DISSERTATION

The response of the cellular immune system to vaccination – focus on COVID-19 vaccination

submitted by

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Graz, 22nd August 2025 *Dr. med. univ. Isabel Hodl, m.p.*

Disclosures

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I hereby confirm that all co-authors have agreed to the inclusion of the data of the above-mentioned publications in this dissertation and have given their written consent. Permission to reproduce illustrations and figures from these two publications has been granted by the respective copyright holders: Frontiers Media SA and Institut Pasteur, Elsevier Masson SAS.

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Abbreviations and Definitions

AID	Autoimmune disease
ALPS	Autoimmune lymphoproliferative syndrome
ANCA	Anti-neutrophil cytoplasmic antibody
APC	Antigen-presenting cells
AUC	Area under the curve
AZA	Azathioprine
BAU	Binding Antibody Units
BCR	B-cell receptor
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CI	Confidence interval
CLL	Chronic lymphocytic leukemia
CMV	Cytomegalovirus
COVID-19	Coronavirus disease 2019
CTD	Connective tissue diseases
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CVID	Common variable immunodeficiency
DMARD	Disease-modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
GC	Germinal center
GvHD	Graft-versus-host disease
GzmB	Granzyme B
HC	Healthy control
HCQ	Hydroxychloroquine
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation

IEI	Inborn error of immunity
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGRT	Immunoglobulin replacement therapy
IIM	Idiopathic inflammatory myopathy
IL	Interleukin
IQR	Interquartile range
IUIS	International Union of Immunological Societies
JAK	Janus kinase
LNP	Lipid nanoparticle
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MM	Multiple myeloma
MMF	Mycophenolate mofetil
MS	Multiple sclerosis
MTX	Methotrexate
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHL	Non-Hodgkin lymphoma
NMOSD	Neuromyelitis optica spectrum disorder
OCR	Ocrelizumab
PBMC	Peripheral blood mononuclear cells
PID	Primary immunodeficiency
PIK3CD	Phosphoinositide 3-kinase catalytic subunit δ
PMA	Phorbol 12-myristate 13-acetate
RA	Rheumatoid arthritis
RBD	Receptor-binding domain
RDA	Research, Documentation and Analysis
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
ROC	Receiver operating characteristic
RSV	Respiratory syncytial virus
RTX	Rituximab
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

SD	Standard deviation
SFCs	Spot-forming cells
SHM	Somatic hypermutation
SID	Secondary immunodeficiency
SLE	Systemic lupus erythematosus
SSc	Systemic sclerosis
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TEMRA cells	Effector memory T cells re-expressing CD45RA
Tfh cell	T follicular helper cell
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
unPAD	Unclassified primary antibody deficiency
VLPs	Virus-like particles
XLA	X-linked agammaglobulinemia

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Zusammenfassung

Immundefiziente Patient*innen sind besonders anfällig für schwere Infektionen und waren während der COVID-19-Pandemie einem erhöhten Risiko ausgesetzt. Als 2021 die ersten COVID-19-Impfstoffe verfügbar wurden, war unklar, ob diese Patient*innen eine effektive Immunantwort entwickeln und von der Impfung profitieren würden.

Zur Beantwortung dieser Frage initiierten wir die prospektive, multidisziplinäre CoVVac-Studie mit Patient*innen mit primären Immundefekten, sekundären Immundefizienzen infolge hämatologischer Malignome oder B-Zell-Depletion bei Autoimmunerkrankungen sowie gesunden Kontrollen. Ziel war es, humorale und zelluläre Immunantworten zu charakterisieren, prädiktive Biomarker zu identifizieren und klinische Impfempfehlungen abzuleiten.

Insgesamt 372 Proband*innen wurden über sechs Studienvisiten begleitet: vor der ersten COVID-19-Impfung (Baseline), nach der ersten sowie zweiten Dosis und bei weiteren Follow-ups. Es wurden Lymphozytensubsets, SARS-CoV-2-Antikörpertiter sowie die T-Zell-Reaktivität mittels IFN-γ ELISpot und Durchflusszytometrie analysiert.

Immundefiziente Patient*innen wiesen signifikant niedrigere Serokonversionsraten im Vergleich zu Gesunden auf, am ausgeprägtesten unter B-Zell-depletierender Therapie. Die Zahl naiver B-Zellen korrelierte stark mit den Antikörpertitern und konnte mit einem Schwellenwert von ≥ 60 naiven B-Zellen pro µl zuverlässig eine robuste humorale Antwort voraussagen. Trotz humoraler Immundefizienz entwickelten Patient*innen mit Autoimmunerkrankungen unter B-Zell-Depletion relevante T-Zell-Antworten; jedoch waren die TNF-α-Produktion durch $CD4^+$- und Granzym-B-Produktion durch $CD8^+$-T-Zellen signifikant reduziert und das Zytokin-Korrelationsmuster gestört. Im Gegensatz dazu zeigten Patient*innen mit primärem Immundefekt vergleichbare T-Zell-Antworten wie gesunde Kontrollen.

Diese Arbeit zeigt, dass SARS-CoV-2-mRNA-Impfstoffe auch bei schwerer humoraler Immundefizienz effektive T-Zell-Antworten induzieren können und daher empfohlen werden sollten. Zudem erwies sich die Zahl naiver B-Zellen als praktischer Biomarker zur Vorhersage der Serokonversion. Weitere Studien sind erforderlich, um krankheitsspezifische Unterschiede sowie Dauer und klinische Wirksamkeit T-Zell-vermittelter Impfantworten zu untersuchen – mit dem Ziel, verlässliche klinische Schutzkorrelate zu definieren.

Abstract

Immunocompromised patients are particularly vulnerable to severe infections and were exposed to an enhanced threat during the COVID-19 pandemic. When the first COVID-19 vaccines became available in 2021, it remained unclear whether these patients would benefit from vaccination by inducing effective immune responses.

To address this, we initiated the prospective, multidisciplinary CoVVac study, enrolling patients with primary immunodeficiencies and secondary immunodeficiency due to hematologic malignancies or B-cell depletion in autoimmune patients, along with healthy controls. Our aims were to characterize humoral and cellular immune responses, to identify predictive biomarkers, and to derive clinical vaccination recommendations.

A total of 372 participants were followed across six study visits: baseline before any COVID-19 vaccination, post-first and -second dose, and longitudinal follow-ups. We performed lymphocyte subset phenotyping, quantification of SARS-CoV-2 antibody titers, and T-cell reactivity assays using IFN-γ ELISpot and flow cytometry.

Immunocompromised patients showed significantly lower seroconversion rates compared to healthy controls, with patients receiving B-cell-depleting therapy exhibiting the lowest anti-SARS-CoV-2 antibody levels. The number of circulating naïve B cells was strongly associated with antibody levels, and a threshold of ≥60 naïve B cells per µL predicted robust humoral responses. Despite humoral deficits, patients with autoimmune diseases under B-cell-depleting therapy mounted notable T-cell responses; however, TNF-α production by $CD4^+$ T cells and Granzyme B production by $CD8^+$ T cells were significantly reduced, and the correlation pattern of cytokines and activation markers was disturbed in these patients. In contrast, patients with primary immunodeficiency exhibited T-cell responses comparable to healthy controls.

This work highlights that even in the presence of severe humoral immunodeficiency, SARS-CoV-2 mRNA vaccinations can elicit notable T-cell responses and should therefore be encouraged. Furthermore, the naïve B-cell count proved to be a practical biomarker for predicting vaccine-induced antibody responses. Future studies are needed to assess the fine differences between disease entities as well as the durability and clinical effectiveness of vaccine-induced T-cell responses, in order to ultimately define correlates of protection.

1. Introduction

1.1. SARS-CoV-2 and its relevance for medicine and society

Since its emergence in late 2019, the COVID-19 pandemic has caused profound health, societal, and economic impacts worldwide. By May 2023, when the World Health Organization (WHO) declared the end of the COVID-19 global health emergency, more than 765 million cases with nearly 7 million deaths had been reported (1). Since then, case numbers have continued to rise; however, due to milder disease courses, among other reasons, only a fraction of infections is still officially registered (2). Beyond the burden of the acute disease, millions of people have experienced – or are still chronically suffering from – post-COVID syndrome, with widely varying estimated prevalence numbers (3).

COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), an enveloped, positive-sense single-stranded RNA betacoronavirus (4). Its viral genome encodes canonical structure proteins, including the spike (S), membrane (M), and envelope proteins, as well as the nucleocapsid (N), which are essential for virus assembly and entry into host cells (4).

Infected individuals may remain asymptomatic or present a variable set of symptoms, most commonly fever and respiratory symptoms, such as dry cough or dyspnea, and fatigue. But also other symptoms, such as loss of taste and smell, myalgia, nausea, and diarrhea, are frequently reported (5). However, it needs to be noted that over the time course of the pandemic, new SARS-CoV-2 virus variants have continuously emerged. As a consequence, typical symptoms have changed and while the transmissibility increased, the overall severity declined (6).

After governments around the globe imposed nation-wide lockdowns and intensified hygiene measures to contain further dissemination of the virus, the demand for an effective and sustainable prophylaxis grew rapidly. As the clinical course of COVID-19 strongly depends on the quality of the host immune response, the goal was to generate a broadly immunogenic vaccine. Within less than a year, researchers developed SARS-CoV-2-specific vaccines, and in December 2020, the first COVID-19 vaccine, Comirnaty® (BioNTech/Pfizer), was authorized for use in both the European Union and the United States of America (7,8).

1.2. Immunological principles of vaccination

1.2.1. Rationale and aims of vaccination

Vaccination aims to induce a controlled and pathogen-specific immune response that mimics natural infection but does not cause the disease. Ideally, it generates a long-lasting immunological memory that protects individuals upon future exposure to the pathogen – without requiring frequent booster doses or posing a high risk of adverse effects (9). Furthermore, economic and practical demands must be met: For worldwide use, vaccines should be cost-effective and stable throughout long transport routes, even under changing environmental conditions such as heat and humidity.

Another key objective of vaccines, particularly relevant in the context of pandemics, is to achieve herd immunity – meaning that further transmission of a disease is limited by reducing the number of susceptible hosts within a population (10). This concept is crucial to protect vulnerable groups who cannot be vaccinated or who may not develop robust immune responses, such as immunodeficient patients or newborns. Epidemiologic estimates for herd immunity thresholds differ by disease and are approximately 92–94% for measles and 50–75% for COVID-19, depending on the virus variant (10).

Finally, vaccination works by either active or passive immunization – with passive immunization meaning the administration of preformed specific antibodies for immediate protection, and active immunization referring to the generation of a long lasting adaptive immune response in the recipient (11).

1.2.2. Components of the adaptive immune response

A functional adaptive immune system is the prerequisite for effective immune responses to many pathogens and also for establishing protection through vaccination. It requires the coordinated involvement of both the humoral and the cellular arms, as outlined in the following.

Antigens, such as virus proteins, whole virus particles or (other) vaccine components, are taken up and processed by dendritic cells in peripheral tissues and subsequently presented to naïve T cells in secondary lymphoid organs via major histocompatibility complex (MHC) molecules (12). If a naïve $CD8^+$ T cell recognizes the presented peptide via MHC class I, it is activated as

a cytotoxic effector T cell (13). If a naïve $CD4^+$ T cell recognizes the presented peptide via MHC class II on the antigen-presenting cell (APC), it is activated as a T helper cell (14). The differentiation of $CD4^+$ T cells into functionally distinct effector subsets, such as Th1, Th2 and Th17 cells, is directed by the local cytokine milieu, which is modulated by the nature of the pathogen/antigen and thus by the innate immune system and their actions (15). Furthermore, activated $CD4^+$ and $CD8^+$ T cells can differentiate into long-lived memory T cells that are capable of mounting rapid responses upon re-exposure to the same antigen (16).

In secondary lymphoid organs, naïve B cells scan for intact antigens using their B-cell receptor (BCR). They may recognize both free native antigens and bound antigens that are presented by follicular dendritic cells (17). Upon recognition and uptake of the corresponding antigen, the B cell presents processed peptides via MHC class II. For full activation of the B cell, additional co-stimulatory signals are required. These are provided by antigen-specific $CD4^+$ T cells, in particular T follicular helper (Tfh) cells, via CD40–CD40L interaction and secretion of cytokines, such as IL-21 (17). Tfh cells also play a central role in germinal center reactions, where they interact with activated B cells to promote affinity maturation through somatic hypermutation and to facilitate immunoglobulin class switching (17). B cells with high-affinity BCRs undergo clonal expansion and differentiate into antibody-secreting plasma cells; a subset is retained as memory B cells that can rapidly respond upon re-exposure to the same antigen (17).

In addition, T-cell independent antibody production can be mediated by marginal zone B cells in the spleen or by B-1 cells in the peritoneal and pleural cavities. This response is mainly restricted to the secretion of low-affinity IgM against repetitive (bacterial) polysaccharide antigens and does not result in a robust immunological memory (17).

1.2.3. Overview of vaccine technologies and their immunological characteristics

The development of vaccines has a long history, beginning with the use of live naturally attenuated pathogens – such as the cowpox virus applied by Edward Jenner in 1796 to protect individuals against smallpox (18). Since then, a variety of vaccine platforms have been established for the prevention of various infectious diseases.

Classic or conventional platforms comprise live-attenuated, inactivated, protein-based subunit, and conjugate vaccines. Live-attenuated vaccines are produced from artificially weakened pathogens, i.e., their virulence is diminished while still mimicking the natural infection (19). Thereby, strong immune responses involving the innate as well as the humoral and cellular adaptive immune system can be elicited and usually one or two doses are sufficient for lifelong immunity (20). However, there is a risk of pathogen amplification and disease outbreak, especially in immunocompromised individuals. Therefore, live-attenuated vaccines are contraindicated in many immunodeficient patients (21). Widely used examples for this vaccine type are the measles, mumps, and rubella virus vaccine MMR-Vax Pro®, the varicella virus vaccine Varivax®, and the yellow fever vaccine Stamaril® (19,22).

The safer counterpart to live-attenuated vaccines are whole inactivated vaccines, in which the pathogens have lost their ability to replicate or mutate (19). Inactivation is typically achieved through physical or chemical treatment, such as exposure to formaldehyde (19). However, a main disadvantage of inactivated vaccines is their limited capacity to stimulate cellular immune responses, as the non-replicating pathogens do not enter the cytosol and therefore fail to elicit efficient $CD8^+$ T-cell responses via MHC class I antigen presentation (23). Nevertheless, immunogenicity can be enhanced by higher antigen doses, repeated booster injections, and the inclusion of adjuvants in the formulation – such as aluminum salts – that activate the innate immune system and promote the migration of antigen-presenting cells (APCs) (19,24). Examples of common inactivated vaccines include the hepatitis A vaccine Havrix® and the Japanese encephalitis vaccine Ixiaro® (19).

A similar concept is pursued by subunit vaccines, which contain only the immunologically relevant components of the pathogen, such as proteins, inactivated toxins, or carbohydrates, usually in combination with adjuvants (20). Widely used examples include the hepatitis B vaccine Engerix®, which contains the hepatitis B surface antigen (HBsAg); the diphtheria, tetanus, and pertussis vaccine Boostrix®, composed of diphtheria and tetanus toxoids and a detoxified pertussis toxin; and the typhoid polysaccharide vaccine Typhim Vi® (19,20,22).

In addition to adjuvants, the immunogenicity of subunit vaccines can be further enhanced by protein conjugation. This approach is especially relevant for polysaccharide antigens, as these are not presented via MHC molecules and therefore do not elicit T-cell responses (19). Moreover, in infants, marginal zone B cells – the primary B-cell subset responsible for reaction

to polysaccharides – are still immature and incapable of mounting sufficient IgM responses (19). Covalently linking a carrier protein to a polysaccharide enables the uptake of both components by a B cell that recognizes the polysaccharide antigen. Through the mechanism of linked recognition, a $CD4^+$ T cell binding a peptide from the carrier protein, presented via MHC II on the same B cell, can provide the co-stimulatory signals required for B cell activation (11). This converts a previously T-cell-independent vaccine into a T-cell-dependent one, thereby also enabling immunization in young children. Widely used examples are the pneumococcal conjugate vaccines Prevenar 20® and Vaxneuvance®, as well as the meningococcal conjugate vaccine Nimenrix® (19,22).

Another conventional vaccine platform is the use of virus-like particles (VLPs). These non-infectious particles are engineered to mimic the morphological structure of native viruses and are produced by transfecting cells of various origins – such as bacterial, yeast, plant, mammalian, or insect cells – with genes encoding selected viral proteins (19). Their design aims to induce potent antibody responses by efficiently targeting B cells and activating $CD4^+$ T cells through MHC class II presentation (19). Moreover, their particulate structure facilitates cross-presentation of VLP-derived peptides via MHC I molecules on antigen-presenting cells, thereby enabling $CD8^+$ T-cell activation (19). Current licensed vaccines containing VLPs are the human papillomavirus vaccines Gardasil 9® and Cervarix® (19,22).

