered alongside RTX. SARS-CoV-2-specific IgG levels post-vaccination were also influenced by the type of chemotherapy. Specifically, post-TP patients who had previously received RTX and bendamustine demonstrated lower or undetectable levels of anti-Spike IgG and anti-RBD IgG compared to post-TP patients treated with RTX and other chemotherapy regimens (Figure 34A-B). Interestingly, when focusing solely on patients treated with RTX and other chemotherapy regimens, a statistically significant positive correlation was observed between anti-Spike and anti-RBD IgG levels and the time elapsed since the last RTX dose, both at one month post-second vaccine dose and one month post-third dose (Figure 34C-D). The T cell-mediated response was also evaluated in relation to the inclusion of bendamustine in the chemotherapy regimen. In contrast to the humoral response, the T cell-mediated response in both TP-ongoing and post-TP patients was not significantly affected by the chemotherapy regimen. In fact, patients treated with RTX and bendamustine exhibited a comparable frequency of CD4+ Spike-specific T cells to those treated with RTX and other chemotherapy regimens not including bendamustine (Figure 34E-G).

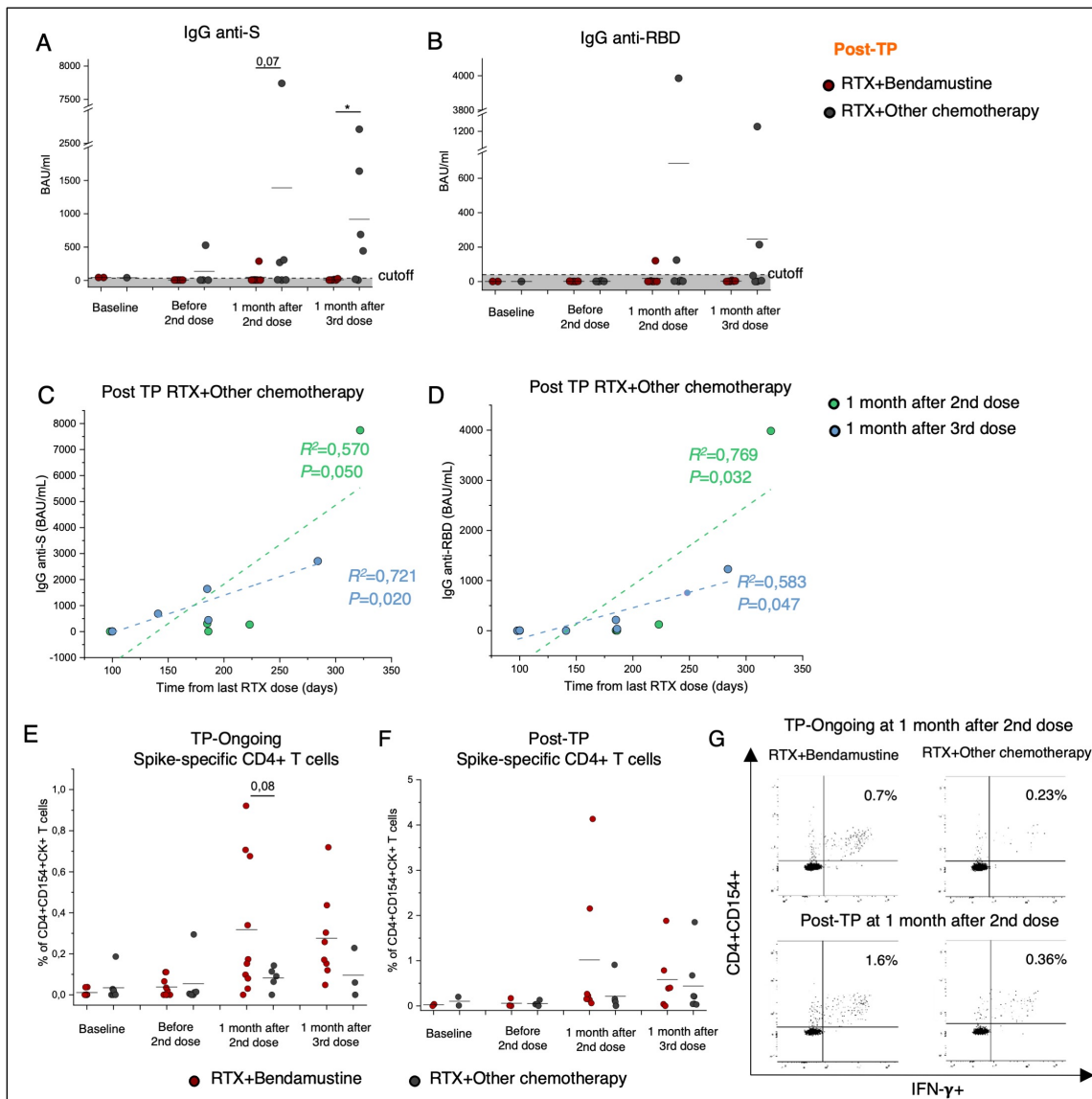


Figure 34 – Evaluation of Spike-specific IgG levels and CD4+ T cells following vaccination in B-cell lymphoma patients who underwent chemotherapy regimens with or without bendamustine.

Evaluation of **(A)** anti-Spike IgG and **(B)** anti-RBD IgG in patients who had been treated with RTX and bendamustine (red dots) at baseline (n=2), before second vaccine dose (n=5), one month after second dose (n=6), and one month after third dose (n=6) and in patients who had been treated with RTX and other chemotherapy (gray dots) at baseline (n=1), before second vaccine dose (n=4), one month after second dose (n=7), and one month after third dose (n=6). Correlation between IgG anti-Spike **(C)** or IgG anti-RBD **(D)** with time from last rituximab dose in patients who had been treated with RTX and other chemotherapy at one month post-second dose (n=7; light green dots) and at one month post-third dose (n=6; light blue dots). Correlations were calculated using Pearson's correlation coefficients. **(E)** Frequency of CD4+ T cells reactive to Spike protein in patients undergoing RTX and chemotherapy including bendamustine (red dots) at baseline (n=7), before second dose (n=9), one month after second dose (n=10), and one month after third dose (n=8) and in TP-ongoing RTX + other chemotherapy-treated patients (gray dots) at baseline (n=7), before second dose (n=6), one month after second dose (n=5), and one month after third dose (n=3). **(F)** Frequency of CD4+ T cells reactive to Spike protein in patients who had been treated with RTX and bendamustine (red dots) evaluated at baseline (n=2), before second dose (n=3), one month after second dose (n=6), and one month after third dose (n=5) and in patients who had been treated with RTX and other chemotherapy (gray dots) evaluated at baseline (n=1), before second dose (n=4), one month after second dose (n=7), and one month after third dose (n=6). **(G)** Representative flow cytometric plots of Spike-specific CD154+IFN-γ+ CD4+ T cells, at one month post-second vaccine dose, in two selected TP-ongoing patients (upper panels) and in two selected post-TP patients (lower panels) treated with RTX+bendamustine or RTX+other chemotherapy (left and right panels, respectively). Black lines in panels A,B,E,F represent mean values. Black asterisks denote unpaired statistics at each time point, calculated with Mann-Whitney U test; * $P < 0.05$

4.4.5 Long-term vaccine-induced immunity in solid-organ transplant recipients

A cohort comprising individuals who had undergone lung transplant due to cystic fibrosis (CFT, n=13), individuals with cystic fibrosis who had not required lung transplant (CF, n=10), and kidney transplant recipients without a diagnosis of cystic fibrosis (KT, n=8) was enrolled. Detailed demographic and clinical characteristics for the recruited patients are summarized in Table 10. The CFT group had a mean age of 42 years, while the CF and KT groups were younger, with mean ages of 20 and 21 years, respectively (Table 10). Time since transplant varied in the CFT and KT groups, with 39% of CFT patients and 25% of KT patients having undergone transplant 12 or more years ago (Table 10). Immunosuppressive regimens in transplanted groups predominantly included antimetabolites, particularly mycophenolate mofetil, with 54% of CFT and all KT patients on antimetabolite therapy (Table 10). A subset of CFT patients received other regimens, including tacrolimus and everolimus (Table 10). All subjects received the original mRNA-1273 vaccine (two doses, 28 days apart) in March 2021. CFT patients underwent blood sample collection before receiving the first vaccine dose (baseline), 28 days after the first dose (before the second dose), and one month after the second dose (Figure 17). For comparison with the CFT group, blood samples from the CF and KT patients were collected one month after receiving the second vaccine dose. All study participants had no previous history of PCR-confirmed SARS-CoV-2 infection and showed undetectable levels of anti-nucleoprotein IgG at baseline or at following time points of analysis.

Table 10 – Basic demographic and clinical characteristics of 21 solid-organ transplant recipients and control group who received vaccination.