Finally, synthetic peptide vaccines represent a conceptual bridge between conventional and modern vaccine designs. In this approach, short immunodominant peptides of a pathogen are synthesized chemically and conjugated to an adjuvant to ameliorate their uptake by antigen-presenting cells and the subsequent immune response (19). The advantages of this platform lie in its safety profile and the ability to rapidly modify peptide sequences. Nevertheless, due to the small size of the peptides and their restriction to a limited number of epitopes, the breadth of the immune response is often diminished (19). In addition, the response to peptides might induce cross-reactive antibodies targeting host tissue (19). Currently, the application of synthetic peptide vaccines remains largely experimental – not only for the prevention of infectious diseases, such as human immunodeficiency virus (HIV), malaria, hepatitis C, or influenza, but also as therapeutic vaccine for cancer or Alzheimer's disease (25).

As classical technologies have not proven effective against all types of pathogens, and as some carry an inherent risk of reversion to higher virulence or require time-consuming production

processes – especially when rapidly evolving variants occur that pose a pandemic threat – new strategies have been developed (19). These include next-generation vaccine platforms, such as viral vector, synthetic DNA, and mRNA-based vaccines.

Viral vector vaccines are based on non-pathogenic or attenuated viruses that are engineered to express genes encoding antigens of the target pathogen, such as the spike protein of SARS-CoV-2 (19). These viral vectors infect cells and may be replication-competent, i.e. capable of inducing an infection, or replication-deficient and thus safer. This strategy generally induces robust humoral and cellular immune responses. However, preexisting immunity against the viral vector may compromise efficacy; therefore, vectors derived from non-human species (e.g., chimpanzees or pigs) are frequently used (19). A rising application of this vaccine type has emerged during the COVID-19 pandemic, in which the viral vector vaccines Jcovden® (Ad26.COV2-S) by Janssen and Vaxzevria® (ChAdOx1-S) by AstraZeneca, both of which are based on adenoviruses engineered to encode the SARS-CoV-2 spike glycoprotein, were administered widely (19,26,27).

Another platform evaluated in clinical trials during the COVID-19 pandemic are synthetic DNA vaccines (19,24). This approach involves the intramuscular injection of plasmid DNA, which may be taken up by myocytes, keratinocytes, and tissue-resident antigen-presenting cells (19). Once internalized, the DNA is transcribed into mRNA, translated into the target proteins, and the resulting antigens are presented on MCH class I and – depending on the cell type – also MHC class II molecules, leading to the induction of T-cell responses (19). B cells can be activated by recognizing the secreted protein in its native form, either freely circulating or displayed on the surface of follicular dendritic cells (19,28). Although highly unlikely, a potential safety concern involves the integration of synthetic DNA into the host genome. Therefore, to date, no vaccine of this type has been licensed for use in humans in the European Union or the United States, despite some veterinary applications (19).

Finally, the novel concept of mRNA vaccines will be described in the following section (1.2.4.), as it represents the technology of the first authorized and widely used COVID-19 vaccine in humans.

1.2.4. SARS-CoV-2 mRNA vaccines

Although the mechanistic basis for mRNA-based therapeutics was already developed in the late 1970s and 1980s (29,30), their breakthrough occurred during the COVID-19 pandemic, when an effective vaccine protecting from severe SARS-CoV-2 infection was urgently needed.

The principle of mRNA-based vaccines lies in the introduction of an mRNA transcript encoding one or more target antigens into host cells, e.g., via intramuscular injection, as in SARS-CoV-2 vaccination, and release of the mRNA from the lipid nanoparticle (LNP) into the cytosol (31,32). The mRNA is translated into polypeptides by host cell ribosomes, and resulting peptides are presented on MHC class I molecules of the expressing cell, while secreted proteins can be taken up by antigen-presenting cells and presented on MHC class II molecules (32). In addition to the induction of adaptive immune responses against the target protein, such as the SARS-CoV-2 spike protein, by activation of B and T cells in the draining lymph nodes (see 1.2.2.), mRNA vaccines are capable of activating multiple cellular pathways of innate immunity reacting to foreign RNA (32). This already occurs shortly after injection of the vaccine, causing local inflammation, which attracts more immune cells, such as neutrophils, monocytes, and dendritic cells, to the injection site. These cells can take up the LNPs containing mRNA, which then stimulates cytosolic pattern recognition receptors, including toll-like receptors (TLRs) 3, 7, and 8, as well as retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), leading to secretion of type I interferons (IFNs) (24,32). Type I IFNs favor the subsequent development of a Th1 rather than a Th2 response, which is particularly effective for antiviral immunity (32,33).

The manufacturing process of mRNA vaccines includes, first, the design of the DNA sequence, followed by high cell-density fermentation and gene synthesis, then harvesting, lysis, and purification of cells to gain DNA plasmids (19). From these, mRNA is produced by linearization of the DNA plasmids and use of an RNA polymerase in an in vitro transcription enzymatic process (19).

Early problems of this technology included instability of the mRNA, reduced translation rates in vivo, and high inflammation, limiting their applicability as therapeutic (19). Over the following years of research, advances in safety and efficacy could be reached by purification processes removing dsRNA, by optimizing RNA sequences and 5’ caps, by including modified nucleosides, and by utilizing LNPs surrounding and protecting the sensitive mRNA (19,24). In

2012, an mRNA vaccine against influenza A was developed and tested in mice, ferrets, and pigs – being the first mRNA vaccine for an infectious disease – and induced B- and T-cell responses (33,34). Experimental mRNA vaccines against various other pathogens, such as Ebola virus, cytomegalovirus (CMV), and human immunodeficiency virus (HIV), followed subsequently (33).

However, the authorization of the first mRNA-based vaccines for human use, Comirnaty® (BNT162b2, by BioNTech/Pfizer) and SpikeVax® (mRNA-1273, by Moderna), in December 2020 marked the greatest milestone so far (7,8), with millions if not billions of administered doses worldwide in the last years (35). Both vaccines consist of LNPs containing an mRNA that encodes the entire SARS-CoV-2 spike protein, but with two proline substitutions in the S2 subunit, which stabilize the spike protein in its prefusion conformation, i.e., the stable and immunogenic conformation the protein adopts before fusing with the host cell membrane (33).

The rapid efficacy of SARS-CoV-2 mRNA vaccines resulted from the induction of high titers of neutralizing antibodies and T-cell responses in wide proportions of the population, including elderly people and other vulnerable patient groups (33). Further advantages of this technology – and also reasons for their rising popularity in vaccine design – are their cost-effectiveness, feasibility of production and rapid adaption, and safety profile with only transient expression of the foreign mRNA (24). However, during the COVID-19 pandemic and their broad administration, many people in the public became skeptical or even refused mRNA vaccination due to personal safety concerns, e.g., due to unjustified concerns about reverse transcription and genomic integration (36) – which has only been observed in vitro in human hepatocellular carcinoma cell lines (37) but is highly biologically implausible in humans in vivo (38) – or fearing side effects such as myocarditis and pericarditis. These inflammatory diseases were reported especially in a small number of young, often male adults following COVID-19 mRNA or viral vector vaccination (33), with an incidence of 0.004–0.007% of vaccinated individuals (39,40). Nevertheless, it was also shown that the risk of myocarditis is greater after SARS-CoV-2 infection than following COVID-19 vaccination (40). Although no major safety concerns for these mRNA vaccines have been confirmed, a rising distrust of vaccination in general has emerged, possibly due to broader societal and political reasons originating from the time of pandemic (41). Another disadvantage of particularly LNP-based vaccines is their low thermostability requiring ultra-cold storage; moreover, the lipids and cholesterol of the LNPs

may easily be degraded by oxidation, further limiting their stability and applicability in some underdeveloped countries (33).

Nevertheless, the rapid reduction in severe disease courses and transmission following the introduction of these vaccines in 2020/21 provides strong proof of concept for mRNA-based vaccines (33).

1.3. Immunodeficiencies and their impact on vaccination

Immunodeficient patients, both children and adults, represent particularly vulnerable patient groups. Depending on their specific underlying disease or immunosuppressive therapy, they may be prone to frequent, severe, and opportunistic infections. Consequently, effective protection against infection is crucial and should be ensured not only through increased hygiene measures but also by prophylactic vaccination strategies. However, not all types of vaccines are suitable for or effective in these patients. This chapter will outline the relevant types of immunodeficiency, focusing on those included in our study, and their implications for vaccine responsiveness.

1.3.1. Definition and classification of immunodeficiencies

Immunodeficiencies are defined as primary or secondary. Primary immunodeficiencies (PID) are a heterogeneous group of diseases also referred to as inborn errors of immunity (IEI), signifying that they are predetermined at birth via known or unknown genetic causes (42). They do not necessarily manifest in childhood but may evolve later in life (42). As they are relatively rare conditions, prevalence data vary considerably due to underreporting, underdiagnosis, and regional differences. However, a recent study evaluating electronic health record data in the United States calculated a prevalence of six IEI cases per 10,000 individuals (0.056%) (43). Of note, there are more than 500 distinct IEIs with widely varying prevalences per specific diagnosis (44). They are classified by the International Union of Immunological Societies (IUIS) Expert Committee, which publishes an update approximately every two years – the most recent version in 2024 (44). This latest update includes 508 causative genes and 17 phenocopies, along with clinically defined IEIs of unknown genetic background. They are categorized into

10 groups with further subdivisions, listed below with the number of entities per main category in brackets (44):

1. Immunodeficiencies affecting cellular and humoral immunity (n = 73)
2. Combined immunodeficiencies with associated or syndromic features (n = 83)
3. Predominantly antibody deficiencies (n = 48)
4. Diseases of immune dysregulation (n = 72)
5. Congenital defects of phagocyte number or function (n = 45)
6. Defects in intrinsic and innate immunity (n = 86)
7. Autoinflammatory disorders (n = 69)
8. Complement deficiencies (n = 36)
9. Bone marrow failure (n = 47)
10. Phenocopies of IEIs associated with autoantibodies or somatic variants (n = 17) (44).

Some of the most prevalent and well-known PIDs include selective immunoglobulin A (IgA) deficiency (category 3), common variable immunodeficiency (CVID, category 3), familial Mediterranean fever (FMF, category 7), X-linked agammaglobulinemia (XLA, category 3), and severe combined immunodeficiency (SCID, category 1) (45,46). Among these, selective IgA deficiency is by far the most common, with a reported prevalence of 1 in 300 to 1 in 500 individuals (45).

In contrast, secondary immunodeficiencies (SID) are acquired impairments of immune function, typically caused by underlying diseases or therapies (42). Common causes of SID are infections such as HIV and measles virus infection, various medications, hematologic malignancies such as multiple myeloma (MM) and chronic lymphocytic leukemia (CLL), malnutrition, gastrointestinal or renal protein loss, and immunosenescence (47). Causative medications include numerous immune-modulating therapies, such as corticosteroids, chemotherapeutic agents, B-cell-targeted therapies – e.g., rituximab, blinatumomab, and belimumab –, as well as other biologics – e.g., TNF-α inhibitor adalimumab and IL-6 receptor antagonist tocilizumab –, and chimeric antigen receptor (CAR) T-cell therapy (42,47,48). In addition, some non-immunosuppressive medications, including antiepileptics (e.g., carbamazepine, valproate) and antipsychotics (e.g., clozapine), may also induce a secondary immunodeficiency (47). Due to the wide range of possible causes, an overall prevalence for SID is difficult to determine. Nevertheless, it is well established that SID is by far more frequent

in adults than PID – with rising incidences, primarily caused by increasing numbers and availability of immunosuppressive drugs and by demographic changes (47). Just as the causes of SID vary, so do the consequences, which may range from a mildly increased susceptibility to specific infections to profound and long-lasting reductions in immune cell numbers and function accompanied by severe infections requiring hospitalization (48).

Intriguingly, in some cases, immunosuppressive therapies leading to a presumed SID may also unmask an underlying PID. This has been reported in patients with lymphoma or autoimmune diseases, both after receiving B-cell-depleting therapy (42).

1.3.2. Selected immunodeficiency subgroups relevant for vaccination

One of the most common primary immunodeficiencies (PIDs) mentioned in the previous section is common variable immunodeficiency (CVID), which typically manifests as an antibody deficiency after the fourth year of life (49). It is characterized by a marked decrease of IgG and IgA levels, sometimes accompanied by low IgM, and usually leads to recurrent upper respiratory and other infections. Some patients also suffer from autoimmunity (such as autoimmune cytopenias), granulomatous disease, and benign or malignant lymphoproliferation (49). Genetic causes of CVID include mutations in the Transmembrane Activator and CAML Interactor (TACI), Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB1 and NFκB2), and Phosphoinositide 3-Kinase Catalytic subunit δ (PIK3CD) but remain elusive in many cases (44). However, most CVID patients exhibit alterations in B-cell maturation and class switch recombination – often reflected by a reduction in switched memory B cells – which limits their capacity to produce high-affinity antibodies in response to vaccination (49). Impaired humoral vaccine responses are therefore considered one of the diagnostic criteria for CVID and are particularly observed after vaccination with polysaccharide-based vaccines, such as the pneumococcal vaccine Pneumovax 23® (PPSV23) (49). However, reduced responses are also seen following T-cell dependent vaccines, including seasonal influenza vaccines or combination vaccines containing diphtheria toxoid (49).

Hematologic malignancies may lead to secondary immunodeficiency (SID) either by mechanisms of the malignancy itself or through immunosuppression caused by necessary treatments, such as high-dose corticosteroids, B-cell-depleting therapies, hematopoietic stem

cell transplantation (HSCT), or other chemotherapeutic treatments – or by a combination of several of these factors (50). Diseases typically inducing SID are multiple myeloma (MM), chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma (NHL), and graft-versus-host disease (GvHD) following allogeneic HSCT (50). Mechanisms contributing to SID involve bone marrow infiltration by malignant cells and subsequent suppression of normal hematopoiesis – particularly in MM, leukemia, and myelodysplastic syndromes – as well as disturbed B-cell maturation due to monoclonal B cell expansion, resulting in a loss of antibody functionality and hypogammaglobulinemia – especially in CLL and MM (51,52). Depending on the extent and nature of immunosuppression by the malignancy and its treatment, vaccine responses in these patients can vary widely. In CLL patients, reduced seroconversion rates have been reported following pneumococcal vaccination (52). Similarly, multiple phase II and III vaccination trials in MM patients have demonstrated reduced protective antibody titers after vaccination against influenza, herpes zoster, pneumococcus (both polysaccharide-based and conjugate vaccines), and *Haemophilus influenzae* type B (53). Of note, although seroconversion rates are generally lower than in healthy individuals, some patients still achieve protective titers, as reported for 19% of MM patients in a trial with a trivalent influenza vaccine (54) and for 60% of MM patients (considering total IgG response) after receiving both a pneumococcal conjugate vaccine (PCV13) and a pneumococcal polysaccharide vaccine (PPV23), although antibody levels for all serotypes declined rapidly in the follow-up period (53,55).

In the majority of autoimmune diseases – such as rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis (MS), and systemic vasculitides – secondary immunodeficiency (SID) may arise not only from immune dysregulation caused by the disease itself but primarily as a consequence of the associated immunosuppressive therapies (48,56). Typical treatments include corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs), calcineurin inhibitors (e.g., ciclosporin), antimetabolites (e.g., azathioprine), and cytostatic agents (e.g., cyclophosphamide) (57). DMARDs can be further subdivided into conventional synthetic DMARDs, such as methotrexate and leflunomide; targeted synthetic DMARDs, such as the Janus kinase (JAK) inhibitors tofacitinib and baricitinib; and biological DMARDs, which comprise an expanding number of monoclonal antibodies directed against cytokines, receptors, and cell surface markers, such as anakinra (anti-IL-1), tocilizumab (anti-IL-6), infliximab (anti-TNF-α), secukinumab (anti-IL-17A), and rituximab (anti-CD20) (48,58). Depending on the mechanism of action, the resulting immunosuppression may be

broad, as in the case of cyclophosphamide, which is cytotoxic to lymphocytes and other dividing cells, or relatively mild and pathway-specific, as in the case of secukinumab, which selectively neutralizes IL-17A and thereby primarily affects Th17-mediated immune responses, leading to an increased susceptibility particularly to mucocutaneous candidiasis (48). Accordingly, vaccination responses may vary depending on the administered therapy. In patients with rheumatoid arthritis, it has been shown that the humoral response to influenza vaccination may be reduced by abatacept, a CTLA-4 immunoglobulin blocking T-cell co-stimulation by APCs, but not necessarily by methotrexate, anti-TNF-α therapy, tocilizumab, or tofacitinib (56). Moreover, in these patients, immunogenicity of pneumococcal vaccines has been reported to be diminished by methotrexate and tofacitinib, but not by tocilizumab or anti-TNF-α agents (56). Regarding pneumococcal vaccination in patients with various autoimmune diseases, including inflammatory bowel disease, rheumatoid arthritis, and spondyloarthritis, studies have shown that patients receiving TNF-α blocking therapy generally exhibit more favorable humoral responses than those under other immunosuppressive treatments (59). Interestingly, the impairment was more pronounced following T-cell dependent pneumococcal conjugate vaccines than after T-cell independent polysaccharide vaccines (59). A special role is played by B-cell-depleting agents, which will be discussed in the following section.