	Lung transplanted patients with cystic fibrosis	Patients with cystic fibrosis	Kidney transplanted patients
No.	13	10	8
Gender			
Females, n (%)	5 (39%)	4 (40%)	6 (75%)
Males, n (%)	8 (61%)	6 (60%)	2 (25%)
Age at vaccination			
Mean age (±SD), years	42 (±13)	20 (±2)	21 (±2)
18-39 years, n (%)	7 (54%)	10 (100%)	8 (100%)
40-59 years, n (%)	6 (46%)	0	0
≥60 years, n (%)	0	0	0
Transplant			
Mean age at transplant (±SD), years	33 (±9)	na	13 (±5)
Mean time from transplant (±SD), years	9 (±7)	na	7 (±5)
<3 years since transplant, n (%)	3 (23%)	na	3 (37.5%)
3-6 years since transplant, n (%)	2 (15%)	na	1 (12.5%)
7-11 years since transplant, n (%)	3 (23%)	na	2 (25%)
≥ 12 years since transplant, n (%)	5 (39%)	na	2 (25%)
Maintenance immunosuppression regimen*			
Include antimetabolite**, n (%)	7 (54%)	na	8 (100%)
mycophenolate mofetil, n	5	na	8
azathioprine, n	2	na	0
Does not include antimetabolite, n (%)	6 (46%)	na	0
tacrolimus only, n	1	na	0
everolimus only, n	1	na	0
tacrolimus + everolimus, n	4	na	0

na denotes not applicable. * All transplant recipients were under low-dose steroids. ** In addition to tacrolimus, except one kidney transplanted patient who was receiving mycophenolate mofetil and cyclosporine.

Additionally, some CFT patients underwent blood sampling before and one month after receiving a third or fourth vaccine dose. Samples following the third vaccine dose were collected between September and October 2021, and those following the fourth dose were collected between April and May 2022.

4.4.6 Antimetabolite therapy weakens vaccine-induced immunity in solid-organ transplant recipients

In patients with cystic fibrosis who had undergone lung transplant (CFT), serum levels of anti-Spike IgG, anti-RBD IgG, and neutralizing antibodies increased significantly following the first vaccine dose, with a further notable rise after the second dose (Figure 35A-C). This increase in antibody levels was mirrored by an increase in the frequency of Spike-specific B cells after the second dose, along with an expansion of Spike-specific CD154+CD4+ T cells producing at least one cytokine among IL-2, IFN- γ , and TNF- α (Figure 35D-E). However, when compared to cystic fibrosis patients who had not undergone transplantation (CF), a reduced humoral response one month post-second vaccine dose was observed in lung transplant recipients (Figure 35F-H). Despite the reduction in the humoral response, the frequencies of Spike-specific B cells and CD4+ T cells were similar between CF and CFT patients, irrespective of transplant status (Figure 35I-J). In non-transplanted CF patients, the humoral and cell-mediated immune responses, assessed one month after the second vaccine dose, were comparable to those observed in naïve healthcare workers at the same time point (Figure 23, Figure 25, Figure 26). Given the variability in immunosuppressive regimens among CFT patients, the group was divided into (1) subjects receiving tacrolimus, everolimus, or both, without antimetabolite therapy (no-antimetabolite immunosuppression group, NA-CFT), and (2) subjects receiving mycophenolate mofetil or azathioprine (antimetabolite immunosuppression group, A-CFT). These two groups were compared to a cohort of kidney transplant recipients (KT) receiving mycophenolate mofetil (A-KT). Serum levels of anti-Spike IgG, anti-RBD IgG, and neutralizing antibodies, measured one month after second vaccine dose, were higher in the no-antimetabolite group compared to the antimetabolite groups (Figure 35K-M). This difference was also observed in the frequency of Spike-specific B cells which was higher in patients not receiving antimetabolites (Figure 35N). On the contrary, even if lower in A-CFT, the frequencies of Spike-specific CD4+ T cells were not statistically different between the groups (Figure 35O).

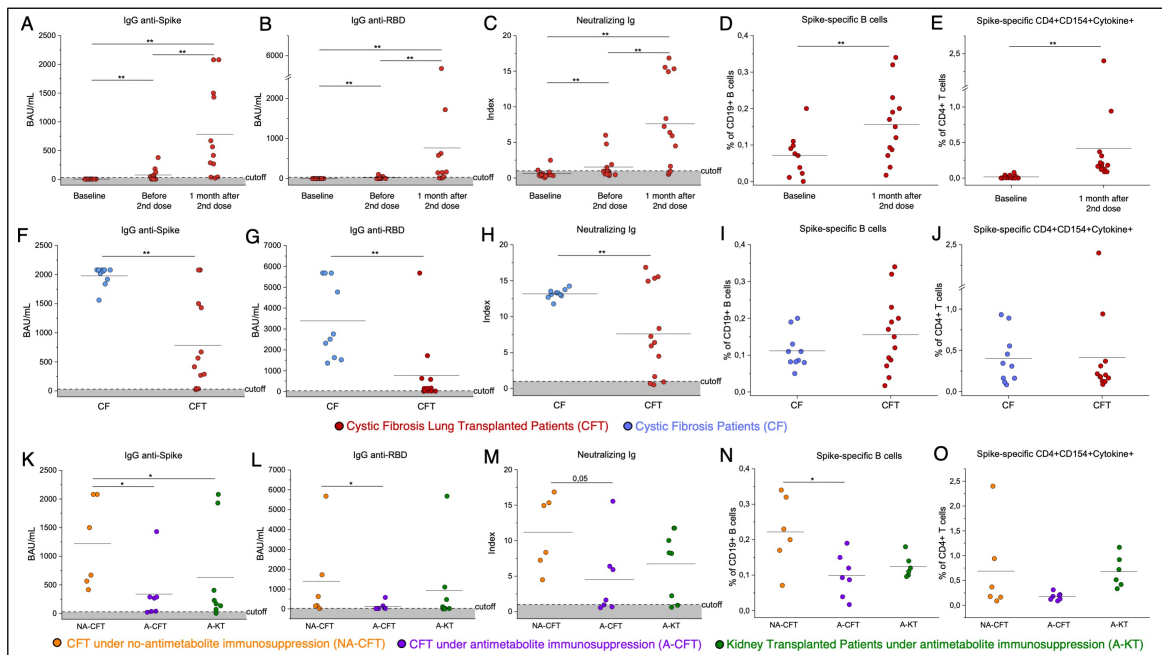


Figure 35 – Evaluation of SARS-CoV-2-specific antibody levels, Spike-specific B cells, and Spike-specific CD4+ T cells in solid-organ transplant recipients following vaccination.

Levels of (A) anti-Spike IgG, (B) anti-RBD IgG, and (C) neutralizing antibodies in patients with cystic fibrosis who had been lung transplanted (CFT, n=13) evaluated before vaccination, before the second vaccine dose (one month after the first dose) and at one month post-second vaccine dose. Frequencies of (D) Spike-specific B cells and (E) CD154+CD4+ Spike-specific T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in 13 CFT patients before vaccination and one month post-second vaccine dose. Asterisks refer to paired statistics at each time point compared with the previous, calculated with Wilcoxon's signed-rank test. * $P < 0.05$; ** $P < 0.01$. Levels of (F) anti-Spike IgG, (G) anti-RBD IgG, and (H) neutralizing antibodies in patients with cystic fibrosis (CF) (blue dots, n=10) and in patients with cystic fibrosis who had been lung transplanted (CFT) (red dots, n=13) evaluated at one month post-second vaccine dose. ** $P < 0.01$ calculated with Mann-Whitney test. Frequencies of (I) Spike-specific B cells and (J) CD154+CD4+ Spike-specific T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in CF patients (blue dots, n=10) and CFT patients (red dots, n=13) evaluated at one month post-second vaccine dose. Levels of (K) anti-Spike IgG, (L) anti-RBD IgG, and (M) neutralizing antibodies in cystic fibrosis lung transplanted patients under immunosuppressive regimen without antimetabolite (NA-CFT) (orange dots, n=6) or under antimetabolite immunosuppression (A-CFT) (violet dots, n=7), and in kidney transplanted patients under antimetabolite immunosuppression (A-KT) (green dots, n=8) evaluated at one month post-second vaccine dose. Frequencies of (N) Spike-specific B cells and (O) CD154+CD4+ Spike-specific T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in NA-CFT (orange dots, n=6) or A-CFT patients (violet dots, n=7), and in A-KT patients (green dots, n=6) evaluated at one month post-second vaccine dose. * $P < 0.05$ calculated with Mann-Whitney test. Black lines represent mean values, dotted lines represent cut-off values.

The impact of antimetabolite therapy on vaccine-induced immunity in solid-organ transplant recipients following booster doses was also evaluated. In CFT patients, serum levels of anti-Spike IgG and neutralizing antibodies, as well as the frequency of Spike-specific B cells increased significantly following the third or fourth vaccine dose (Figure 36A-C). However, there was no observable difference in the frequency of Spike-specific CD4+ T cells before and after the booster doses (Figure 36D). When examining the effect of the booster dose within the no-antimetabolite and antimetabolite groups, a general increase in antibody titers and Spike-specific B cells was observed, but no significant effect was detected for Spike-specific CD4+ T cells (Figure 36E-H). In comparing the no-antimetabolite and antimetabolite groups, serum levels of anti-Spike IgG and

neutralizing antibodies were lower in the antimetabolite group both before and one month after the booster dose, reaching statistical significance only pre-booster (Figure 36E-F). Similarly, the frequencies of Spike-specific B cells and Spike-specific CD4+ T cells were lower in the antimetabolite group compared to the no-antimetabolite group, though these differences did not reach statistical significance (Figure 36G-H). Consequently, the regimen including antimetabolites impaired mainly the humoral, but also the cell-mediated vaccine-induced immune response, even after the administration of booster doses. Notably, no correlation was found between the antibody levels and the frequency of Spike-specific B cells.