1.3.3. Impact of B-cell depletion on immune responses

In recent years, one of the key immunosuppressive mechanisms has been the depletion of B cells to impair the ongoing production of pathogenic autoantibodies. This approach is applied not only in various autoimmune diseases, such as rheumatoid arthritis, systemic sclerosis, and multiple sclerosis, but also in primary immunodeficiencies with autoimmune manifestations, such as CTLA-4 haploinsufficiency (60,61). Moreover, B-cell-depleting agents are routinely used in hematologic malignancies, particularly in B-cell lymphomas, to eliminate aberrant monoclonal cells (62).

Widely used B-cell-depleting drugs include the monoclonal anti-CD20 antibodies rituximab, ocrelizumab, and ofatumumab (60). Their mechanisms of action comprise complement-dependent cytotoxicity, antibody-dependent cellular cytotoxicity, and the induction of apoptosis following binding to CD20 on the B cell surface (63,64). As a result, peripheral B cells at different maturation stages – including transitional, naïve, and memory B cells – are

depleted, whereas plasma cells, which lack CD20 expression, remain unaffected (60). After termination of B-cell-depleting therapy, repopulation of B cells typically requires 6 to 20 months, or even longer, depending on the underlying disease and treatment regimen (60).

An unfavorable adverse effect of B-cell depletion is the impairment of protective antibody production, particularly in response to novel antigens, increasing susceptibility to infection in many recipients (64). Additionally, B-cell depletion poses a major challenge to vaccination, as a sufficient number of functional (especially naïve) B cells is required for an effective primary vaccination response (64,65). This phenomenon has been studied extensively in B-cell-depleted patients receiving influenza vaccinations. In patients with rheumatoid arthritis (RA), it has been shown that those patients treated with rituximab 4–8 weeks or 6–10 months prior to influenza vaccination had lower seroconversion and seroprotection rates than RA patients treated with methotrexate or than healthy controls (66). A systematic review and meta-analysis from 2021 including 905 patients from international cohorts treated with anti-CD20 therapy for hematologic malignancies, autoimmune diseases such as multiple sclerosis, RA, and immune thrombocytopenia, or following transplantation, assessed both humoral and cellular vaccine responses (67). Humoral responses to vaccines against influenza, hepatitis A and B, tetanus, pertussis, diphtheria, polio, *Haemophilus influenzae* B, and pneumococcus were compared across studies and showed markedly reduced seroconversion and seroprotection rates in these patients compared to healthy or disease controls (67). Particularly poor seroconversion rates – ranging from 0 to 25% – were observed in patients under active anti-CD20 therapy (67). The included meta-analysis of influenza vaccination studies yielded a pooled estimate of seroconversion rates of only 3% (95% CI, 0–9%) after a single vaccine dose when administered within three months of anti-CD20 treatment, with improved rates up to 50% at longer intervals since the last treatment (67).

1.3.4. Challenges and strategies for vaccination in immunodeficient patients

When vaccinating immunodeficient patients to prevent infectious diseases, various aspects need to be considered to ensure both safety and effectiveness. First, live vaccines are contraindicated in many of these patients, such as those with primary immunodeficiencies or in patients receiving rituximab, as these vaccines contain attenuated pathogens and may cause disease in immunocompromised patients who are unable to fully control replication of the pathogen

(21,57). As a result, vaccine options in these patients are limited, particularly because comparable inactivated vaccine alternatives are not available for all pathogens. Second, even when vaccination is possible, as it is the case for – among others – whole inactivated or subunit vaccines, a reduced vaccine response must be expected, the extent of which depends on the nature of the underlying immunodeficiency (see 1.3.2 and 1.3.3). Thus, effective protection against certain pathogens may not be achieved despite vaccination. To assess vaccine-induced immunity, it may be beneficial to measure post-vaccination antibody titers in immunodeficient patients. However, in contrast to, for instance, influenza vaccination, correlates of protection or standardized reference values do not exist for all vaccines, which further impedes a reliable interpretation (67,68). Third, immunodeficient patients may require modified vaccination schedules, including additional booster doses or altered dosages, to enhance immunogenicity (69). But to date, guidance is primarily based on expert consensus, which can serve as a basis for individualized clinical decisions (57,69,70). Widely accepted strategies include to perform vaccinations, if possible, before initiating certain immunosuppressive treatments in patients with autoimmune diseases or hematologic malignancies who are at risk of developing a secondary immunodeficiency, e.g., following B-cell-depleting therapy (69). Furthermore, in some treatment settings, postponing vaccination for some months may be appropriate to improve immunogenicity: For instance, after hematopoietic stem cell transplantation (HSCT), it is generally recommended to delay administration of inactivated vaccines for at least 3 to 6 months to enable an immune response (71). However, in recent years, the evaluation of T-cell responses to vaccination has gained increasing attention, as these may confer a degree of protection in the absence of measurable antibody responses for some vaccines (72).

Finally, if protective immunity cannot be achieved through active vaccination, alternative strategies remain, including short-term passive immunization using specific immunoglobulins, antibiotic prophylaxis against certain bacterial infections, and the implementation of enhanced hygiene precautions (57,70,73).

1.4. Aim of the dissertation and hypothesis

In 2021, when the first COVID-19 vaccines became available, it remained unclear whether immunocompromised patients would benefit from vaccination by eliciting sufficient immune responses. To address this question, we initiated the prospective, multidisciplinary CoVVac

study, which included patients with primary immunodeficiency, secondary immunodeficiency due to hematologic malignancies or B-cell depletion in autoimmune patients, as well as healthy controls. The aims of the study were to characterize both humoral and cellular immune response to SARS-CoV-2 vaccinations in these disease entities, to identify potential predictive factors for favorable responses, and to derive clinical recommendations for vaccination strategies.

The dissertational part of the described CoVVac study covered the first year of the study, starting with study design, submission to ethics committee and regulatory authorities, and implementation of the study, then conduction of the first three visits, and extending up to visit 4 and the time point of booster vaccination. We hypothesized that patients with predominantly humoral immunodeficiencies might still benefit from COVID-19 vaccination by mounting robust T-cell responses.

2. Material and Methods

2.1. Study design and participants

Starting in January 2021, we designed a prospective, phase IV, monocentric, multidisciplinary cohort study at the Medical University of Graz in Austria, called *"Humoral and cellular immune response to COVID-19 vaccines in immunocompromised and healthy individuals – The CoVVac study"*.

In the following, the study was registered in the EU clinical trials register (EudraCT number 2021-001040-10) and at the Austrian Federal Office for Safety in Healthcare (BASG). Approval by the ethics committee of the Medical University of Graz (33-314 ex 20/21) was gained by April 2021.

The preparation and conduction of the study occurred at the Division of Rheumatology and Immunology in cooperation with the Division of Hematology, the Institute of Hygiene, Microbiology and Environmental Medicine, the Department of Blood Group Serology and Transfusion Medicine, the Institute of Immunology, and the Biobank of the Medical University of Graz.

Patients with inborn errors of immunity (primary immunodeficiencies), hematological malignancies, autoimmune diseases under B-cell-depleting therapy, as well as healthy controls were included prior to their first SARS-CoV-2 vaccination. For details on diagnoses of participants, see 2.1.2. Inclusion criteria below. We aimed to recruit 195 patients and 195 healthy controls by sample size calculation, which is described in 2.8.1.

2.1.1. Schedule of the CoVVac study

Initially, six visits over a time course of 25–29 months were planned. For an overview, see Figure 1.

At visit 1, before the participants' first SARS-CoV-2 vaccination, patients and healthy controls first signed the informed consent form of the clinical trial. Inclusion and exclusion criteria were checked, and medical history was assessed. Blood was taken for measurement of anti-SARS-

CoV-2 antibody levels, T-cell immunity, T-cell aging, and immune status. Moreover, patients delivered urine for a pregnancy test (female patients only) and saliva. Details on the performed assays are described in 2.3–2.7. All relevant data were documented in a structured manner via RDA (Research, Documentation and Analysis) tool. After completion of visit 1, participants received their first dose of COVID-19 vaccination (an mRNA vaccine) and the Vienna Food Record questionnaire for completion at home during the week before visit 3.

Visit 2 was conducted as a telephone visit for safety monitoring. Adverse events occurring within two weeks after vaccination were documented. An appointment for the second COVID-19 vaccination, four weeks (if mRNA-1273, Moderna) or three weeks (if BNT162b2, BioNTech/Pfizer) after the first one, and also for the third visit was scheduled.

Visit 3 took place 21–28 days after the second vaccination to assess vaccine reactions and changes in medical status. Blood (for serology and T-cell immunity) and saliva were taken. Female participants delivered urine for a second pregnancy test. In addition, the Vienna Food Record questionnaire was collected for nutritive assessment, and body fat composition was measured by ultrasound.

Visit 4 was scheduled as a follow-up six months after the second vaccination, including safety monitoring and taking of blood and saliva for SARS-CoV-2 serology.

Visits 5 and 6 were scheduled as follow-ups 12 and 24 months after the second vaccination, respectively. As the protocol had to be amended prior to visit 4 due to changes in the Austrian vaccination strategy, details of visits 4–6 are described separately in paragraph 2.1.4.

Figure 1 – Overview of the initial schedule of the CoVVac study.

Time points are shown above visit numbers, tasks and materials of each visit below. IC: informed consent.

2.1.2. Inclusion criteria

For enrollment in the study, participants had to be at least 18 years of age, able to understand the study procedures, and able to provide written informed consent.

If these requirements were fulfilled, one of the following inclusion criteria had to be met additionally:

1. Noninfectious immunocompetent participants (i.e., healthy participants) as determined by medical history and clinical judgement
   or
2. Patients with primary immunodeficiencies
   or
3. Patients with B-cell-depleting therapy due to autoimmune disease
   or
4. Patients with benign and malignant hematological diseases receiving specific treatments with known immunosuppressive effects, including cytotoxic agents, systemic corticosteroids, monoclonal antibodies, and targeted therapies
   or
5. Patients with active hematological diseases and secondary immunoglobulin deficiency (e.g., CLL, MM) currently not receiving specific treatment
   or
6. Patients >3 months but <12 months after autologous HSCT
   or
7. Patients >3 months but <12 months after allogeneic HSCT
   or
8. Recipients of HSCT >12 months after allogeneic HSCT but under immunosuppressive therapy.
   or
9. Patients with chronic GvHD and persistent immunodeficiency.

All inclusion and exclusion criteria can be found in our study protocol, version 4.0 (29.09.2021, see Appendix).

2.1.3. Exclusion criteria

Exclusion criteria were defined for healthy subjects and patients separately.

Healthy participants could not be enrolled if they met one of the following criteria:

1. Presence of diseases or therapies that are likely to interfere with the immune response to vaccination
2. Presence of a disease requiring change in therapy during four weeks before enrollment
3. Any contraindications to the vaccine planned to receive as listed in the product characteristics
4. Lack of willingness to undergo serial blood draws and attend follow-up appointments
5. Women who are pregnant or breastfeeding
6. Previous vaccination with any coronavirus vaccine
7. Persons who are not willing to sign the informed consents (biobank informed consent and study specific informed consent).

Immunodeficient patients were excluded if they met one of these criteria:

1. Patients with hematological diseases within three months from B-cell-depleting immunotherapy (rituximab, ofatumumab, obinutuzumab, blinatumomab, CAR T cells)
2. Patients with hematological malignancies in remission and >12 months after end of specific therapy
3. Patients within three months from HSCT
4. Any contraindications to the vaccine planned to receive as listed in the product characteristics
5. Lack of willingness to undergo serial blood draws and attend follow-up appointments
6. Women who are pregnant or breastfeeding
7. Previous vaccination with any coronavirus vaccine (exception: if serum prior to vaccination is available from the biobank)
8. Patients who are not willing to sign the informed consents (biobank informed consent and study specific informed consent).

2.1.4. Protocol adaption due to recommendation of a 3rd SARS-CoV-2 vaccination

In September 2021, a third (booster) COVID-19 vaccination was recommended by the U.S. Food and Drug Administration and the Austrian national vaccination board for immunocompromised patients, health care workers and elder people (>65 years of age) six to nine months after their first two vaccinations.

We adapted the study protocol (version 4.0, 29.09.2021, see Appendix) as follows:

- At visit 4, participants with a low anti-SARS-CoV-2 antibody titer (<1000 U/ml at visit 3) donated blood for serology, blood group, immune status, and T-cell immunity.
- Participants eligible for a third vaccination could receive a third mRNA vaccination shot up to four weeks after visit 4, with female participants performing a pregnancy test beforehand.
- Visit 5 was scheduled earlier – three to six weeks after booster vaccination or after visit 4 (for those not having received the booster) – with a blood draw for serology and T-cell immunity, acquisition of saliva, and assessment of possible vaccine adverse events.
- Visit 6 was also planned earlier, six months after visit 4, and comprised blood sampling for serology and T-cell immunity, saliva collection, completion of the Vienna Food Record for a second time, ultrasound examination of body fat composition, and safety monitoring.

By this, the individual study period was shortened to 13–15 months.

However, this thesis is based on data collected up to and including visit 4.

2.2. Sampling and Storage

The following sample volumes were required from each participant at the respective visits:

- Visit 1: 8 mL of blood for serology, 60 mL of blood for cellular immunity, 19 mL of blood for immune status, 20 mL of blood for T-cell aging, 1.5 mL of saliva, and urine of female participants for pregnancy testing (detection of human chorionic gonadotropin beta)
- Before second vaccination: urine of female participants for pregnancy testing

- Visit 3: 8 mL of blood for serology, 60 mL of blood for cellular immunity, 1.5 mL of saliva
- Visit 4: 8 mL of blood for serology, 60 mL of blood for cellular immunity and 19 mL of blood for immune status (only patients without seroconversion at visit 3), 3 mL of blood for blood group testing, 1.5 mL of saliva
- Before booster vaccination: urine of female participants for pregnancy testing
- Visit 5: 8 mL of blood for serology, 60 mL of blood for cellular immunity, 1.5 mL of saliva
- Visit 6: 8 mL of blood for serology, 60 mL of blood for cellular immunity, 1.5 mL of saliva

All blood samples that were not immediately analyzed or not fully consumed by analyses were stored at the Biobank of the Medical University of Graz. Serum samples were aliquoted and stored at −80 °C. Peripheral blood mononuclear cells (PBMCs), after isolation from whole blood directly after blood draw, were frozen in a DMSO-based medium and kept in liquid nitrogen.

Handling of saliva samples was managed at the Institute of Hygiene, Microbiology and Environmental Medicine. Therefore, samples were centrifuged with 10.000G at 4 °C for 15 min in order to remove cell debris. The supernatant was stored at −80 °C, and later the samples were jointly transferred to the Natural and Medical Sciences Institute (NMI) at the University of Tübingen, Germany, for conduction of specific antibody assays.

2.3. Antibody assays

Serum antibody assays were performed at the Institute of Hygiene, Microbiology and Environmental Medicine by comparative utilization of two immunoassays (nowadays) used in routine diagnostics:

- The Elecsys® Anti-SARS-CoV-2 S electrochemiluminescence immunoassay was performed at a fully automated cobas® e 801 analytical unit (both Roche Diagnostics GmbH, Mannheim, Germany) allowing high throughput analyses. The assay measures

IgG antibodies targeting the receptor-binding domain (RBD) of the spike (S) protein of SARS-CoV-2 with a quantification range from 0.4 to 250 U/ml. Values of ≥0.8 U/ml are interpreted as reactive, those below as non-reactive.

- The LIASON® SARS-CoV-2 TrimericS IgG assay was conducted at the LIAISON® XL immunoassay analyzer (both DiaSorin, Saluggia, Italy), which also allowed high throughput diagnostics in a fully automated way. In contrast to the Roche assay, the test measures IgG antibodies not only targeting the S1 subunit of the spike protein but the whole trimeric spike protein. Thus, coverage of a broader variety of possibly neutralizing anti-SARS-CoV-2 spike antibodies is enabled. It quantifies concentrations from 4.81 to 2080 BAU/ml (binding antibody units per milliliter) with a cut-off of ≥33.8 BAU/ml discriminating positive from negative results.

Initially, the study protocol included performing SARS-CoV-2 live virus neutralization assays in the biosafety level 3 laboratory of the Institute. However, due to the high number of samples within a short timeframe and the extensive biosafety requirements, it was not feasible to process all samples using this method. As previous studies demonstrated a strong correlation between the results of the Roche assay and those of live virus neutralization tests (74,75), we applied the Roche assay as a surrogate measure of neutralizing capacity of the anti-SARS-CoV-2 spike protein antibodies.

If concentrations exceeded the upper quantification limit, automatic sample dilutions were performed according to the manufacturer's protocol in selected cases.

2.4. Lymphocyte phenotyping

Lymphocyte phenotyping was conducted following routine assays at the immunology laboratory of the Division of Rheumatology and Immunology at the Medical University of Graz. Within four hours after blood draw (at visit 1), ethylenediaminetetraacetic acid (EDTA) whole blood was stained with the antibodies CD3-FITC, CD4-APC, CD8-PE, CD45-PerCP, CD16+56-PE, and CD19-APC (BD Biosciences, Franklin Lakes, NJ, USA) and measured by flow cytometry (BD FACSLyric™ cytometer) for discrimination of main lymphocyte subpopulations.

For B-cell subphenotyping, PBMCs were first isolated from lithium heparin blood after Ficoll gradient density centrifugation. One million cells were incubated with the antibodies CD19-VioGreen, anti-IgD-VioBlue, anti-IgM-PE, CD24-PerCP-Vio700, CD27-APC, CD86-PEVio770, CD21-APC-Vio770, and CD38-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) and measured by flow cytometry (BD FACSLyric™ cytometer). Data analysis was performed using FACSSuite (BD Biosciences).

If total $CD19^+$ counts were 1 cell per µL or lower, subphenotyping was not conducted.

2.5. IFN-γ ELISpot assay

PBMCs had been frozen at the day of retrieval and needed to be thawed for parallel conduct of experimental, newly established SARS-CoV-2 ELISpot and reactive T-cell assays (see 2.6. below). After resuspension in AIM V medium, cells rested for two hours at 37 °C and were counted. 3.3 x 10^5 cells were added to each well of the ELISpot plate (Oxford Immunotec Ltd, Oxfordshire, United Kingdom) and either stimulated with 2 µg/mL PepTivator® SARS-CoV-2 Prot_S Complete (Miltenyi Biotec, Bergisch Gladbach, Germany) or PMA/Ionomycin (as a positive control) or AIM V media (as a negative control). Subsequently, cells incubated for 16–20 hours at 37 °C. T cells previously sensitized to SARS-CoV-2 or its spike protein (through vaccination) responded by production of interferon gamma (IFN-γ), which was subsequently captured by immobilized antibodies on the bottom membrane of the wells. After removal of the cells by washing, diluted conjugate solution, containing a secondary antibody to IFN-γ conjugated with alkaline phosphatase (ELISpotPro kit, Mabtech), was added to each well. Incubation for one hour at 4 °C followed. After washing, substrate solution (5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, [ELISpotPro kit, Mabtech]) was added to each well and incubated at room temperature in the dark for seven minutes. Reactions were stopped by adding distilled water. The supernatant was removed, and plates dried for 24 hours. Bound IFN-γ had formed visible spots, which were scanned using an AID Classic Robot ELISpot Reader and analyzed by AID ELISpot 7.0 software (both AID Autoimmun Diagnostika, Straßberg, Germany).

2.6. SARS-CoV-2 reactive T-cell assay

Reactive T-cell assays served for characterization of the following intracellular and surface markers and cytokines: CD3, CD4, CD8, CD45RA, CD107a, CD137, CD154, CXCR5, CCR7, PD-1, IFN-γ, IL-2, TNF-α, IL-17, and Granzyme B. Two separate panels, named "surface" and "cytokine" panel, were designed accordingly and conducted in parallel.

Frozen PBMCs were thawed and rested in AIM V medium for two hours, as described above (in 2.5.). Two million cells were added to each well of a 96-well U-bottom plate, along with either 2 µg/mL PepTivator® SARS-CoV-2 Prot_S Complete (Miltenyi Biotec, Bergisch Gladbach, Germany) or AIM V medium (negative and positive controls) and/or T Cell TransAct (Miltenyi Biotec, positive controls of the surface panel). Cells were subsequently cultured for 16–20 hours at 37 °C. For the last four hours of incubation, first Cell Stimulation Cocktail (Thermo Fisher, Waltham, USA) was added to those wells designated for positive controls of the intracellular panel; second, Protein Transport Inhibitor Cocktail (Thermo Fisher) was added to all wells of the intracellular panel in order to stop Golgi transport within the cells. Next, cells were transferred to two separate V-bottom plates for individual conduction of cytokine and surface panel. After centrifuging and washing, Fixable Viability Dye (Thermo Fisher) was given to all wells and cells incubated for 15 minutes in the dark. Once centrifuged and washed, antibody master mixes for surface staining (different for both panels) were added to all wells and incubated for 15 minutes in the dark. Following centrifugation and washing, permeabilization and fixation of the cells in the cytokine panel was performed by using the respective solution of the Cytofix/Cytoperm kit (BD Biosciences). Cells incubated for 20 minutes at 4 °C in the dark and were centrifuged and washed in permeabilization buffer. Next, the master mix for intracellular staining was added to these cells in permeabilization buffer and they incubated for another 20 minutes in the dark at room temperature. After completion of the centrifugation and washing steps, cells from both panels were resuspended in staining buffer and ready for measurement using a BD FACSLyric™ cytometer (BD Biosciences). Data analysis was performed with FlowJo software version 10.5.3 (FlowJo LLC).

Antibodies for "cytokine panel"	CD3-APC-H7 (surface marker)
	CD4-APC (surface marker)
	CD8-V500 (surface marker)
	INF-γ-FITC
	TNF-R718
	Hu IL-2-BB700
	Granzyme B Brilliant Violet 421
	CD107a-PE-Cy7
	IL-17-PE
Antibodies for "surface panel"	CD3-APC-H7
	CD4-PE-Cy7
	CD8-V500
	Hu CXCR5-R718-RF8B2
	Hu CCR7-APC
	CD45RA-BB515
	CD137-PE
	Hu CD154-BB700-TRAP1
	PD-1 Brilliant Violet 421

Table 1 – Overview of staining antibodies used for either "cytokine" or "surface panel".
All antibodies were obtained from Becton Dickinson, San Diego, USA.

2.7. Saliva antibody assays

After preparation of saliva samples by the Institute of Hygiene, Microbiology and Environmental Medicine, frozen samples were transferred to the Natural and Medical Sciences Institute (NMI) at the University of Tübingen, Germany. Measurements of SARS-CoV-2 IgG and IgA directed to the Spike trimer, the RBD, subunit S1, S2, or the nucleocapsid (N) protein were performed according to established protocols by the NMI. Moreover, specific IgG and IgA against endemic coronaviruses, including HCoV-OC43 (S1 and N protein), HCoV-HKU1 (S1 and N), HCoV-NL63 (S1 and N), and HCoV-229E (S1 and N), were measured.

2.8. Statistical Analyses

2.8.1. Sample size calculation

For sample size calculation, we considered the primary endpoint of the CoVVac study, i.e., anti-SARS-CoV-2 IgG antibody levels after the second vaccination. The calculation was performed using nQuery, version 8.6.1 (Statistical Solutions Inc.), based on previous studies examining seroconversion rates in immunodeficient patients following influenza vaccination (76). A Mann–Whitney U test was applied with a significance level of 0.01 and a power of 95%. This resulted in a required sample size of 175 subjects per group (immunodeficient patients versus healthy controls). To account for an estimated dropout rate of approximately 10%, we aimed to recruit 195 participants per group.

2.8.2. Analysis of humoral vaccination responses in immunocompromised patients

The first interim analysis of the CoVVac study was performed for humoral vaccination responses at visit 3 in comparison to baseline (visit 1) using Stata 16.1 (Stata Corp., Houston, TX, USA) and IBM SPSS Statistics Version 29 (IBM Corp., Armonk, NY, USA). We depicted continuous data as medians with interquartile ranges (25^{th}–75^{th} percentile) and categorical data as absolute frequencies and percentages (%). By application of Spearman's rank correlation coefficients, rank-sum test, and χ^2 tests, correlations and associations of antibody responses with other tested variables were calculated. Multiple linear regression models with the antibody response as dependent variable were established for calculation of coefficients of determination (R^2). Receiver operating characteristic (ROC) analysis and Youden's index were used for determination of an optimal cut-off value for predicting sufficient vaccination responses. Univariate and multivariate modeling of vaccination responses were conducted using logistic regression models. In order to compare continuous variables across three or more treatment groups, we applied Kruskal–Wallis test and post hoc analysis for determination of differences between groups.

2.8.3. Analysis of T-cellular vaccination responses in B-cell-depleted patients

SARS-CoV-2-specific T-cell responses were statistically analyzed using IBM SPSS Statistics, version 27 (IBM Corp., Armonk, NY, USA) and R, version 4.1.1 (https://www.r-project.org/). We examined the distribution of continuous variables with the Kolmogorov–Smirnov test: If variables showed a parametric distribution, data were described as mean and standard deviation (SD), and the two-sided Student's t-test (for comparison of two groups) was performed. If variables showed a non-parametric distribution, they were reported as median and range, and Mann–Whitney U test as well as Kruskal–Wallis test were executed. The χ^2 test was applied to analyze the distribution of categorical data. Correlation matrix calculation and visualization were performed with psych (version 2.1.9), stats (version 3.6.2), and corrplot (version 0.92) packages in R. Spearman's rank correlation coefficient was calculated for correlation analyses with application of Bonferroni correction for multiple testing. Graphs displaying mean values and standard error of the mean were generated using GraphPad Prism (GraphPad Software, San Diego, USA).

2.8.4. Analysis of T-cellular vaccination responses in patients with primary immunodeficiencies

For this statistical analysis, IBM SPSS Statistics, version 29 (IBM Corp., Armonk, NY, USA) and RStudio, version 2023.12.0+369 "Ocean Storm" for Windows, were utilized. In the descriptive statistics, the Shapiro–Wilk test was applied to assess the distribution of continuous variables. Variables following a parametric distribution were described as mean and standard deviation (SD); if variables showed a non-parametric distribution, they were reported as median and interquartile range (IQR). Since the majority of variables deviated from normality, non-parametric methods were applied for all subsequent analyses: The Mann–Whitney U test was used for two-group comparisons and the Kruskal–Wallis test for comparisons among more than two groups. Multivariate analysis of variance (MANOVA), including Pillai's trace, Wilks' lambda, Hotelling's trace, Box's test of equality of covariance matrices, Levene's test of equality of error variances, and tests of Between-Subjects Effects, was conducted to further examine differences in T-cell responses between patients and controls.

Spearman's rho and Kendall's tau rank correlation coefficient were calculated for correlation analyses with application of Bonferroni correction for multiple testing using RStudio. Corresponding plots and heatmaps were created with the R packages ggplot2 (version 3.5.1), ggpubr (version 0.6.0), and pheatmap (version 1.0.12). Boxplots of the comparison of T-cell responses were also created using RStudio.

3. Results

3.1. Recruitment of study population

After successful initiation of the CoVVac trial, immunodeficient patients meeting the inclusion criteria (see 2.1.2.) were recruited by the Division of Rheumatology and Immunology and by the Division of Hematology. Healthy controls were recruited by the Department of Blood Group Serology and Transfusion Medicine.

In total, we recruited 67 patients at the Rheumatology and Immunology outpatient clinic, of whom 25 had a primary immunodeficiency (PID) and 42 suffered from autoimmune diseases and were therefore treated with B-cell-depleting therapies. Among these 42 patients, 10 had neurological conditions, specifically multiple sclerosis or neuromyelitis optica spectrum disorder (NMOSD). One of the 42 patients had to be excluded from further analyses, as it became evident in retrospect that she had never received B-cell-depleting therapy.

Moreover, 132 hematologic patients were included, predominantly with multiple myeloma (MM), acute and chronic leukemia, or myelodysplastic syndrome (MDS), some being post hematopoietic stem cell transplant (HSCT).

Included healthy participants were either COVID-naïve (n = 90) or had already been infected with and recovered from SARS-CoV-2 prior to visit 1 (n = 83). One participant of the COVID-naïve cohort had to be excluded after visit 1 due to being newly diagnosed with chronic lymphocytic leukemia (CLL), another one was excluded due to COVID-19 infection at visit 1.

Table 2 gives an overview of the whole initial study cohort.

For the following analyses, patients of interest and a selection of age- and sex-matched healthy controls were included.

Cohort	Number of partici-pants	Mean age [years]	Gender (female/male)	Diagnosis	Diagnosis frequency
Rheumatology and Immunology: PID	25	53.32	14 (56%) / 11 (44%)	CVID	11 (44%)
				Antibody (or subclass) deficiency	11 (44%)
				Other PID	3 (12%)
Rheumatology and Immunology: AID	42	53.12	29 (69%) / 13 (31%)	Arthritis (RA or PsA)	6 (14%)
				Systemic sclerosis	8 (19%)
				Idiopathic inflammatory myopathy	5 (12%)
				Multiple sclerosis or NMOSD	9 (21%)
				Other connective tissue disease (SLE, pSS)	8 (19%)
				Other AID	5 (12%)
				Other	1 (2%)
Hematology	132	63.61	65 (49%) / 67 (51%)	Multiple Myeloma	43 (33%)
				Acute myeloid leukemia	18 (14%)
				GvHD after alloHSCT	14 (11%)
				Lymphoid neoplasms	27 (20%)
				Myelodysplastic syndrome	7 (5%)
				Myeloproliferative neoplasms	25 (19%)
				Other hematologic disease	13 (10%)
Healthy: COVID-19 naïve	90	47.64	52 (58%) / 38 (42%)	-	-
Healthy: COVID-19 convalescent	83	46.72	39 (47%) / 44 (53%)	-	-

Table 2 – Overview of the initial recruitment status of the CoVVac study.

Note that some hematologic patients are represented by more than one diagnosis category. The reference date for age was 1st April 2021. AID, Autoimmune disease; CVID, Common variable immunodeficiency; GvHD, Graft-versus-host disease; HSCT, Hematopoietic stem cell transplantation; NMOSD, Neuromyelitis optica spectrum disorder; PID, Primary immunodeficiency; PsA, Psoriatic arthritis; pSS, Primary Sjögren's syndrome; RA, Rheumatoid arthritis; SLE, Systemic lupus erythematosus.

3.2. Analysis of humoral vaccination responses in immunocompromised patients

3.2.1. Selected study population

For the first interim analysis of the study, we selected 199 participants who had completed their full vaccination schedule (i.e., two vaccinations). 120 of them were immunocompromised and 79 were healthy participants of the COVID-19 naïve group. Almost the whole cohort received the Moderna vaccine mRNA-1273; only two healthy participants were vaccinated with the BioNTech/Pfizer vaccine BNT162b2. No severe adverse events related to the vaccinations occurred. Table 3 displays the baseline characteristics of the selected cohort.

Variable	Overall (n = 199)	Healthy (n = 79)	Immunocompromised (n = 120)	*p*
Age (years)	54 [43–62]	51 [36–56]	58 [50–65]	**<0.001**
Female gender (%)	110 (55)	45 (57)	65 (54)	0.698
Body mass index (kg/m²)	24.4 [22.2–27.0]	23.7 [21.6–26.4]	24.8 [22.7–27.8]	**0.039**
Immunodeficiency group				n/a
Primary immunodeficiency (%)			25 (21)	
Autoimmune disease (%)			39 (32)	
Hematologic disease (%)			56 (47)	
Vaccine				0.080
mRNA-1273 (Moderna, %)	197 (99)	77 (97)	120 (100)	
BNT162b2 (BioNTech/Pfizer, %)	2 (1)	2 (3)	0 (0)	
B-cell-depleting therapy				n/a
None (%)	123 (62)		44 (37)	
Rituximab (%)	35 (18)		35 (29)	
Ocrelizumab (%)	6 (3)		6 (5)	
HSCT (%)	35 (17)		35 (29)	
Days since depletion			166 [69–545]	
Antibody Response				
Roche, any response	159 (80)	79 (100)	80 (67)	**<0.001**
Roche, stringent response	117 (59)	79 (100)	38 (32)	**<0.001**
Roche in U/mL	2020 [4–2500]	2500 [2500–2500]	67 [0–1947]	**<0.001**

DiaSorin, any response	177 (89)	79 (100)	98 (82)	**<0.001**
DiaSorin, stringent response	105 (53)	79 (100)	26 (22)	**<0.001**
DiaSorin in BAU/mL	2080 [142–2080]	2080 [2080–2080]	233 [12–1760]	**<0.001**

Table 3 – Baseline characteristics of the selected study population of the 1st interim analysis and antibody response.