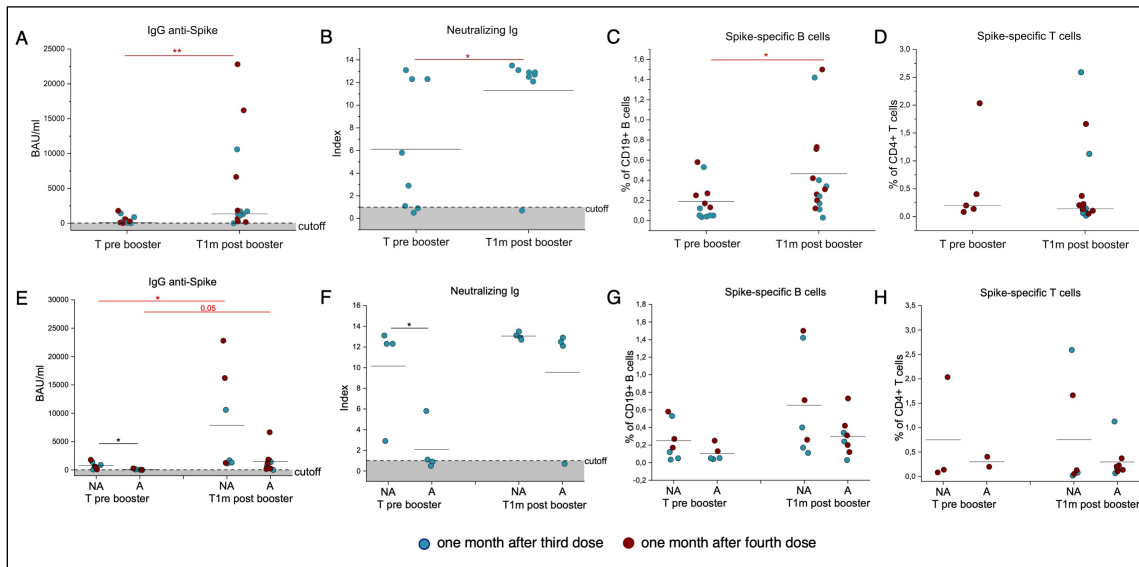


Figure 36 – Evaluation of SARS-CoV-2-specific antibody levels, Spike-specific B cells, and Spike-specific CD4+ T cells in solid-organ transplant recipients following boosters.

Levels of **(A)** anti-Spike IgG and **(B)** neutralizing antibodies in patients with cystic fibrosis who had been lung transplanted (CFT) evaluated before and one month after the third (blue dots, n=8 pre and post) or fourth (red dots, n=5 pre, n=8 post) vaccine dose. Neutralizing antibodies were only assessed following the third dose. Frequencies of **(D)** Spike-specific B cells in CFT patients evaluated before and one month after the third (blue dots, n=7 pre and post) or fourth (red dots, n=5 pre, n=8 post) vaccine dose. Frequencies of **(E)** CD154+CD4+ Spike-specific T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in CFT patients evaluated before and one month after the third (blue dots, n=6 pre and post) or fourth (red dots, n=5 pre, n=8 post) vaccine dose. Levels of **(F)** anti-Spike IgG and **(G)** neutralizing antibodies in CFT patients under immunosuppressive regimens without (NA) or with antimetabolites (A), assessed before and one month after the third (blue dots, NA n=4 pre and post, A n=4 pre and post) or fourth vaccine dose (red dots, NA n=3 pre and post, A n=2 pre and n=5 post). Neutralizing antibodies were only assessed following the third dose. Frequencies of **(H)** Spike-specific B cells in CFT patients under immunosuppressive regimens without (NA) or with antimetabolites (A), evaluated before and one month after the third (blue dots, NA n=4 pre and post, A=3 pre and post) or fourth (red dots, NA n=3 pre and post, A n=2 pre and n=5 post) vaccine dose. Frequencies of **(I)** CD154+CD4+ Spike-specific T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in CFT patients under immunosuppressive regimens without (NA) or with antimetabolites (A), evaluated before and one month after the third (blue dots, NA n=3 pre and post, A=3 pre and post) or fourth (red dots, NA n=3 pre and post, A n=3 pre and n=5 post) vaccine dose. Black lines represent mean values and dotted lines indicate cut-off values. Red asterisks refer to paired statistics (Wilcoxon's signed-rank test) within each group relative to the pre-booster time point, while black asterisks indicate unpaired statistics (Mann-Whitney test) between the NA and A groups. * $P < 0.05$, ** $P < 0.01$

The incidence and severity of breakthrough SARS-CoV-2 infections in the study population was prospectively assessed during a one-year follow-up period post-vaccination. Among all recruited patients, breakthrough infections were reported in 21 out of 31 subjects (occurring from 3 to 12 months after receiving booster doses) (Table 11). Notably, only one patient in the CFT cohort, who was not on antimetabolite therapy, developed severe COVID-19 that required hospitalization, while all other cases were mild to moderate (Table 11). It is important to note that two CFT patients on antimetabolite therapy (A06, A07), along with one patient not receiving antimetabolite (NA02), received prophylactic treatment with tixagevimab and cilgavimab. Another CFT patient not receiving antimetabolite (NA01) received tixagevimab and cilgavimab as a therapeutic intervention following SARS-CoV-2 infection in August 2022. None of the other study participants received monoclonal antibody therapy.

Table 11 – Breakthrough SARS-CoV-2 infection in 21 solid-organ transplant recipients and control group.

Subject (ID)	SARS-CoV-2 infection after vaccination	No. of breakthrough infections	COVID-19 severity
Lung transplanted patients with cystic fibrosis receiving immunosuppression without antimetabolite therapy			
CFT-NA01	Yes	2	1;1
CFT-NA02	Yes	1	1
CFT-NA03	Yes	1	3
CFT-NA04	No	0	na
CFT-NA05	Yes	1	1
CFT-NA06	Yes	1	1
Lung transplanted patients with cystic fibrosis receiving immunosuppression with antimetabolite therapy			
CFT-A07	Yes	1	1
CFT-A08	Yes	1	1
CFT-A09	Yes	2	1;1
CFT-A10	No	0	na
CFT-A11	No	0	na
CFT-A12	No	0	na
CFT-A13	No	0	na
Kidney transplanted patients			
KT-A01	Yes	1	1
KT-A02	No	0	na
KT-A03	Yes	1	1
KT-A04	Yes	1	1
KT-A05	No	0	na
KT-A06	Yes	1	1
KT-A07	Yes	1	1
KT-A08	No	0	na
Patients with cystic fibrosis			
CF01	No	0	na
CF02	Yes	1	1
CF03	Yes	1	0
CF04	Yes	2	2
CF05	Yes	1	1
CF06	Yes	1	0
CF07	Yes	1	1
CF08	Yes	1	2
CF09	No	0	na
CF10	Yes	1	1

na denotes not applicable.

4.4.7 Transient boost in Spike-specific immune response after fourth vaccine dose in people living with HIV

To evaluate the long-term effect of vaccine-induced immunity, 8 people living with HIV (PLWH) who received a fourth dose of the original BNT162b2 mRNA vaccine in March 2022 were evaluated up to 2 months post-vaccination (Figure 17). Main demographic and clinical characteristics are summarized in Table 12. Most participants had very low or undetectable blood levels of HIV viremia. Three individuals had previously developed AIDS and AIDS-related complications, while 5 out of 8 presented comorbidities (Table 12). All patients were undergoing or had undergone antiretroviral therapy (ART) and received the fourth vaccine dose 3 to 5.5 months after the third dose was administered (Table 12). Notably, 6 participants had CD4+ T cell counts below 500 cells/mm³, along with a lower CD4/CD8 ratio compared to average healthy individuals. All individuals tested negative for anti-nucleoprotein IgGs at the baseline. At the 2-month follow-up, two individuals (V7 and V8) tested positive for anti-nucleoprotein IgGs, indicating exposure to SARS-CoV-2 during the last month of the study. In both cases, the infection was asymptomatic, and neither individual was tested with a nasopharyngeal swab.

Table 12 – Basic demographic and clinical characteristics of 8 people living with HIV (PLWH) who received vaccination.

PLWH	Age (years)	Age at HIV diagnosis (years)	Time from HIV diagnosis to ART start (days)	CD4+ T cell nadir count (cell/mm ³)	HIV-1 RNA zenith (copies*10 ³ /mL)	AIDS (age at onset)	Complications	Comorbidities	Latest CD4+ T cell count (cell/mm ³)	Latest HIV-1 RNA (copies/mL)	Adherence to ART	Vaccine type (1 st +2 nd +3 rd +4 th dose)	Time between 3 rd and 4 th vaccine dose (days)	CD4/CD8 ratio
V1	47	22	29	355	>500	NA	No	Allogeneic HSCT in AML at 41 years	1300	TND	Yes	S+S+C+C	167	1.7
V2	64	31	1034	300	ND	NA	No	Cardiovascular disease	989	TND	Yes	S+S+C+C	160	1
V3	64	50	42	8	227	Yes (50)	Cryptococcal meningitis and pneumonia; MAC infection	Major depressive disorder	172	<20	No	S+S+S+C	107	0.4
V4	65	30	1461	15	232	Yes (60)	<i>Pneumocystis jirovecii</i> pneumonia; CMV infection	Chronic hepatitis C	253	22000	No	S+S+C+C	108	1
V5	48	45	3	3	1420	NA	No	None	210	<20	Yes	S+S+S+C	101	0.4
V6	58	48	28	28	>10000	Yes (55)	Wasting syndrome; esophageal candidiasis	None	243	58	Yes	S+S+S+C	94	0.8
V7	51	31	128	103	449	NA	No	Severe obesity	390	TND	Yes	S+S+C+C	117	0.1
V8	71	70	7	186	2810	NA	No	None	829	84	Yes	S+S+S+C	94	0.7
Mean	58.5	48.8	657.1	124.7	-	-	-	-	548.2	-	-	-	119	0.8
SD	9	15.2	956.3	140.3	-	-	-	-	430.8	-	-	-	29	0.5

AIDS: acquired immunodeficiency syndrome; AML: acute myeloid leukemia; C: original BNT162b2 vaccine; ART: antiretroviral therapy; HSCT: hematopoietic stem cell transplantation; MAC: mycobacterium avium complex; NA not applicable; ND not determined; S: original mRNA-1273 vaccine; TND: target not detected.

The levels of anti-Spike IgM resulted below the cut-off at every time point (Figure 37A), while anti-Spike IgGs showed an increasing trend from baseline to one month after the fourth dose, followed by a significant decline 2 months post-vaccination (Figure 37B). Patient V8 experienced a peak in anti-Spike IgG levels 7 days after receiving the fourth dose, followed by a similar decrease to that observed in other participants (Figure 37B). All subjects demonstrated higher concentrations of neutralizing antibodies than the cut-off value at all time points (Figure 37C). The levels of anti-RBD IgG increased significantly in all subjects from baseline to 7 days post-vaccination, followed by a decline at 1 month (Figure 37D). Two months after the fourth dose, most patients showed a marked decrease in anti-RBD IgGs (Figure 37D). However, individuals V6 and V7 exhibited an increase, while V8 maintained stable levels of anti-RBD IgGs (Figure 37D). Participants V7 and V8 tested positive for IgGs targeting SARS-CoV-2 Nucleoprotein two months after the injection, suggesting prior exposure to the virus, while in V6 anti-Nucleoprotein IgG titers remained below the cut-off value, as in all the other remaining individuals. Similarly, the frequency of Spike-specific B cells increased variably among study participants during the first month after the fourth vaccine dose, but showed a significant decline 2 months post-vaccination (Figure 37E). Longitudinal analysis revealed that the frequency of Spike-specific CD4+ T cells generally increased between 7 days and 1 month after the fourth vaccine dose, followed by a decrease at the 2-month follow-up (Figure 37F).

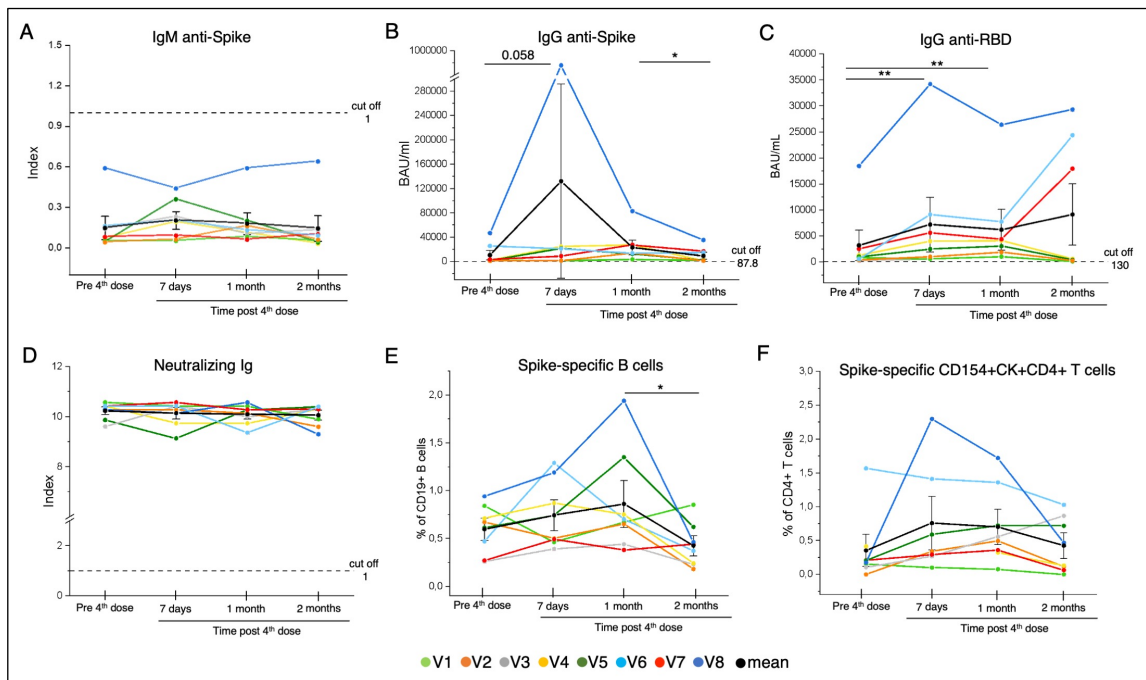


Figure 37 – Evaluation of Spike-specific antibody levels, Spike-specific B cells and Spike-specific CD4+ T cells in people living with HIV following fourth vaccine dose administration.

Levels of **(A)** anti-Spike IgM, **(B)** anti-Spike IgG, **(C)** anti-RBD IgG, and **(D)** neutralizing antibodies in people living with HIV (n=8), evaluated at baseline (before the fourth vaccine dose), 7 days, one month, and two months post-vaccination. Frequencies of **(E)** Spike-specific B cells and **(F)** CD154+CD4+ Spike-specific T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in people living with HIV (n=8), evaluated at baseline (before fourth vaccine dose), 7 days, one month, and two months post-vaccination. Each color represents a different subject, black dots represent mean values. Error bars represent standard error. refer to paired statistics at each time point compared with the previous, calculated with Wilcoxon's signed-rank test. * $P \leq 0.05$; ** $P \leq 0.005$.

At this time point, PBMCs were stimulated with three separate peptide pools: one covering the entire Spike protein of the SARS-CoV-2 Wuhan strain (wild type, WT), one covering the Wuhan Spike portion mutated in the B.1.1.529 (Omicron) variant (WT Spike reference pool), and one covering only the mutated region (Omicron Spike mutated pool) (Figure 38A). The frequency of CD154+CD4+ T cells producing at least one effector cytokine (IL-2, TNF- α , or IFN- γ) reactive to the reference pool or the mutated pool was significantly lower than the frequency of CD4+ T cells activated by the total Wuhan Spike protein (Figure 38B). These CD4+ T cells specific for the mutated regions represented only a minor portion of the overall Spike-specific response across all subjects. In comparing CD4+ T cells specific to the mutated sequences with those reactive to the corresponding region in the wild-type strain, no significant differences were observed, though CD4+ T cells reactive to WT reference pool and producing at least one effector cytokine were slightly more frequent (Figure 38B). To assess the potential cross-reactivity of Spike-specific B cells to the Omicron variant, the subset of CD27+ B cells capable of binding the Omicron Spike within the WT Spike-specific population was identified (Figure 38C). Among 5 PLWH analyzed at 2 months post-vaccination, the frequency of Wuhan Spike-specific CD27+ B cells that could bind both WT and Omicron Spike protein was significantly higher than the frequency of CD27+ B cells that exclusively bound the WT Spike (Figure 3D).