Data are reported as medians [25th–75th percentile] and absolute frequencies (%). Significant p-values are highlighted in bold type. Any response, any seroconversion. DiaSorin stringent response, DiaSorin SARS-CoV-2 TrimericS IgG ≥2000 BAU/mL. Roche stringent response, Roche anti-SARS-CoV-2 S total antibody titer ≥1000 U/mL. Adapted from Schulz, Hodl et al., 2021 (77).

3.2.2. Assessment of humoral vaccination responses

Antibody responses of both groups at visit 3 (three to four weeks after the second vaccination) were analyzed using the Roche and DiaSorin assays, as described above. All healthy controls showed positive responses in both assays, whereas only 67% (Roche) and 82% (DiaSorin) of immunocompromised patients did so. Median antibody titers among patients were 67 U/mL (Roche) and 233 BAU/mL (DiaSorin) in contrast to 2500 U/mL and 2080 BAU/mL, respectively, in the healthy controls. As patient responses were significantly lower and no established quantitative correlate of protection exists, we additionally defined a "stringent response" for both assays. It reflects the lowest antibody titer observed among healthy participants, i.e., ≥1000 U/ml (Roche) and ≥2000 BAU/ml (DiaSorin), and was considered likely to confer protection. Only 32% (Roche) and 22% (DiaSorin) of patients reached this threshold. The difference in response rates between the two assays was not statistically significant ($p = 0.108$). The results are summarized in Table 3.

Figure 2 displays the Roche anti-SARS-CoV-2 antibody titers of healthy controls and patient subgroups, of which patients under anti-CD20 therapy showed the lowest rate of stringent response (≤10%).

For comparability with other studies, we concentrated in the following on the widely used Roche assay. In our study cohort, both assays also correlated excellently with each other ($\rho = 0.914$, $p < 0.001$, $R^2 = 0.887$; Figure 3a).

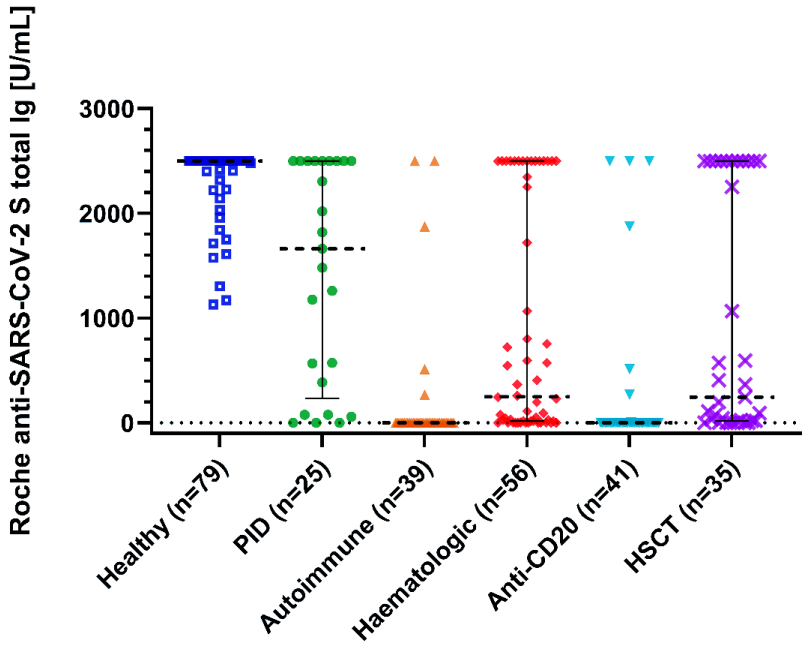


Figure 2 – Roche anti-SARS-CoV-2 S total antibody titers following SARS-CoV-2 mRNA vaccination.

Antibody titers are shown for healthy controls, patients with primary immunodeficiencies (PID), autoimmune diseases, hematologic malignancies, anti-CD20 therapy, and post-HSCT. Lines are medians with interquartile range; $p < 0.001$ between all groups calculated with Kruskal–Wallis test and post hoc analysis. Note: Some patients belong to more than one category. Figure is adapted from Schulz, Hodl et al., 2021 (77).

3.2.3. Correlation of antibody levels with B-cell subsets

To investigate potential predictors of post-vaccination antibody levels, we analyzed B cells and their subsets prior to vaccination (at visit 1). In the patient cohort, total B-cell counts and all tested B-cell subsets correlated positively with the vaccination-induced antibody titers (Table 4). In particular, naïve B cells demonstrated the highest correlation ($\rho = 0.761$, $p < 0.001$, $R^2 = 0.153$). This correlation could also be confirmed in the whole study cohort ($\rho = 0.636$, $p < 0.001$, $R^2 = 0.123$; Figure 3b). However, in the whole cohort, positive correlations of all other B-cell subsets were not statistically significant (Table 4). Of note, the interval from last B-cell depletion to vaccination in the patient cohort also showed a positive correlation.

Variable	Whole cohort			Patient cohort		
	ρ	p	R^2	ρ	p	R^2
DiaSorin SARS-CoV-2 TrimericS IgG	0.914	**<0.001**	0.887	0.915	**<0.001**	0.841
Age	-0.209	**<0.001**	0.057	0.091	0.321	0.001
Body mass index	0.003	0.438	0.003	0.169	0.076	0.004
Days since last B-cell depletion	0.595	0.078	0.041	0.595	**<0.001**	0.041
Interval in days from last B-cell-depleting therapy to vaccination up to 365 days	0.481	0.096	0.096	0.481	**0.001**	0.096
IgA	0.264	**<0.001**	0.095	0.042	0.651	0.015
IgG	0.343	**<0.001**	0.105	0.065	0.481	0.031
IgM	0.386	0.573	0.002	0.386	**<0.001**	0.002
Lymphocytes abs.	0.289	0.802	<0.001	0.222	0.018	0.002
$CD3^+$ cells abs.	0.169	0.028	0.025	0.112	0.236	0.034
$CD3^+CD8^+$ cells abs.	0.128	0.836	<0.001	0.170	0.071	0.005
$CD3^+CD4^+$ cells abs.	0.192	**<0.001**	0.068	0.025	0.789	0.062
$CD3^-CD16^+CD56^+$ NK cells abs.	0.039	0.329	0.005	-0.014	0.880	0.014
$CD19^+$ abs.	0.670	0.638	0.001	0.739	**<0.001**	0.001
$CD45^+$ abs.	0.295	0.762	<0.001	0.227	0.015	0.002
Transitional B cells abs.	0.444	0.284	0.006	0.491	**<0.001**	0.033
Naïve B cells abs.	0.636	**<0.001**	0.123	0.761	**<0.001**	0.153
IgD^+ memory B cells abs.	0.600	0.034	0.024	0.657	**<0.001**	0.004
IgD^- memory B cells abs.	0.616	0.270	0.006	0.710	**<0.001**	0.003
$CD21^-$ cells abs.	0.484	0.636	0.001	0.640	**<0.001**	0.001
Plasmablasts abs.	0.438	0.485	0.003	0.580	**<0.001**	0.001

Table 4 – Correlations of the Roche anti-SARS-CoV-2 S total antibody results with different variables in the whole and in the patient study population.

Correlations were calculated using Spearman's rank-based rho and corrected for multiple testing. Significant p-values are highlighted in bold type. Note: Correlations of the DiaSorin SARS-CoV-2 TrimericS IgG results with the respective variables were comparable and showed the same or similar significance levels. Abs., absolute. Adapted from Schulz, Hodl et al., 2021 (77).

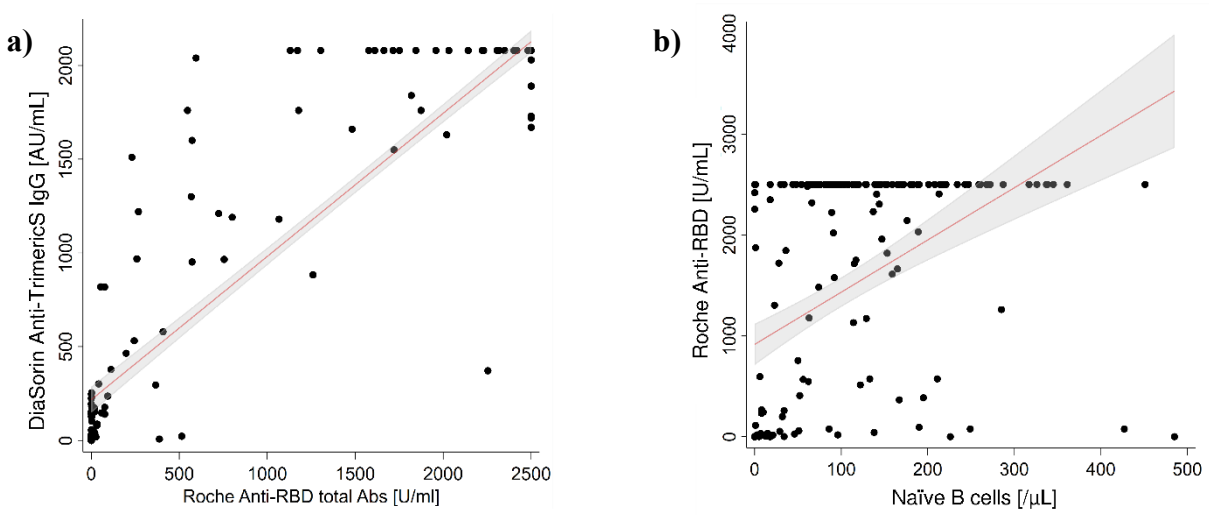


Figure 3 – Correlations of Roche anti-SARS-CoV-2 total antibody titers after 2nd vaccination with (a) DiaSorin SARS-CoV-2 TrimericS IgG titers and with (b) naïve B-cell count before vaccination in the whole study cohort.

The scatter plots show a linear regression line including a 95% confidence interval. Adapted from Schulz, Hodl et al., 2021 (77).

3.2.4. Potential predictors for SARS-CoV-2 vaccination responses

To complement the correlation analysis and to assess the predictive potential of several laboratory markers, we first performed receiver operating characteristic (ROC) analyses and calculated the corresponding areas under the curve (AUC) based on the Roche assay results for stringent responses. According to this model, the total number of B cells as well as naïve B cells, pre-switched, and post-switched memory B cells could serve as predictors for the whole study cohort, as they showed the best discrimination (Table 5).

Variable	Stringent Response	
	AUC	**95% CI**
Age	0.36	0.28–0.45
Body mass index	0.46	0.37–0.54
IgA	0.68	0.59–0.76
IgG	0.66	0.57–0.74
IgM	0.76	0.68–0.83
Lymphocytes abs.	0.65	0.57–0.72
$CD3^+$ cells abs.	0.61	0.52–0.70
$CD3^+CD8^+$ cells abs.	0.54	0.44–0.63

$CD3^+CD4^+$ cells abs.	0.67	0.59–0.75
$CD3^-CD16^+CD56^+$ NK cells abs.	0.54	0.45–0.63
$CD19^+$ abs.	**0.87**	0.79–0.92
$CD45^+$ abs.	0.68	0.60–0.76
Transitional B cells abs.	0.60	0.50–0.69
Naïve B cells abs.	**0.82**	0.73–0.88
IgD^+ memory B cells abs.	**0.83**	0.76–0.89
IgD^- memory B cells abs.	**0.85**	0.79–0.91
$CD21^-$ B cells abs.	0.76	0.67–0.84
Plasmablasts abs.	0.69	0.59–0.77

Table 5- Prediction of SARS-CoV-2 stringent vaccination response by different variables for the whole study population.

The calculation is based on results of the Roche anti-SARS-CoV-2 S assay. AUCs >0.8 are considered to demonstrate excellent discrimination and are highlighted in bold type. Stringent response, Roche anti-SARS-CoV-2 S total antibody titer ≥1000 U/ml. Abs., absolute. Adapted from Schulz, Hodl et al., 2021 (77).

Furthermore, we performed univariate and multivariate linear regression for any and stringent responses to test the association of all prior variables with the vaccination response.

In univariate linear regression of the patient cohort, counts of naïve B cells (OR: 1.17, 95% CI: 1.07–1.28, $p = 0.001$), pre-switched memory B cells (OR: 1.78, 95% CI: 1.09–2.92, $p = 0.021$), plasmablasts (OR: 7.95, 95% CI: 2.68–23.4, $p < 0.001$), and the interval from last B-cell-depleting therapy to vaccination (OR: 1.41, 95% CI: 1.13–1.76, $p = 0.002$) demonstrated significant associations with any response. The same parameters, except for pre-switched memory B cells, were also significantly associated with stringent responses (Table 6). In respect of the whole cohort, all of these four parameters showed significant associations for both stringent (Table 6) and any response. Some other parameters, including IgG, IgA, and IgM levels, T-helper-cell count, and age, did not show congruent results, as they were only significant for either stringent or any response. Table 6 compares the univariate linear regression results for stringent responses in patients and in the whole cohort.

Variable	Patient cohort			Whole cohort		
	OR	**95% CI**	***p***	**OR**	**95% CI**	***p***
Age (per 10 years)	1.02	0.76–1.38	0.880	0.69	0.54–0.87	**0.001**
Body mass index (per 5 points)	1.11	0.71–0.73	0.633	1.03	0.94–1.13	0.565
Interval in days from last B-cell-depleting therapy to vaccination up to 365 days (per 30 days)*	1.31	1.02–1.67	**0.035**	1.31	1.02–1.67	**0.035**
IgA (per 1g/L)	1.30	0.87–1.93	0.199	2.15	1.51–3.05	**<0.001**
IgG (per 5g/L)	1.43	0.90–2.27	0.127	3.00	1.79–5.04	**<0.001**
IgM (per 1g/L)	0.98	0.92–1.05	0.677	0.98	0.93–1.04	0.512
Lymphocytes abs. (per 1 G/L)	1.03	0.89–1.18	0.686	1.01	0.87–1.17	0.867
T cells abs. (per 10/µL)	1.00	0.99–1.01	0.101	1.00	0.99–1.01	0.071
Cytotoxic T cells abs. (per 10/µL)	1.00	0.99–1.01	0.846	0.99	0.98–1.00	0.417
T helper cells abs. (per 10/µL)	1.01	1.00–1.02	0.009	1.02	1.00–1.03	**<0.001**
NK cells abs. (per 10/µL)	1.01	0.99–1.03	0.180	1.01	0.99–1.03	0.232
B cells abs. (per 10/µL)	0.99	0.99–1.01	0.931	0.99	0.99–1.00	0.651
$CD45^+$ cells abs. (per 10/µL)	1.00	0.99–1.01	0.653	1.00	0.99–1.00	0.823
Transitional B cells abs. (per 1/µL)	1.05	0.96–1.15	0.233	1.03	0.95–1.12	0.416
Naïve B cells abs. (per 10/µL)	1.09	1.04–1.14	**<0.001**	1.15	1.09–1.20	**<0.001**
Pre-switch memory B cells abs. (per 10/µL)	1.03	0.95–1.11	0.480	1.30	1.09–1.55	**0.004**
Switched memory B cells abs. (per 10/µL)	0.99	0.99–1.00	0.957	0.99	0.99–1.00	0.425
$CD21^-$ B cells abs. (per 10/µL)	1.00	0.99–1.01	0.746	0.99	0.99–1.01	0.686
Plasmablasts abs. (per 1/µL)	1.85	1.24–2.74	**0.002**	2.85	1.88–4.32	**<0.001**

Table 6 – Univariate linear regression analysis for stringent vaccination responses in the patient and in the whole cohort.

The analysis is based on stringent responses (≥1000 U/mL) in the Roche anti-SARS-CoV-2 S assay. Significant p-values are highlighted in bold type. *B-cell-depleted patients only (n = 53). Abs., absolute. Adapted from Schulz, Hodl et al., 2021 (77).

In the multivariable analysis of stringent responses in the entire cohort, naïve B-cell counts remained significantly associated in the context of all tested variables (OR: 1.14 per 10 µL increase, 95% CI: 1.08–1.20, $p < 0.001$), indicating that they were the only independent predictor (see Table 7).

Multivariable Model for stringent response	Variable	Multivariable OR	95% CI	*p*
1st (n = 189)	Naïve B cells abs. (per 10/µL)	1.14	1.08–1.20	**<0.001**
	Age (per 10 years)	0.85	0.65–1.11	0.234
2nd (n = 189)	Naïve B cells abs. (per 10/µL)	1.15	1.09–1.21	**<0.001**
	IgA (per 1g/L)	2.22	1.48–3.35	**<0.001**
3rd (n = 189)	Naïve B cells abs. (per 10/µL)	1.14	1.08–1.20	**<0.001**
	IgG (per 5g/L)	5.59	1.78–17.50	0.003
4th (n = 189)	Naïve B cells abs. (per 10/µL)	1.14	1.09–1.20	**<0.001**
	$CD3^+CD4^+$ cells abs. (per 10/µL)	1.02	1.01–1.03	0.003
5th (n = 189)	Naïve B cells abs. (per 10/µL)	1.14	1.08–1.20	**<0.001**
	IgD^+ memory B cells abs. (per 10/µL)	1.03	0.88–1.20	0.732
6th (n = 189)	Naïve B cells abs. (per 10/µL)	1.15	1.09–1.20	**<0.001**
	Plasmablasts abs. (per 1/µL)	1.00	0.90–1.11	0.979
7th (n = 189)	Naïve B cells abs. (per 10/µL)	1.14	1.08–1.20	**<0.001**
	Age (per 10 years)	0.94	0.70–1.28	0.723
	IgA (per 1g/L)	1.81	1.13–2.88	**0.013**
	IgG (per 5g/L)	2.76	0.74–10.20	0.129
	$CD3^+CD4^+$ cells abs. (per 10/µL)	1.02	1.00–1.03	**0.006**
	IgD^+ memory B cells abs. (per 10/µL)	1.00	0.89–1.11	0.981
	Plasmablasts abs. (per 1/µL)	1.02	0.91–1.14	0.764

Table 7 – Multivariable linear regression models testing the independent effect of naïve B-cell counts on stringent vaccination responses in the entire study population.

The analysis is based on stringent responses (≥1000 U/mL) in the Roche anti-SARS-CoV-2 S assay and variables from the univariate linear regression. Interval from last B-cell-depleting therapy to vaccination was not included because this variable is neither applicable to all patients nor to the controls. Significant p-values are highlighted in bold type. Abs., absolute. Adapted from Schulz, Hodl et al., 2021 (77).

3.2.5. Cut-off calculation for stringent vaccination response

We further aimed to define a cut-off value of naïve B cells before vaccination that would be required for a stringent antibody response, as this parameter was the only independent predictor

in the multivariable regression analysis. By ROC analysis and Youden's index (= 0.704), we estimated a cut-off of ≥60 naïve B cells per µL.

Moreover, we applied ROC analysis to determine the minimum interval between the last B-cell-depleting therapy and vaccination needed for any (in contrast to no) vaccination response in the patients, and Youden's index (= 0.543) yielded a cut-off of >115 days. Stringent response was not a sufficient marker in this setting, with only 7 of 53 participants achieving this threshold and the maximum Youden's index reaching only 0.487, although formally calculating the same cut-off of 115 days. Figure 4 displays the three ROC curves for these analyses.

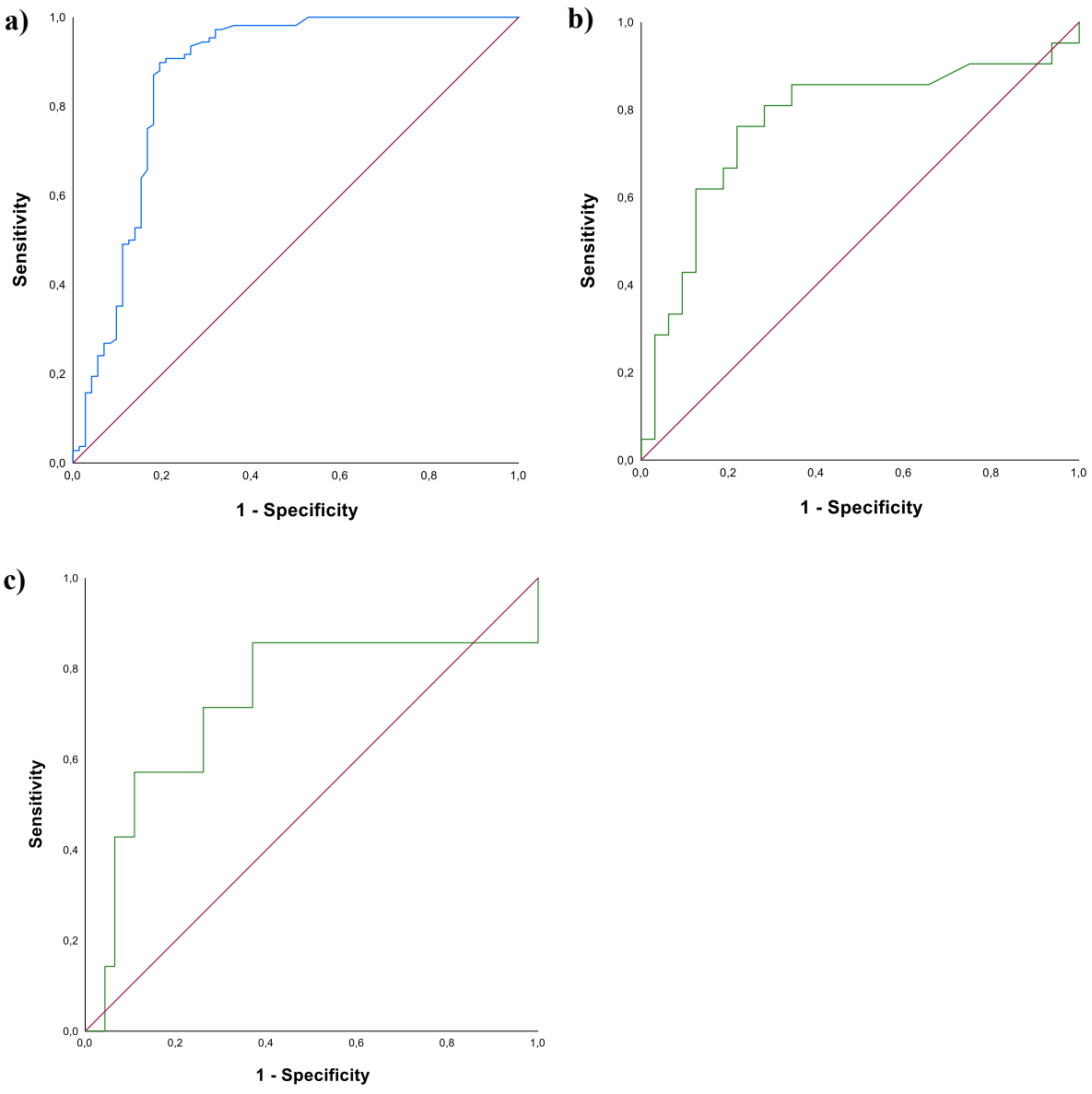


Figure 4 – ROC analysis for cut-off calculation of naïve B cells and interval from last B-cell-depleting therapy.

a) ROC curve for naïve B cells before vaccination discriminating stringent antibody response versus no or any response measured by Roche anti-SARS-CoV-2 S assay. Calculation based on 180 participants of the interim analysis, AUC = 0.866.
b) ROC curve for interval between last B-cell-depleting therapy and vaccination (up to 365 days) differentiating any versus no vaccination response. Calculation based on 53 patients, AUC = 0.772.
c) ROC curve for interval between last B-cell-depleting therapy and vaccination (up to 365 days) differentiating stringent versus no or any vaccination response. Calculation based on 53 patients, AUC = 0.727.

3.3. Analysis of T-cellular vaccination responses in B-cell-depleted patients

3.3.1. Selected study population

For the second interim analysis of the study, we selected 103 participants after completion of their full vaccination schedule (i.e., two vaccinations). 40 of them were patients with autoimmune diseases (AID) receiving B-cell-depleting therapy, and 63 were healthy controls of the COVID-19 naïve group. Almost the whole cohort received the Moderna vaccine mRNA-1273; only two healthy participants were vaccinated with the BioNTech/Pfizer vaccine BNT162b2. Table 8 displays the baseline characteristics of the selected cohort.

Variable	HCs	AIDs	*p*
Number	63	40	
Age [years]*	49.8 (23–77)	54 (27–76)	0.260
Female gender, n (%)	37 (58.7)	28 (70)	0.298
Body mass index [kg/m²]*	24 (18.1–38.4)	24.6 (16.8–39.4)	0.252
Vaccine			
mRNA-1273 (Moderna), n (%)	61 (96.8)	40 (100)	
BNT162b2 (BioNTech/Pfizer), n (%)	2 (3.2)	0	
Diagnosis			
Arthritis, n (%)		6 (15)	
Systemic sclerosis, n (%)		7 (17.5)	
Multiple sclerosis, n (%)		10 (25)	
Idiopathic inflammatory myopathies, n (%)		5 (12.5)	
Other connective tissue diseases, n (%)		8 (20)	
Other, n (%)		4 (10)	
Disease parameters°			
Remission/inactive disease, n (%)		23 (82.1)	
Low disease activity, n (%)		4 (14.3)	

Moderate/high disease activity, n (%)		1 (3.6)	
Disease duration [years]*		4 (0–35)	
B-cell-depleting therapy			
Rituximab, n (%)		33 (82.5)	
Ocrelizumab, n (%)		7 (17.5)	
Days since B cell depletion*		69 (0–603)	
Concomitant medication			
None, n (%)		17 (42.5)	
Corticosteroids, n (%)		2 (5)	
HCQ, n (%)		5 (12.5)	
MMF, n (%)		9 (22.5)	
MTX, n (%)		2 (5)	
AZA, n (%)		1 (2.5)	
Other, n (%)		4 (10)	

Table 8 – Baseline characteristics of the selected population of the 2nd interim analysis.
*median (range); °disease activity was assessed in 28 AID patients. HC, Healthy Control; AID, Patient with Autoimmune Disease; HCQ, Hydroxychloroquine; MMF, Mycophenolate mofetil; MTX, Methotrexate; AZA, Azathioprine. Adapted from Hodl et al., 2022 (78).

3.3.2. Comparison of T-cellular immune responses to COVID-19 vaccination in AID patients and healthy controls

In line with the analysis of humoral vaccination responses described in 3.2, we analyzed T-cell responses of both groups at visit 3 (three to four weeks after the second vaccination), utilizing PBMCs for ELISpot and flow cytometry as described above (see 2.5–2.6).

Although patients under B-cell-depleting therapy demonstrated significantly lower antibody responses than healthy participants (see 3.2 and Figure 2), we did not observe any differences in T-cell responses (i.e., IFN-γ secretion) measured by IFN-γ ELISpot assay (Figure 5a). Flow cytometry enabled a more detailed analysis of a broader variety of effector cytokines and activation markers of both cytotoxic T cells and T helper cells. However, the results revealed significantly lower numbers only for TNF-α^+ T helper cells and GzmB$^+$ cytotoxic T cells in the patient cohort (Figure 5d, g). No significant differences between the two cohorts were observed for IFN-γ, IL-2, and CD137 – in neither cytotoxic nor T helper cells – or for TNF-α production, CD107a, and CD154 (= CD40 ligand) expression of cytotoxic T cells. CD107a is a lysosomal membrane protein serving as marker for degranulation of these T cells. The results are depicted graphically in Figure 5. Of note, IFN-γ results showed moderate correlations between ELISpot

(total T cells) and flow cytometry (T helper and cytotoxic T cells separately): Correlation coefficients were 0.431 for T helper and 0.538 for cytotoxic T cells (both $p < 0.001$).

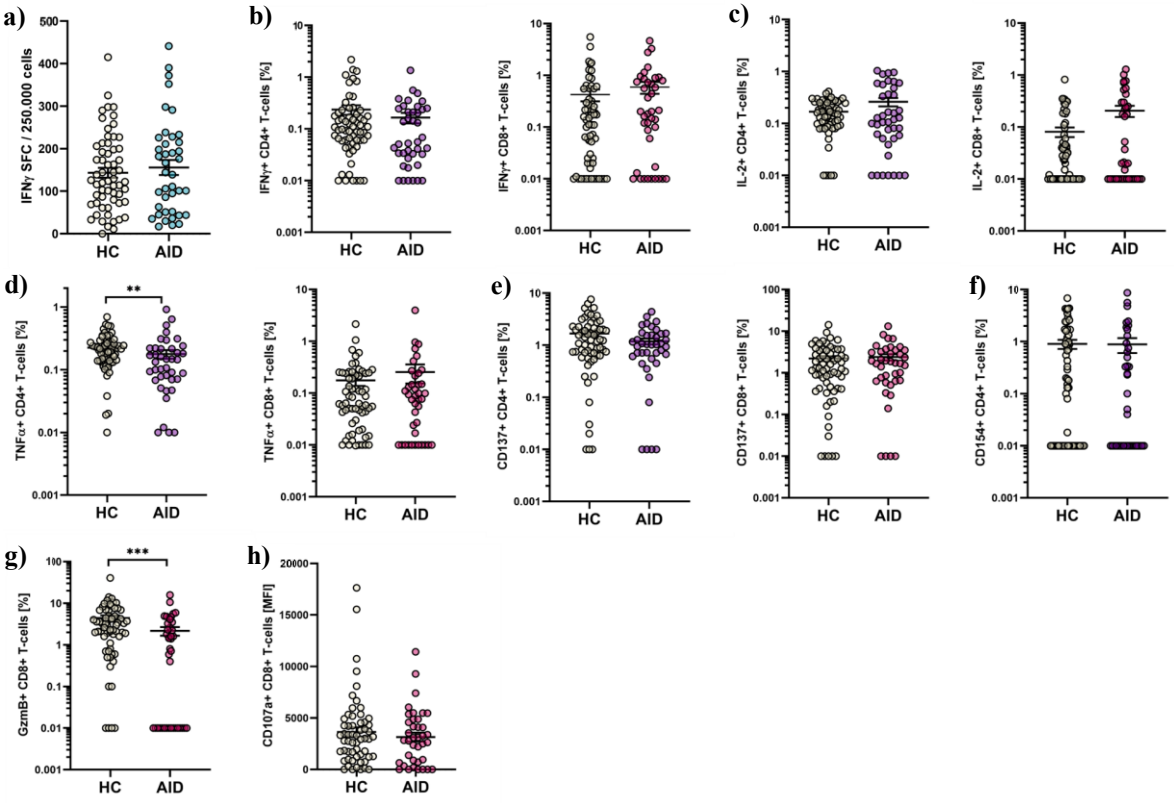


Figure 5 – T-cell responses to COVID-19 spike protein in healthy controls (HC) and patients (AID) after two COVID-19 vaccinations.

Graph a) shows the number of IFN-γ spot-forming cells (SFCs) in healthy controls (HC) and patients with autoimmune diseases (AID) in the ELISpot analysis. Graphs b), c), d), and g) depict the frequencies of IFN-γ-, IL-2-, TNF-α-producing $CD4^+$ and $CD8^+$ T cells, and GzmB-producing $CD8^+$ T cells in HCs and AIDs determined by flow cytometry. Graphs e), f) and h) present the upregulation of surface markers CD137, CD154, and CD107a on $CD4^+$ and/or $CD8^+$ T cells in HCs and AIDs determined by flow cytometry. $^{**}p < 0.01$; $^{***}p < 0.001$. Adapted from Hodl et al., 2022 (78).

Furthermore, we wanted to investigate if patients and healthy controls expressed different activation profiles in effector memory subsets. In both groups, we observed a higher expression of the activation marker CD137 in effector memory and TEMRA cells than in central memory and naïve T cells, as expected (Figure 6). Healthy participants had a higher proportion of activated $CD8^+$ and $CD4^+$ T cells in the effector memory cells, whereas the patients showed higher numbers of activated $CD8^+$ T cells in their TEMRA cell pool. The results of this analysis are displayed in Figure 6.