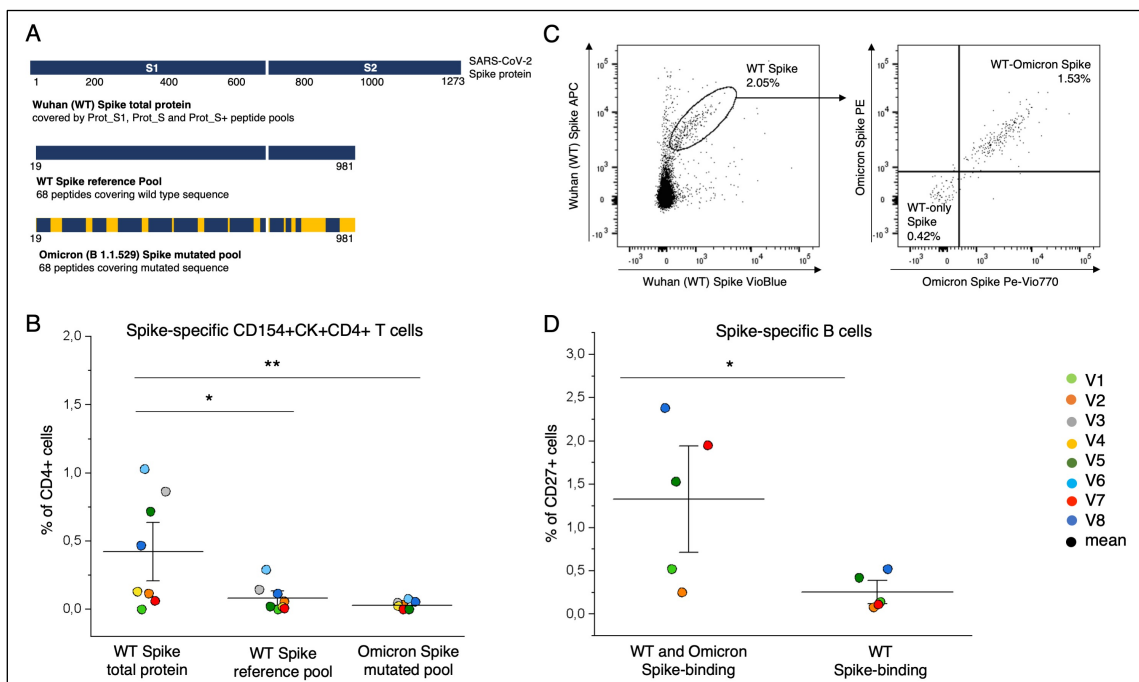


Figure 38 – Evaluation of CD4+ T cells and CD27+ B cells specific to the Wuhan SARS-CoV-2 Spike and the Omicron (B.1.1.529) BA.1 variant in people living with HIV following fourth vaccine dose.

(A) Schematic overview of alignment of different peptide pools. **(B)** Frequencies of CD154+CD4+ T cells producing at least one cytokine among IL-2, IFN- γ and TNF- α in response to stimulation with Spike peptide pools from the wild-type (Wuhan) and Omicron (B.1.1.529 BA.1) Spike covering the mutated sequence, in people living with HIV (n=8) evaluated at 2 month post-fourth vaccine dose. **(C)** Representative flow cytometry plots identifying Wuhan Spike-specific cells labeled with APC-PB and Omicron Spike-specific B cells labeled with PE-PeVio770 within the Wuhan Spike-specific pool. **(D)** Frequencies of memory B cells binding to both Wuhan and Omicron Spike proteins, and CD27+ B cells binding exclusively to the Wuhan Spike. Each color represents a different subject. Black lines represent mean values. * $P \leq 0.05$, ** $P \leq 0.005$. Error bars represent standard error (SE).

5. DISCUSSION

The study of the immune response against an infectious pathogen is essential to understand the interaction between the host and that specific pathogen. The emergence of SARS-CoV-2 in the community prompted the investigation of the immune response against this novel virus. Shortly thereafter, the development of anti-SARS-CoV-2 mRNA vaccines raised questions about the immune response elicited by these novel vaccines [175]. In the endemic phase of COVID-19, the active monitoring of long-term immunity both to natural infection and vaccination is thus critical to guide public health interventions and inform vaccination strategies. At the start of the global vaccination campaign in December 2020, a two-dose mRNA vaccine series was recommended for all eligible individuals, regardless of prior SARS-CoV-2 infection. Despite the initial phase of the vaccination programme being limited to a minority of the global population, the priority was to protect as many people as possible, given the constraints of the vaccine supply [176]. The differences in Spike-specific immune response elicited after the first and second mRNA vaccine doses between healthcare workers with or without prior SARS-CoV-2 infection contributed to the rationale for limiting mRNA vaccine doses in previously infected individuals [109,177]. In infection-naïve individuals, anti-Spike neutralizing antibodies and Spike-specific B and T cell frequencies only significantly increased after the second vaccine dose, confirming the necessity of the second dose in this group, as evidenced in clinical trials [178,179]. In contrast, individuals who recovered from COVID-19, showed a reduction in Spike-specific B and T cell responses after the second vaccine dose, suggesting a limited benefit of a second dose for this population. Krammer et al. and Bradley et al. similarly reported that a single dose of mRNA vaccine in previously exposed individuals rapidly increased anti-Spike IgG levels compared to naïve individuals [180,181]. Anichini et al. performed neutralization assay on VeroE6 cells and demonstrated that previously infected individuals produced higher neutralizing antibody titers after a single dose than those observed after the second dose in naïve individuals [182]. These findings align with studies by Prendecki et al. and Lozano-Ojalvo et al., which indicated that one vaccine dose induced robust humoral and cellular responses in individuals with history of SARS-CoV-2 infection, whereas naïve individuals exhibited only modest T cell responses and low levels of neutralizing antibodies after one vaccine dose [183,184]. In the current project, the majority of participants with prior COVID-19 had mild to moderate disease, so it was not possible to assess immune responses based on disease severity. However, symptomatic SARS-CoV-2 infection often correlates with higher antibody levels and more robust T cell-mediated response compared to asymptomatic cases [185,186,187,188]. It is important to consider that vaccine-induced immune responses in previously infected individuals may vary depending on COVID-19 severity, given that a considerable proportion of global SARS-CoV-2 infections are asymptomatic [189,190]. Indeed, individuals who were asymptomatic during the infection exhibited a diminished antibody response and neutralization capacity following

vaccination in comparison to those who had recovered from symptomatic COVID-19 [191]. Considering previously infected symptomatic individuals, the first vaccine dose induces a rapid reactivation of Spike-specific B cells, leading to a strong antibody response. However, following the second dose, Spike-specific B cell frequencies decline more rapidly compared to naïve subjects. Mechanisms of senescence, deletion, functional exhaustion, or others might be involved in causing this steady reduction of circulating Spike-specific B cells in the long-term [192]. Neutralizing antibodies are primarily responsible for blocking SARS-CoV-2 infection, and their development is likely a key correlate of protection against severe COVID-19 upon re-exposure [193]. In the current project, in contrast to the potential decline in T and B cell responses observed after the second vaccine dose in individuals who recovered from COVID-19, humoral responses showed minimal variation. Specifically, neutralizing antibody titers increased after the second dose in individuals with prior infection, indicating that the additional dose did not result in any detrimental effects and may not have significant clinical consequences for people previously infected. Hammerman et al. evaluated vaccine effectiveness after COVID-19 recovery in a large retrospective cohort, demonstrating that a single dose of the BNT162b2 vaccine was sufficient to confer protection from reinfection, and that receiving more than one dose did not enhance vaccine effectiveness in individuals with prior SARS-CoV-2 infection [194]. By administering only one vaccine dose to individuals who had recovered from COVID-19, the availability of doses for the naïve population significantly increased, thereby accelerating the achievement of herd immunity. In addition, studies have demonstrated that extending the interval between infection and vaccination may enhance the vaccine-induced immune response [195,196]. In previously infected individuals, vaccination can be deferred for up to 3 months from symptom onset or a positive test in case of asymptomatic infection [106, 197]. After 8 months post-vaccination, antibody levels declined significantly, particularly in individuals without prior SARS-CoV-2 infection. However, a decrease in antibody quantity does not necessarily indicate a reduction in the quality of the humoral response. Despite the overall decline in total antibody levels, affinity maturation may select B cells that produce antibodies with improved antigen-binding capacity, maintaining the overall functionality [198]. This is supported by the gradual reduction in neutralizing antibodies observed in vaccinated individuals, regardless of their infection history, suggesting sustained protection. Accordingly, Goel et al. showed that neutralizing antibodies declined slower than total anti-Spike antibodies in both naïve and COVID-19 recovered individuals [199]. They also found that memory B cells specific to both the full-length Spike protein and the RBD are efficiently primed by mRNA vaccination and detectable in all SARS-CoV-2 naïve subjects after the second vaccine dose, though the memory B cell response showed a slight decline with age. In individuals who have recovered from infection, antibody and memory B cell responses are significantly boosted after the first vaccine dose. However, no further increases in circulating antibodies, neutralizing titers, or antigen-specific memory B cells are observed following the second dose. Goel et al. found that this robust boost after the first dose strongly correlates with pre-existing levels of memory B cells in recovered individuals, highlighting the critical role that these cells play in mounting recall responses to SARS-

CoV-2 antigens [199]. Despite the limited sample size and analysis primarily focusing on the initial weeks post-vaccination, their observations indicate that pre-formed Spike-binding memory B cell clones do not exhibit significant increases in somatic hypermutation (SHM) [199]. This suggests that the B cell clones enhanced by mRNA vaccination in individuals recovered from SARS-CoV-2 maintain stable SHM profiles. Nevertheless, these clones may evolve and undergo additional SHM over time, similar to what occurs following natural SARS-CoV-2 infection [200,201,202]. The pronounced early increase in circulating Spike-specific memory B cells in COVID-19 recovered individuals is followed by a substantial contraction phase within the first month post-vaccination. In contrast, naïve individuals showed a gradual increase in the frequency of Spike-specific B cells from 1 month to 8 months after their primary vaccination series. Notably, by 8 months post-vaccination, the frequency of Spike-specific memory B cells becomes comparable between naïve and COVID-19 recovered subjects. Importantly, throughout the study period, all recruited individuals did not experience reinfection, as confirmed by the kinetics of anti-Nucleoprotein antibodies and medical history evaluation. The frequencies of Spike-specific CD4⁺ T cells in naïve and recovered COVID-19 subjects were comparable following vaccination, with similar kinetics observed in both groups. Spike-reactive CD4⁺ T cells exhibited similar frequencies in both groups at one month post-vaccination, with levels gradually declining in parallel up to 8 months. In terms of effector functionality, although the frequencies of vaccine-induced CD4⁺ T cells are comparable at 8 months, COVID-19 recovered individuals produced significantly higher levels of IFN- γ in response to stimulation with Spike protein compared to naïve individuals receiving vaccination.

To assess the long-term durability of natural immunity following SARS-CoV-2 infection, a cohort of unvaccinated individuals, enrolled during the first wave of the COVID-19 pandemic, was monitored for up to 12 months after hospital discharge. These individuals were hospitalized for moderate to severe COVID-19. Since vaccination was not available at that time, this cohort is rather unique because it allows monitoring of long-term effects of SARS-CoV-2 infection regardless of vaccination. The waning of both humoral and cell-mediated immune responses was similar compared to vaccine-induced immunity. Anti-Nucleoprotein IgGs showed the shortest durability, with levels falling below the cut-off value 12 months after infection. Neutralizing antibodies declined more slowly than anti-Spike IgG and anti-RBD IgG levels, with the reduction in humoral immunity potentially contributing to reinfections [203,204]. However, Spike-specific B cells and CD4⁺ T cell responses remained generally stable up to one-year post-infection, indicating long-term persistence of memory B and T cells. These findings align with studies on the original SARS-CoV, where memory cells were detectable up to 17 years after the 2003 outbreak [205]. It is important to note that the severity of infection impacts the magnitude of immunological memory, nonetheless in a cohort of unvaccinated subjects with mostly mild COVID-19, Kratzer et al. showed similar humoral decay at 10 months post-infection [206].

At the onset of the vaccination campaign in 2020, it was anticipated that COVID-19 vaccines would prevent infection and inhibit onward transmission before the emergence of viral variants [175]. This expectation stemmed from the observation of high neutralizing antibody titers

at the peak of immunity following vaccination. However, the COVID-19 pandemic landscape changed significantly, prompting a recalibration of vaccination goals in light of reduced neutralizing antibody titers and the global rise of VOCs. During the Delta wave, a noticeable decrease in protection against symptomatic disease was observed, alongside a rise in severe cases [207,208]. Additionally, over time antibody levels declined following two-dose mRNA vaccination [209,210]. The administration of a third (booster) vaccine dose restored protection against symptomatic disease [211,212,213]. Bar-On et al. reported that, at least 12 days following the booster dose, the rates of confirmed infection and severe illness were reduced by factors of 11.3 and 19.5, respectively, in individuals receiving a third vaccine dose compared to the non-booster group [212]. These findings led to the initiation of booster campaigns in 2021. There has been debate regarding whether the booster dose should be viewed as an enhancement of a vaccine regimen with waning efficacy or as an integral part of the original vaccination series. Many vaccines, such as those for hepatitis B virus, *Haemophilus influenzae* type B, inactivated poliovirus, pneumococcal conjugate, diphtheria, tetanus, and pertussis (DTaP), comprise multi-dose primary series [214,215]. In this project, the impact of a third mRNA vaccine dose on both humoral and cellular responses in a cohort of healthcare workers with or without a history of SARS-CoV-2 infection prior to receiving the primary vaccine series was assessed, as well as in immunocompromised individuals. A significant boost in anti-Spike antibody levels and a more rapid reactivation of Spike-specific B and CD4+ T cell responses were observed after the third vaccine dose, particularly in naïve individuals compared to COVID-19 recovered subjects. However, antibody levels remained significantly higher in the latter group. Tartof et al. demonstrated that the effectiveness of mRNA vaccines against infections declined from 88% during the first month after two-dose vaccine series to 47% after 5 months, but effectiveness against hospitalization remains high up to 6 months [216]. Similarly, Levin et al. reported that 6 months after the second dose of the BNT162b2 vaccine, the humoral response significantly decreased, particularly in men, people aged 65 years or older, and those immunocompromised [217]. Chin et al. evaluated the effectiveness of two or three doses of the BNT162b2 and mRNA-1273 vaccines, as well as prior infection, in reducing the risk of infection against the SARS-CoV-2 Omicron variant [218]. They found that the estimated effectiveness of prior infection against Omicron among unvaccinated individuals who had been infected before or during the Delta wave ranged from 16% to 49% [218]. Depending on previous infection status, vaccination effectiveness (compared to unvaccinated naïve individuals) ranged from 17% to 83% with two doses and from 41% to 88% with three doses [218]. Incremental effectiveness estimates for the third dose (compared to two doses) ranged from 25% to 58% among individuals without prior documented infection or infected before the Delta wave [218]. Notably, these estimates focused on confirmed infections rather than other critical outcomes, such as symptomatic infections or severe disease. Nonetheless, substantial increases in protection against variants after a booster dose were observed, particularly among naïve individuals [219,220,221]. Recommendations for additional vaccine doses vary based on age, vaccination history, and immune status. Gilboa et al. demonstrated sustained anti-Spike humoral immunity up to 5 months post-booster dose [222]. The

waning of anti-Spike IgG and neutralizing antibody levels was found to be slower following the third dose compared to the second dose [222]. Notably, the long-term kinetics of neutralizing antibodies after the third dose remained stable and differed from the kinetics observed after the second dose, where a significant decrease continued beyond 70 days [222]. Interestingly, although neutralizing antibodies significantly decreased one week post-booster in COVID-19 recovered individuals, levels remained above the cut-off limit. Despite lower titers of anti-Spike IgG, anti-RBD IgG, and neutralizing Ig levels in naïve individuals compared to COVID-19 recovered subjects before the booster dose, titers were comparable one week post-vaccination, regardless of prior SARS-CoV-2 infection. Consequently, the third vaccine dose had minor effects on individuals who had recovered from COVID-19, aligning with the immunologic effects observed following the second vaccine dose. COVID-19 recovered individuals exhibited high levels of anti-Spike antibodies, as well as memory B cells and CD4⁺ T cells, even at 8 months post-vaccination (Figure 39). This suggests that regulatory mechanisms might limit an excessive anti-Spike immune response in these individuals. Additionally, Spike-specific B and CD4⁺ T cells may present an exhausted phenotype, potentially reducing their capacity for reactivation after antigen stimulation.

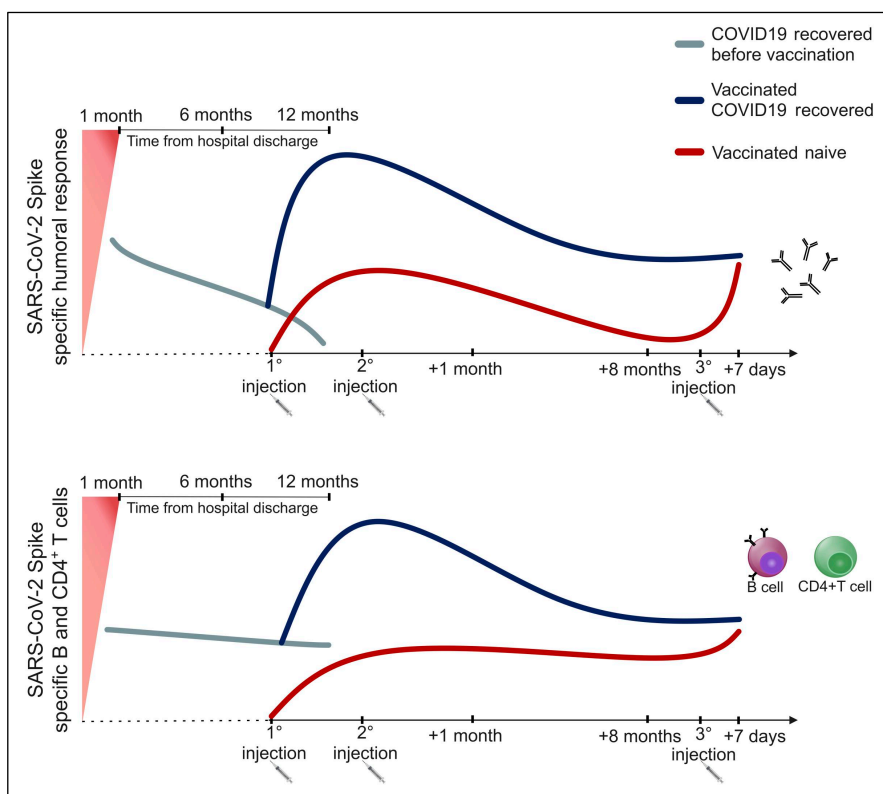


Figure 39 – Schematic overview of long-term SARS-CoV-2 Spike-specific vaccine-induced and natural adaptive immune responses.