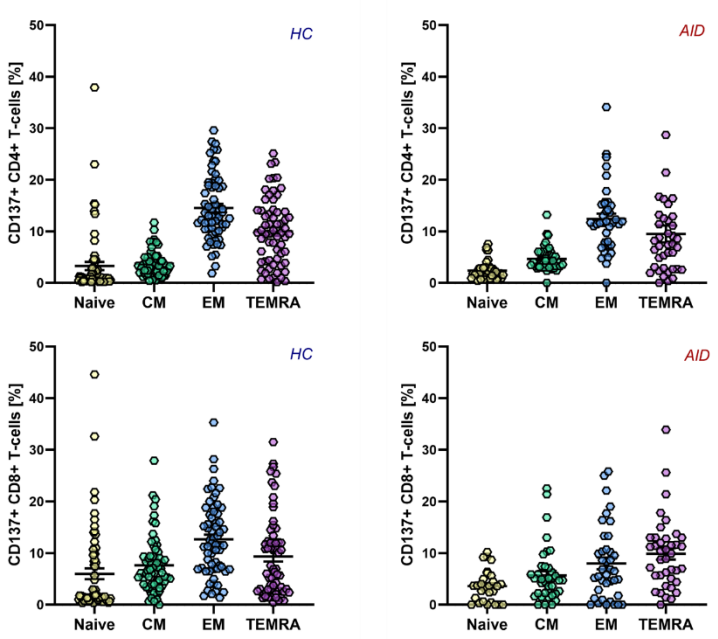


Figure 6 – Upregulation of activation marker CD137 in T-cell subsets.
Graphs display the frequencies of $CD137^{+}CD4^{+}$ (top) or $CD137^{+}CD8^{+}$ T cells (bottom) in naïve, central memory (CM), effector memory (EM) T cells, and terminally differentiated effector memory cells re-expressing CD45RA (TEMRA). Results of the healthy controls (HC) are on the left, those of the patients (AID) on the right. Adapted from Hodl et al., 2022 (78).

Taking all cellular responses into consideration, all tested effector cytokines and activation markers correlated positively with each other in the healthy controls, indicating a coordinated process of T-cell activation upon stimulation with the COVID-19 spike protein. In contrast, in the patients, this pattern appeared to be disturbed. In particular, GzmB and the activation markers CD137 and CD154 showed only low or even negative correlations with the other tested markers and did not reach significance (Figure 7). Additionally, differences in $CD4^{+}$ T cells producing IL-2, TNF-α, or IFN-γ and in $CD8^{+}$ T cells producing TNF-α or IFN-γ became evident; these shared significant correlations with the healthy cohort only to a limited extent.

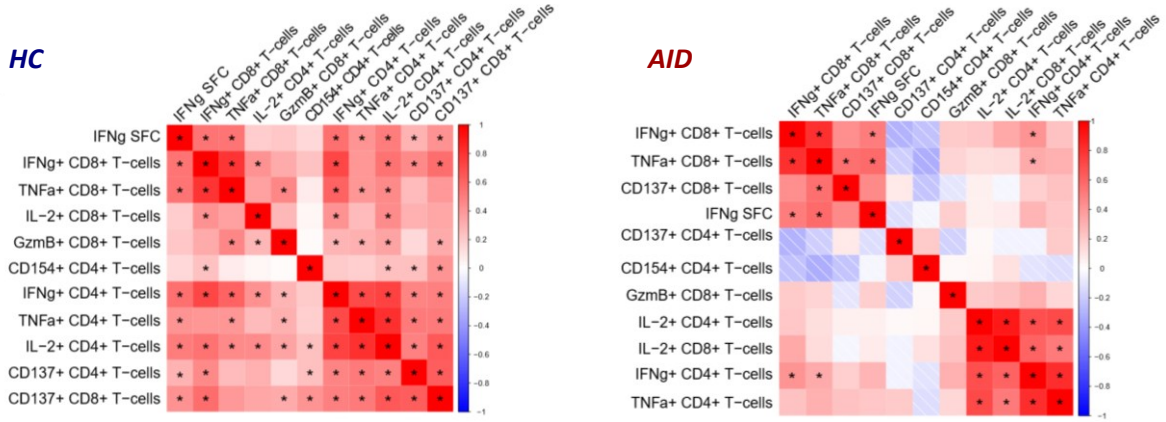


Figure 7 – Correlation matrices of effector cytokine and activation marker expression in healthy controls (HC) compared to the patients (AID).

Results for all markers are shown for both T helper and cytotoxic T cells, if applicable. CD154 and GzmB are mainly expressed by either T helper or cytotoxic T cells, respectively. *$p < 0.05$. Matrices are published in Hodl et al., 2022 (78).

3.3.3. Investigation of a possible impact of humoral on cellular responses

Next, we examined whether seroconversion influenced the cellular responses described above by dividing the patients into two groups according to their humoral responses: no seroconversion vs. any seroconversion. We observed positive trends in patients with seroconversion, namely a higher production of IFN-γ by T cells in ELISpot and of TNF-α by T helper cells in flow cytometry. However, flow cytometry revealed no difference in IFN-γ production in either cytotoxic or T helper cells depending on the seroconversion status, nor in IL-2 levels. Additionally, in cytotoxic T cells, there was no detectable difference in GzmB, TNF-α, or the surface marker CD137 between the seroconversion groups. In T helper cells, expression of CD154 was also comparable between groups. Only CD137 showed a significantly higher expression in this T-cell subset in the group with seroconversion (Figure 8).

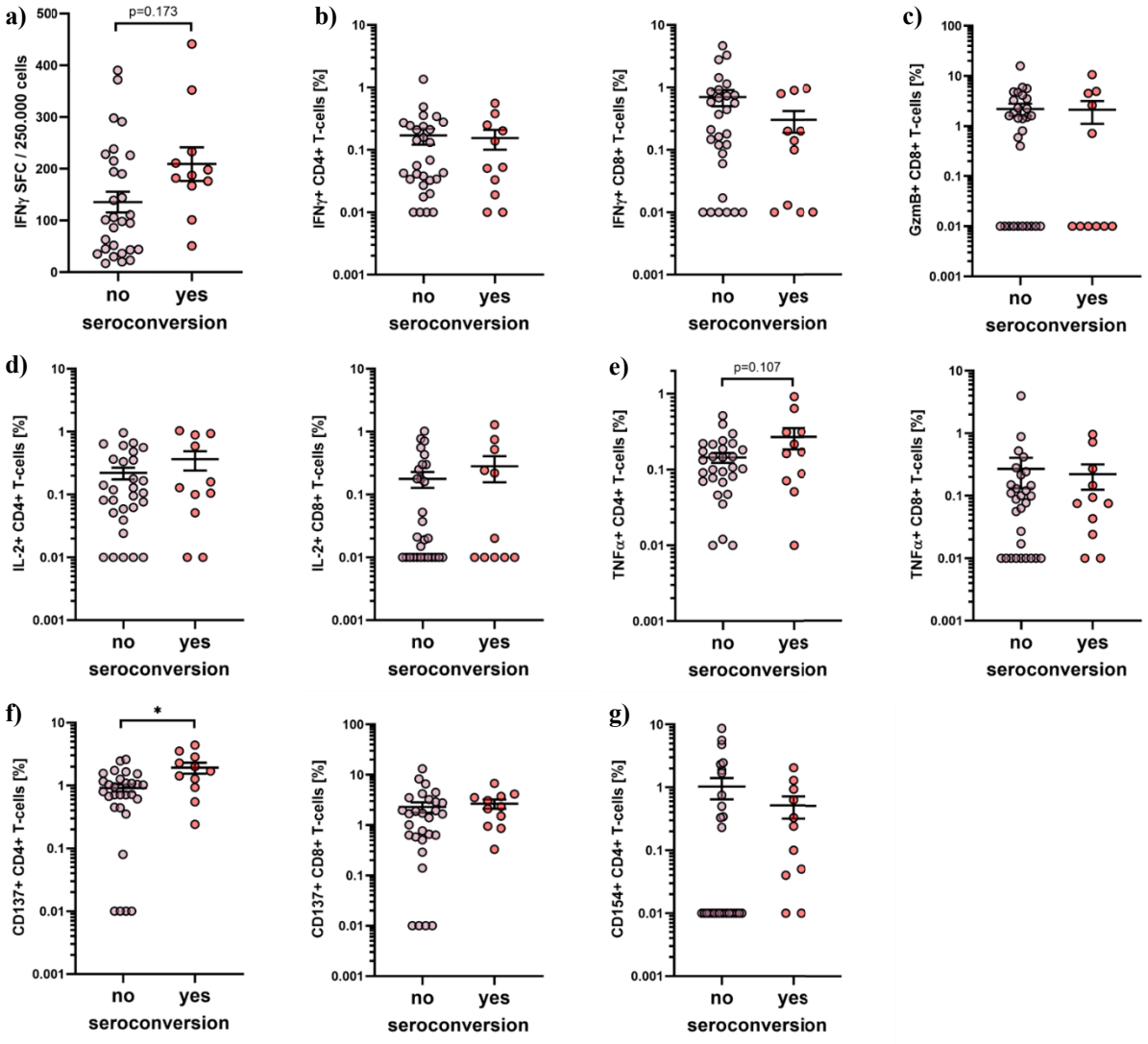


Figure 8 – Comparison of T-cell results between AID patients with and without seroconversion after two COVID-19 vaccinations.

Graph a) shows the number of IFN-γ spot-forming cells (SFCs) in AID patients with and without seroconversion following vaccination in the ELISpot analysis. Graphs b), c), d), and e) depict the frequencies of IFN-γ-, IL-2-, TNF-α-producing $CD4^+$ and $CD8^+$ T cells, and GzmB-producing $CD8^+$ T cells determined by flow cytometry. Graphs f) and g) present the upregulation of surface markers CD137 and CD154 on $CD4^+$ and/or $CD8^+$ T cells in HCs and AIDs determined by flow cytometry. *$p < 0.05$. Adapted from Hodl et al., 2022 (78).

3.3.4. Correlation between immune cell composition and cellular immunity

As numbers of naïve B cells before vaccination had demonstrated a predictive capacity for humoral vaccination responses in our first interim analysis, we subsequently investigated the correlation of lymphocyte subpopulations with the observed cellular responses. For B cells and

their subsets, this analysis was only conducted in healthy controls since it was not applicable to all patients due to B-cell depletion. In the healthy cohort, total B cells and naïve B cells each correlated negatively with GzmB$^+$ cytotoxic T cells ($\rho = -0.328$, $p = 0.009$; and $\rho = -0.457$, $p < 0.001$, respectively). Moreover, T follicular helper (Tfh) cells showed a mild positive correlation with the anti-spike antibody titer post-vaccination (measured by Roche assay; $\rho = 0.279$, $p = 0.028$) – an effect we did not observe in the patient cohort. In the patients in particular, we detected negative correlations of total T cells, T helper cells, and cytotoxic T cells with GzmB$^+$ cytotoxic T cells ($\rho = -0.464$, $p = 0.003$; $\rho = -0.477$, $p = 0.002$; and $\rho = -0.314$, $p = 0.055$, respectively), although the latter was not significant. Moreover, cytotoxic T cells correlated negatively with IFN-γ$^+$ T helper cells ($\rho = -0.337$, $p = 0.038$). Figure 9 displays the results of this correlation analysis.

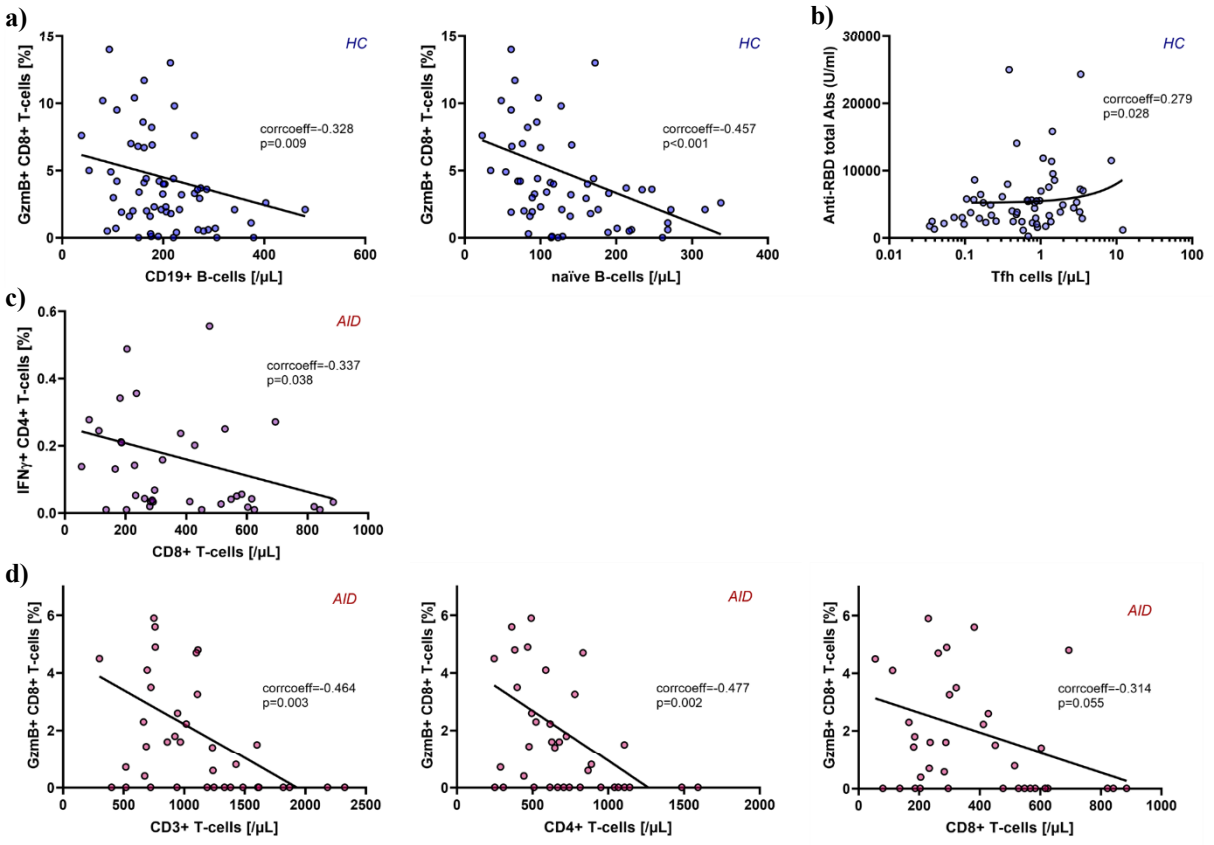


Figure 9 – Correlation of selected lymphocyte subpopulations with T-cell responses to the SARS-CoV-2 spike.

Graph a) shows correlations of the frequency of GzmB-producing CD8$^+$ T cells with absolute numbers of total and naïve B cells in HCs; b) the anti-RBD antibody titers with the total number of circulating Tfh cells in HCs; c) the frequency of IFN-γ-producing CD4$^+$ T cells with absolute numbers of CD8$^+$ T

cells in AIDs; d) the frequency of GzmB-producing $CD8^+$ T cells with absolute numbers of total T cells, $CD4^+$ T cells, and $CD8^+$ T cells in AIDs. Adapted from Hodl et al., 2022 (78).

3.3.5. Effects of concomitant immunosuppressive medications and underlying diseases on cellular immunity

As the patients received different treatments and had different underlying diseases, we next investigated whether these factors might have influenced our markers. Interestingly, patients receiving B-cell-depleting therapy with ocrelizumab showed enhanced cytokine responses in $CD4^+$ T cells, significantly shown for IFN-γ and IL-2, and with a trend for TNF-α, in comparison to those receiving rituximab, although their levels of B cells and the time point of their last treatment dose were comparable (Figure 10). IL-2 also appeared to be expressed at higher levels in $CD8^+$ T cells of patients receiving ocrelizumab but did not reach significance ($p = 0.58$).

Additional immunosuppressive medications, i.e., corticosteroids, hydroxychloroquine, mycophenolate mofetil, methotrexate, azathioprine, and others (leflunomide, sulfasalazine), caused only a small reduction in IL-2^+ $CD4^+$ T cells, particularly in patients receiving hydroxychloroquine ($p = 0.073$; Figure 11d). All of the other tested markers were neither significantly affected by these therapies, nor did they demonstrate a clear trend; therefore, the detailed graph for single medications in Figure 11 is only shown for IL-2^+ $CD4^+$ T cells. The frequency of these cells was also the one most affected by disease entities, i.e., inflammatory arthritis, systemic sclerosis, multiple sclerosis, idiopathic inflammatory myopathies, other connective tissue diseases, and others (e.g., ANCA-associated vasculitis). The only observable trend was that patients with inflammatory arthritis exhibited the lowest proportions of IL-2^+ $CD4^+$ T cells ($p = 0.065$; Figure 12). Taken together, none of the tested markers were significantly affected by additional therapies or diseases. The results are displayed in Figure 11 and Figure 12.