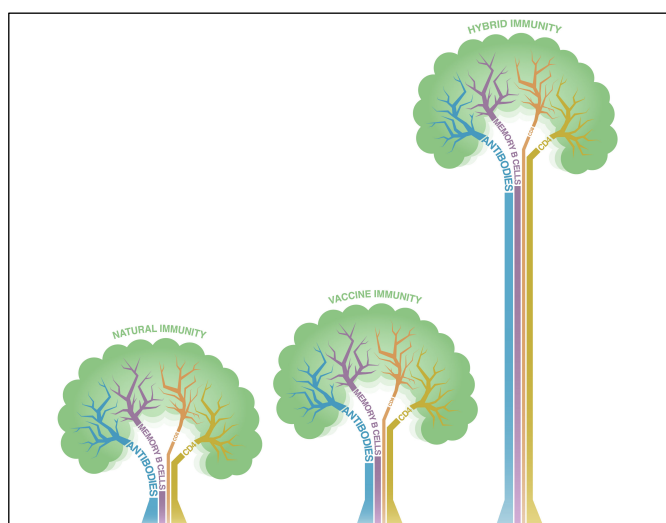
Furthermore, the observation that infection followed by two vaccine doses leads to longer-lasting anti-Spike antibody levels suggests that a third vaccine dose might similarly result in prolonged antibody persistence in previously uninfected healthy individuals. It has been shown that the vaccine-induced T cell response remains largely conserved against SARS-CoV-2 variants [223,224].

Tarke et al. found that the overall reactivity against viral variants is comparable in terms of both magnitude and frequency of response [225]. Skelly et al. confirmed that the majority of T cell responses in recipients of two doses of the BNT162b2 vaccine are driven by epitopes that are invariant between the Wuhan strain and Alpha or Beta variant [226]. In a subsequent study, Tarke et al. assessed T cell responses at 3-4 months and 6 months after vaccination using distinct Spike peptide pools derived from prominent SARS-CoV-2 variants, including Omicron (B.1.1.529) [227]. They compared variant-specific T cell responses to those elicited by the ancestral strain Spike pool and discovered that over 80% of both CD4+ and CD8+ T cell responses were able to cross-recognize the different Spike protein variants, regardless of the vaccine platform used [227]. Notably, at 6 months post-vaccination, 84% (CD4+) and 85% (CD8+) T cell responses were preserved against Omicron [227]. Mazzoni et al. showed that CD4+ T cell response to the Omicron peptide pool in vaccinated individuals accounted for 20% of the total Spike-specific CD4+ T cell response after the second dose and 30% after the third dose [228]. In contrast, stimulation with the mutated Omicron pool triggered a much lower response, being 65% lower after the second dose and 61% lower after the third dose [228]. Overall, 87% of the total Spike-specific CD4+ T cell response remained effective against Omicron after the second dose, and 82% after the third dose [228]. In addition, T-cell responses elicited by vaccination were confirmed to be cross-reactive against Omicron sub-variants, even prior to Omicron infection [229,230,231].

Overall, vaccination in individuals who have recovered from COVID-19 generates “hybrid immunity”, characterized by stronger humoral and cellular responses and a broader antibody repertoire compared to immunity from natural infection or vaccination alone in previously uninfected individuals (Figure 40).

Figure 40 - Hybrid immunity with COVID-19 vaccination.

Hybrid vigor, or heterosis, occurs when two different plant lines are bred together, producing a hybrid that exhibits enhanced strength and vitality compared to its parents. A similar phenomenon is observed in immunology when natural immunity from infection is combined with vaccine-induced immunity, often referred to as “hybrid immunity”. This results in significantly stronger immune responses driven by memory B cells and CD4+ T cells and broader protection against viral variants compared to either natural infection or vaccination alone. Figure by Prof. Shane Crotty on Science 2021 [232].



Notably these observations derive from healthy individuals, but persistence of immunological memory may vary according to conditions of immune impairment. For this reason, the project also focused on assessing the characteristics and durability of long-term vaccine-induced immunity in three main cohorts of immunocompromised individuals: patients with B-cell

lymphoma, solid-organ transplant recipients and people living with HIV. Understanding how immunosuppressive regimens impact the persistence of vaccine-induced immunity is essential for optimizing vaccination strategies, including the timing and dosage of booster vaccinations, to ensure sustained immunity against SARS-CoV-2. Patients with B-cell lymphoma are at increased risk for severe COVID-19, often with fatal consequences, as hematological malignancies are an independent risk factor for COVID-19 mortality [233,234]. Anti-CD20 monoclonal antibody therapy is associated with poor or absent humoral responses to the vaccine, due to treatment-related B-cell depletion [235,236,237]. By binding to CD20, RTX – a chimeric monoclonal antibody – triggers mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and induction of apoptosis leading to B-cell depletion, with exclusion of pro-B cells, plasmablasts and plasma cells. Individuals who were vaccinated at least 3 months after stopping RTX therapy showed higher antibody responses, likely due to the gradual reconstitution of the B-cell compartment after treatment discontinuation [127]. However, no correlation between time since last RTX administration and antibody titers was observed across the entire cohort, except in patients who did not receive bendamustine in conjunction with RTX. While Ishio et al. demonstrated that antibody titers correlated with the time elapsed from the last anti-CD20 antibody treatment to vaccination in patients with B-cell lymphoma, they also found a correlation with the period since the last bendamustine treatment and serum IgM levels [238]. These findings suggest that both RTX and the type of chemotherapy influence the recovery of B cells and subsequent humoral responses to vaccination. Bendamustine, frequently used in combination with RTX for treating indolent B-cell lymphoma, has been associated with more profound myelosuppression, including lymphopenia, compared to other chemotherapy regimens like R-CHOP [239,240,241,242,243]. Bendamustine is a chemotherapeutic agent combining alkylating and antimetabolite properties [244]. While its alkylating effects resemble those of cyclophosphamide and chlorambucil, its benzimidazole ring structure is similar to cladribine. Unlike conventional alkylators, bendamustine disrupts DNA repair and cell cycle progression more effectively and induces cell death through both apoptotic and non-apoptotic pathways in rapidly dividing cells, including lymphocytes. Patients treated with RTX and bendamustine exhibited lower or absent humoral responses to the mRNA vaccine and lacked the correlation between time since RTX administration and antibody titers observed in patients treated with other chemotherapy regimens. This observation indicates a more prolonged and potent immunosuppressive effect on the B-cell compartment in patients receiving bendamustine, even after a prolonged period following treatment. Robust Spike-specific CD4⁺ T cell responses were observed after two vaccine doses, comparable to those seen in healthy subjects. In a subset of patients tested, CD4⁺ T cell responses against the Omicron variant were found to be maintained. Interestingly, the T cell response was not significantly impacted by RTX or chemotherapy regimens, including those involving bendamustine. In fact, patients treated with RTX and bendamustine showed similar frequencies of Spike-specific CD4⁺ T cells compared to those treated with other regimens, suggesting that T cell responses may partially compensate for the lack of humoral immunity in these patients. However, the T cell

response was measured in terms of frequency rather than absolute cell numbers, which may be lower in immunocompromised patients due to the lymphopenia commonly induced by bendamustine. This may provide an explanation for why patients treated with RTX and bendamustine are more susceptible to severe COVID-19 and other infectious pathogens, despite having comparable T cell responses, as their overall immunosuppression is more profound [245].

Similarly to patients with hematological malignancy treated with bendamustine, solid-organ transplant recipients under antimetabolite maintenance therapy (mycophenolate mofetil or azathioprine) displayed reduced antibody response after vaccination. Spike-specific B and CD4+ T cell responses appeared to be unaffected by immunosuppressive therapies. However, lung transplanted patients not receiving antimetabolite therapy exhibited higher Spike-specific humoral and cell-mediated immune responses 1 month after the second vaccine dose compared to kidney or lung transplanted patients on antimetabolite treatment. Antimetabolites inhibit the proliferation of lymphocytes by interfering with nucleotide synthesis, thereby impairing B-cell expansion and leading to reduced antibody production following vaccination. Considering additional vaccine doses, despite the observed increase in antibody levels and Spike-specific B cell response after the third or fourth vaccine dose, no significant changes were detected in Spike-specific CD4+ T cells up to one month post-booster. Although both humoral and cell-mediated vaccine-induced immune responses were lower in patients receiving antimetabolite therapy, the incidence and severity of breakthrough infections were comparable irrespective of the treatment received. Previous studies have addressed the blunted vaccine-induced immunity in solid-organ transplant recipients, highlighting the improved immunogenicity of additional vaccine doses and the significant impact of mycophenolate compared to calcineurin inhibitors and mTOR inhibitors [122,246,247]. The novelty of the current study is its focus on a specific cohort of patients with cystic fibrosis, either post-lung transplantation or prior to needing organ replacement. Cystic fibrosis is a genetic disease caused by mutations in cystic fibrosis transmembrane conductance regulator (CFTR) gene, primarily affecting the lungs and leading to recurrent respiratory infections, progressive lung damage due to muco-obstructive disease, and, in advanced stages, the need for lung transplantation [248,249]. The age difference between the two groups of patients, exceeding 20 years, corresponds to a longer disease duration leading to end-stage lung disease and the need for transplantation, which likely contributes to greater frailty in the transplanted group. In comparison to lung-transplanted cystic fibrosis patients, non-transplanted patients exhibited higher antibody levels, though this was not mirrored by a corresponding increase in Spike-specific B cells; indeed, antibody production reflects the functional activity of B cells and may not always directly correlate with the quantity of B cells. Comprehensively, these observations demonstrate that the inclusion of antimetabolites in immunosuppressive regimens reduces the ability to mount both humoral and cell-mediated responses to vaccination, even after a booster dose. Nonetheless, vaccination proved to be effective in preventing severe disease in immunocompromised individuals. Despite the limited sample size, the differences observed between immunosuppression regimens (specifically the use of antimetabolites) warrant careful consideration when developing vaccination strategies, with the need for additional interventions to

improve vaccine-induced immunity in immunocompromised patients. Potential strategies may include adjusting the vaccination schedule, administering additional vaccine doses, or modified vaccination schedules in relation to the immunosuppressive treatment. However, further research is needed to determine whether these interventions would enhance protection. Vaccination efficacy improves as the level of immunosuppression decreases. However, suspending or interrupting maintenance therapy in solid-organ transplant recipients is generally not feasible, making the administration of additional COVID-19 vaccine doses a recommended strategy to enhance immunity. Patients treated with chemotherapeutic regimens that include bendamustine are particularly at risk for severe COVID-19 infection, hence they necessitate close monitoring for early diagnosis and prompt management in case of exposure. Additional COVID-19 vaccine doses are recommended in patients with prior or active hematological malignancies, as they may have a suboptimal vaccine response due to both disease-associated and therapy-related immune dysfunction.

Together with iatrogenic immunosuppression, one of the most common causes of secondary immunodeficiency is HIV infection [250]. Considering the increased risk of developing severe COVID-19 in people living with HIV (PLWH), additional vaccine doses have been proposed during the pandemic in this population [152]. This project focused on the evaluation of the effects of a fourth vaccine dose in a cohort of PLWH with no previous history of infection. Anti-Spike IgG and neutralizing antibodies levels resulted above the cut-off value at all time points of analysis, suggesting that humoral immunity in PLWH persists over time. Interestingly, while initial increases in anti-RBD IgG levels were observed, a decline was noted two months after the fourth dose in the majority of cases. In detail, two patients who showed an increase in anti-RBD IgGs after the fourth dose were also positive for anti-Nucleoprotein IgGs, indicating likely SARS-CoV-2 infection within the study period. Despite this observation, their total anti-Spike IgG levels followed the same downward trend as the rest of the cohort. However, anti-Spike IgGs were higher than anti-RBD IgGs, likely reflecting a broader antibody response targeting the entire Spike protein. Notably, these patients did not report any symptoms, underscoring the effectiveness of the additional vaccine dose in preventing severe COVID-19. A rapid reactivation of Spike-specific B and CD4⁺ T cells was observed 7 days after the fourth vaccine dose, followed by a contraction 2 months post-vaccination. Given that circulating PBMCs were analyzed, it is possible that the CD19⁺ B cells responding to vaccination or infection migrated to secondary lymphoid organs, particularly in individuals positive for anti-Nucleoprotein IgGs. The evaluation of the adaptive immune responses following a fourth vaccine dose revealed a robust Spike-specific immune reactivation in PLWH. Despite the emergence of new variants of concern, including Omicron, vaccinated individuals generally experienced milder disease courses compared to unvaccinated subjects [251]. This suggests that while vaccine-induced immunity may not fully prevent infection or its early resolution, it offers protection against severe COVID-19. T cell reactivity to Spike epitopes containing Omicron-specific mutations was evaluated two months after the fourth dose, showing that CD4⁺ T cells primarily responded to conserved regions of the Spike protein [228]. Minimal activation occurred in response exclusively to mutated

antigens, indicating a low frequency of cross-reactive T cells capable of recognizing mutated Spike epitopes. To assess B cell memory, a staining protocol to identify memory B cells capable of binding both the Wuhan and Omicron Spike proteins was used. A high frequency of memory B cells bound to both the Wuhan and Omicron Spike proteins, while a smaller percentage bound only to the WT Spike. Although isolated mutated Spike regions were not tested, these findings suggest that vaccine-induced memory B cells may recognize both conserved and possibly mutated regions of the Omicron Spike protein, consistent with previous reports [252].

The evolution of mRNA COVID-19 vaccines reflects a strategic response to the emergence of SARS-CoV-2 variants. Initially, mRNA vaccines were monovalent, targeting the ancestral Wuhan SARS-CoV-2 strain. In September 2022, bivalent formulations were introduced, incorporating antigens from both the wild-type strain and the BA.4/BA.5 Omicron subvariants, addressing the increased prevalence of Omicron-lineage variants. These bivalent vaccines became the exclusive mRNA formulations by April 2023. In September 2023, the strategy shifted back to a monovalent approach, targeting the XBB.1.5 Omicron variant [253]. By August 2024, updates were made to target the KP.2 strain, a descendant of the JN.1 sublineage, which had been circulating during the summer and early fall of 2024. The COVID-19 vaccines for the 2024-2025 season are monovalent JN.1-lineage vaccines [254,255].

New infections with SARS-CoV-2 continue even five years after the initial outbreak, despite significant advancements in vaccines, antiviral therapies, and preventive public health measures [256]. This project underscores the critical importance of actively monitoring immunity against SARS-CoV-2 to inform and optimize vaccination strategies. Despite limitations, such as the relatively small sample size, the follow-up duration and the focus on the original monovalent mRNA vaccines, the project provides a thorough evaluation of both humoral and cell-mediated immune responses induced by SARS-CoV-2 mRNA vaccines across diverse populations, including immunocompetent and immunocompromised individuals. The focus on the two arms of adaptive immunity distinguishes this work from the majority of studies, which predominantly focus on antibody responses. By adopting this broader perspective, the study contributes a holistic understanding of vaccine-induced immunity across time as well as its relevance to varied patient populations, including vulnerable individuals. It provides critical insights into the durability of vaccine-induced immunity in terms of circulating antibodies and Spike-specific T and B cells analyzing the effect of additional vaccine doses. Comprehensively, this work contributes to the existing body of research by (a) evaluating populations with diverse immune status, addressing a critical knowledge gap in vaccine efficacy and optimization for at-risk groups; (b) identifying factors, such as immunosuppressive therapies, that offer actionable insights into managing vaccination schedules and treatment plans by developing personalized vaccination approaches; (c) emphasizing the importance of third and fourth vaccine doses, particularly in naïve and immunocompromised individuals, ensuring more precise and evidence-based guidance to evolving booster dose recommendations and public health policies; (d) providing detailed description of Spike-specific T cell-mediated immunity, demonstrating its significance in populations with impaired humoral

response. Continuous assessment of immune responses is essential to ensure the effectiveness of both humoral and cell-mediated immunity, particularly in the context of emerging variants of concern and in immunocompromised populations. Future research should prioritize the evaluation of the dynamic interplay between antibody-mediated and T cell-mediated responses by establishing long-term surveillance systems. The potential for immune evasion by viral variants as well as the assessment of immunity elicited by adapted mRNA vaccines need to be taken into consideration. Future studies will be instrumental in refining vaccination protocols, including the timing and composition of booster doses, to maintain robust and comprehensive protection against SARS-CoV-2.

6. CONCLUSIONS

Anti-SARS-CoV-2 mRNA vaccines induce long-term adaptive immunity. Immunological memory induced by vaccination shows similar decay kinetics to that observed following natural infection. In individuals who have recovered from COVID-19, particularly those who had symptomatic infections, one mRNA vaccine dose is sufficient to reactivate immunological memory. However, naïve individuals, with no prior history of SARS-CoV-2 infection, require two mRNA vaccine doses to generate a strong immune response. Additionally, a third vaccine dose is important for naïve individuals, as it not only restores but also enhances anti-Spike immunity to levels similar to those observed in vaccinated COVID-19 recovered individuals. In patients with B-cell lymphoma receiving rituximab therapy, anti-SARS-CoV-2 mRNA vaccination induces a weak or absent humoral response, but a strong Spike-specific CD4+ T cell response. Bendamustine-containing chemotherapy further impairs the ability to mount a humoral response, even after prolonged discontinuation of treatment, and it is associated with the severity of breakthrough infections in patients with B-cell lymphoma. Similarly, in solid-organ transplant recipients, immunosuppressive regimens containing antimetabolites lead to diminished humoral and cell-mediated immune responses following vaccination. In people living with HIV and lower CD4+ T cell counts, a fourth vaccine dose transiently boosts Spike-specific adaptive immunity with cross-reactive CD4+ T cell and B cell responses against the Omicron variant. In conclusion, the active long-term monitoring of immunity against SARS-CoV-2, whether induced by infection or vaccination, has proven to be essential for informing and optimizing vaccination strategies in both immunocompetent and immunocompromised individuals.

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