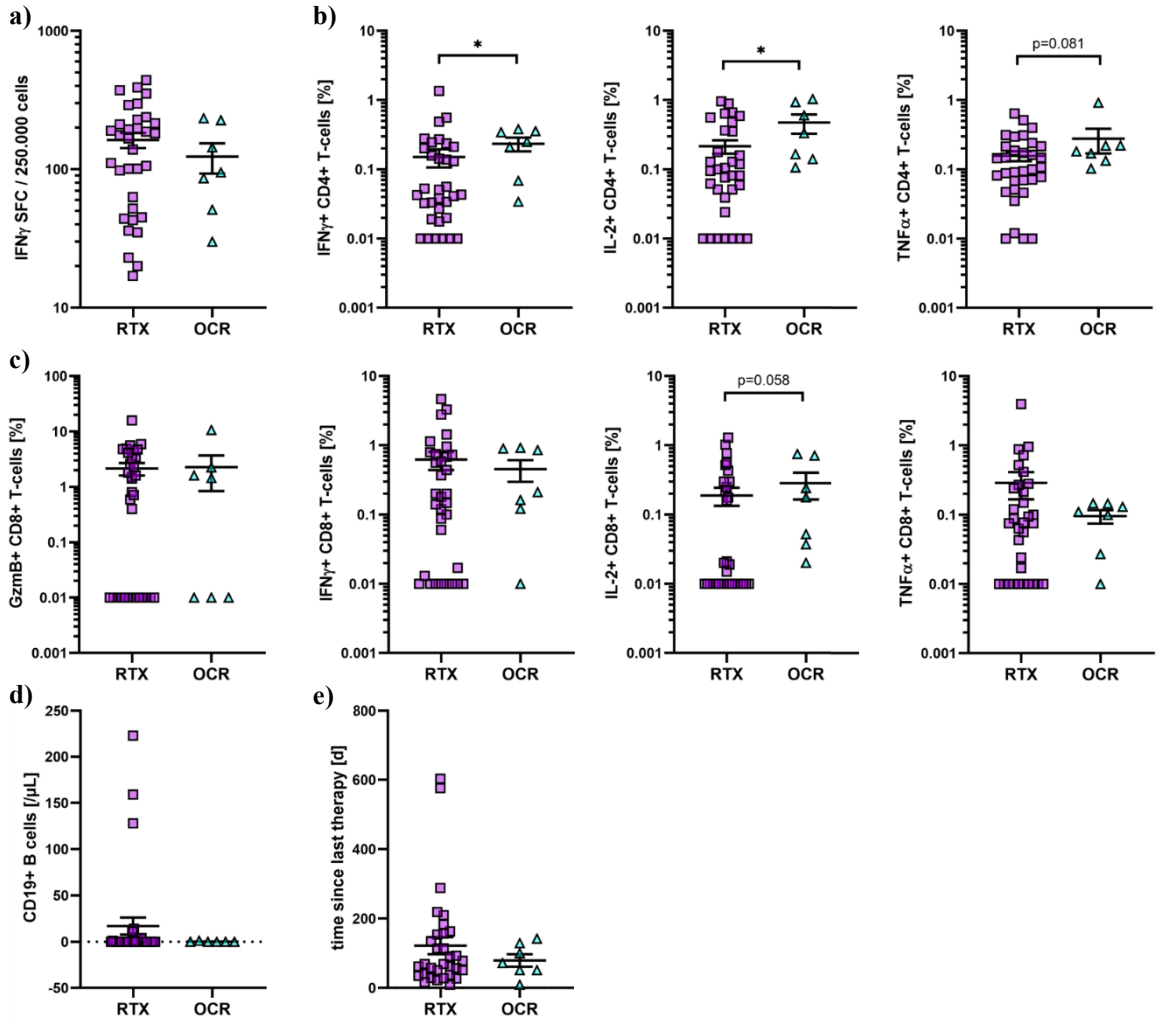


Figure 10 - Comparison of parameters between AID patients receiving rituximab or ocrelizumab.

Graph a) shows the number of IFN-γ spot-forming cells (SFCs) in AID patients treated with rituximab (RTX) or ocrelizumab (OCR) in the ELISpot analysis after two COVID-19 vaccinations. Graph b) depicts the frequencies of IFN-γ-, IL-2-, and TNF-α-producing $CD4^+$ T cells determined by flow cytometry, graph c) the frequencies of GzmB-, IFN-γ-, IL-2-, and TNF-α-producing $CD8^+$ T cells. Graph d) depicts the total B-cell counts in the two groups and e) the time since last infusion of RTX or OCR. *$p < 0.05$. Adapted from Hodl et al., 2022 (78).

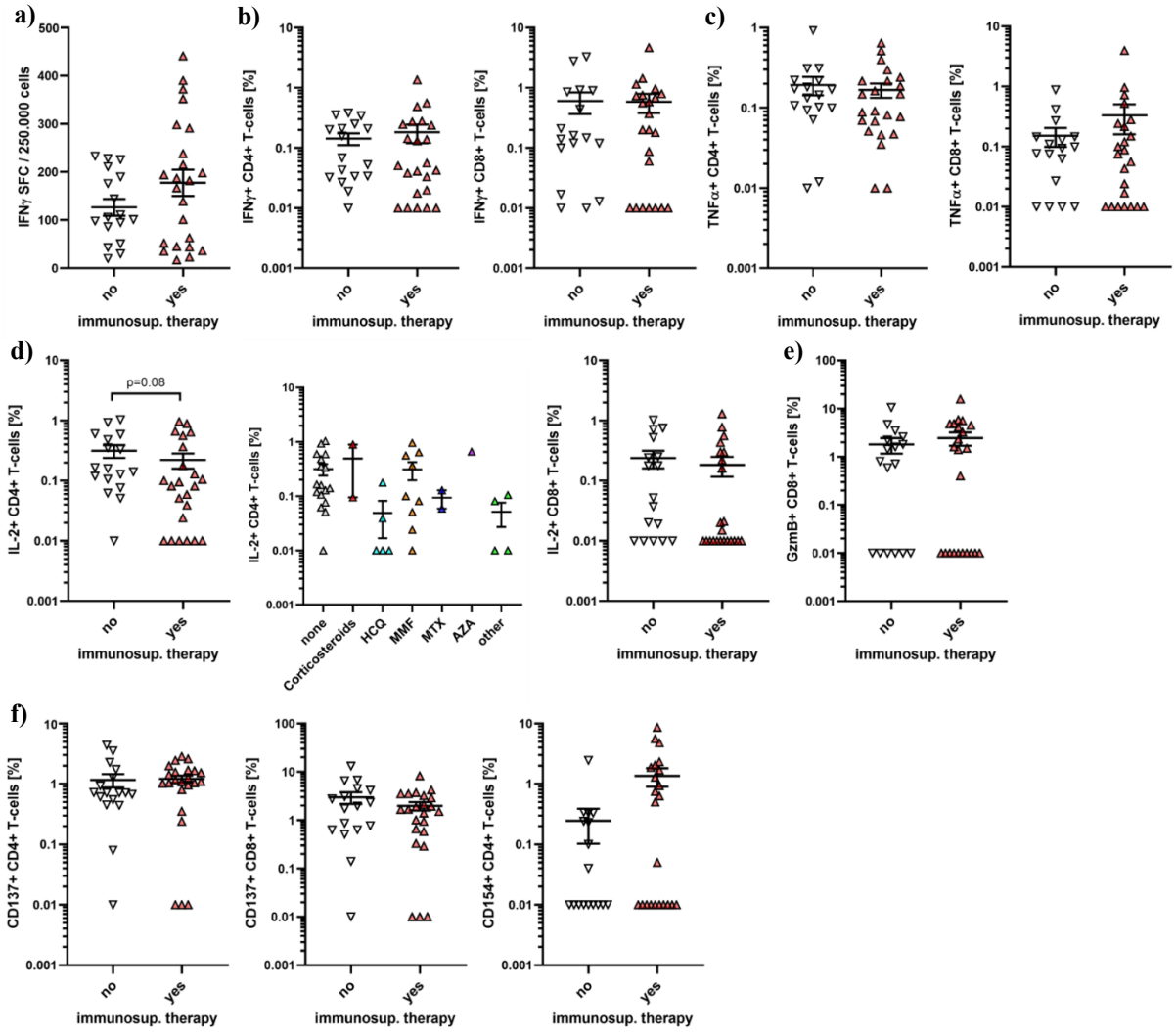


Figure 11 – Comparison of T-cell results between AID patients receiving additional immunosuppressive therapies (yes) or B-cell depletion only (no).

Graph a) shows the number of IFN-γ spot-forming cells (SFCs) in AID patients with or without additional immunosuppressive medications measured by ELISpot after two COVID-19 vaccinations. Graphs b), c), d), and e) depict the frequencies of IFN-γ-, TNF-α-, IL-2-producing $CD4^+$ and $CD8^+$ T cells, and GzmB-producing $CD8^+$ T cells determined by flow cytometry. As IL-2^+ $CD4^+$ T cells showed a trend towards lower expression in patients with additional therapies, the detailed results for these therapies are shown in d) as well. Graph f) presents the upregulation of surface markers CD137 and CD154 on $CD4^+$ and/or $CD8^+$ T cells determined by flow cytometry. All results are $p > 0.05$. HCQ, hydroxychloroquine; MMF, mycophenolate mofetil; MTX, methotrexate; AZA, azathioprine. Adapted from Hodl et al., 2022 (78).

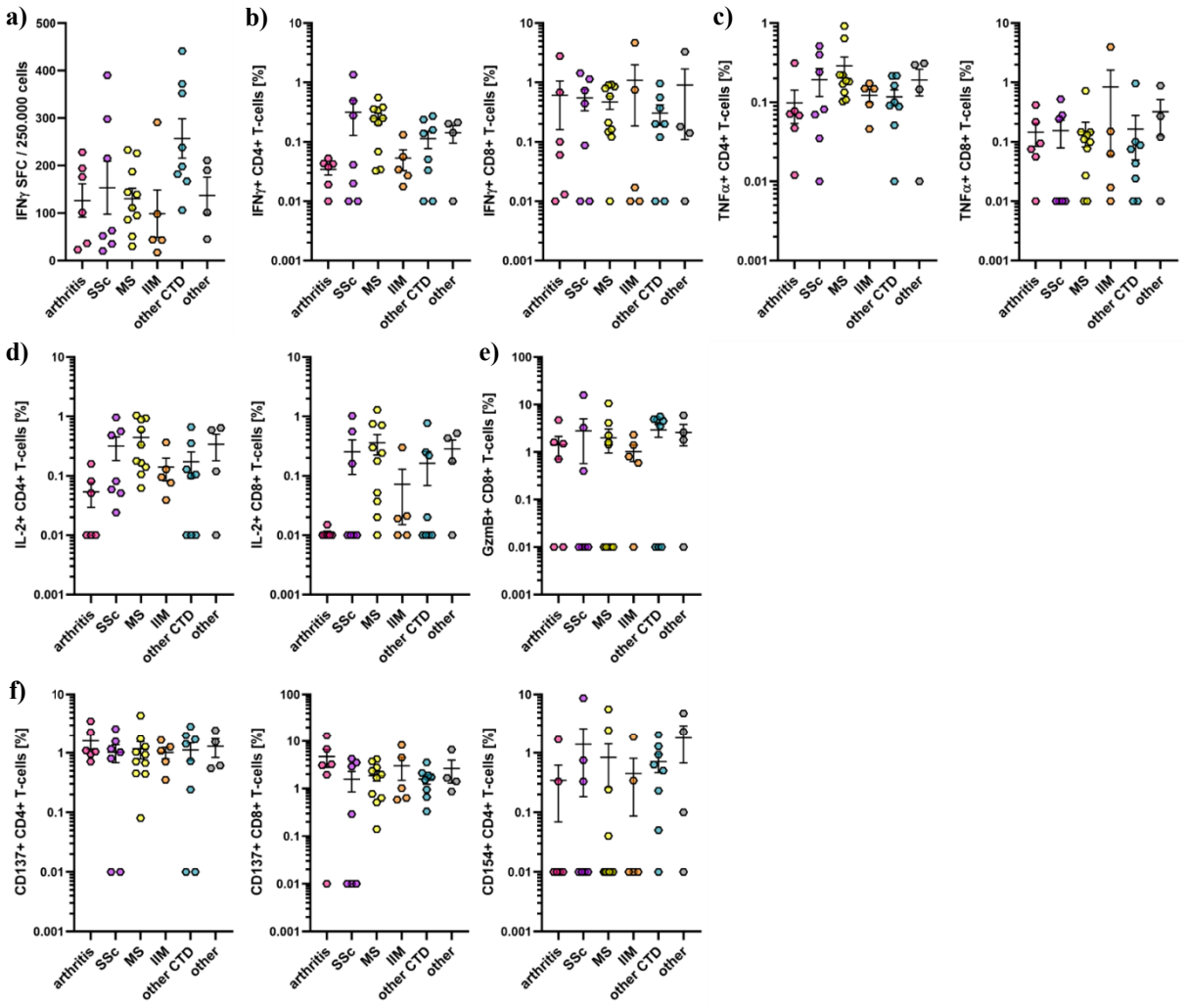


Figure 12 – Comparison of T-cell results between AID patients according to their underlying disease. Graph a) shows the number of IFN-γ spot-forming cells (SFCs) in the different disease entities measured by ELISpot after two COVID-19 vaccinations. Graphs b), c), d), and e) depict the frequencies of IFN-γ-, TNF-α-, IL-2-producing $CD4^+$ and $CD8^+$ T cells, and GzmB-producing $CD8^+$ T cells determined by flow cytometry. Graph f) presents the upregulation of surface markers CD137 and CD154 on $CD4^+$ and/or $CD8^+$ T cells determined by flow cytometry. All results are $p > 0.05$. SSc, systemic sclerosis; MS, multiple sclerosis; IIM, idiopathic inflammatory myopathies; CTD, connective tissue diseases. Adapted from Hodl et al., 2022 (78).

3.3.6. Sustainability of the anti-SARS-CoV-2 antibody titers

In order to investigate whether aspects of the cellular immune response affected the sustainability of anti-SARS-CoV-2 antibodies, we compared antibody titers three to four weeks after the second vaccination and after six months, i.e., before the third vaccination was due. As expected, we detected a significant decline in the healthy cohort (Figure 13a). Due to the small number of seroconverted patients, a reliable analysis of the patient data was not possible.

However, the healthy participants showed a strong correlation between their total B-cell count, and in particular between their naïve B cells, and the anti-RBD antibody titer before the third vaccination (Figure 13b). This was in concordance with the results we found in our first analysis (see 3.2) for patients exhibiting strong antibody responses after the second vaccination.

Regarding T-cell responses, we found positive correlations for higher SARS-CoV-2 antibody titers after six months in those healthy participants who had a higher reactivity in ELISpot and higher counts of IFN-γ- or TNF-α-producing $CD4^+$ T cells in the initial analysis after the second vaccination, as shown in Figure 13c.

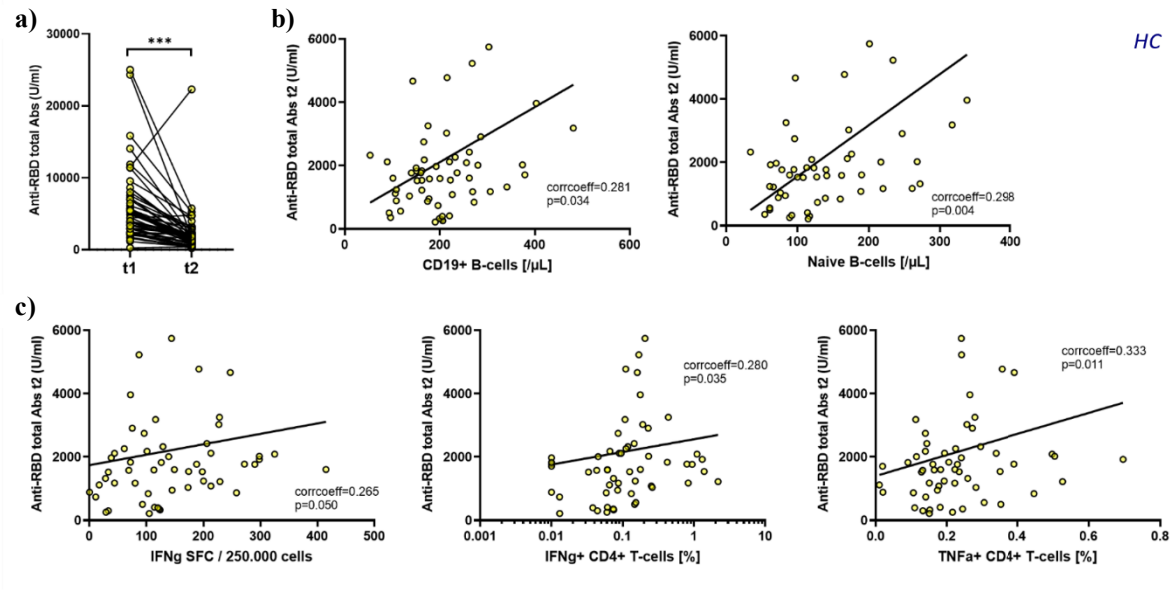


Figure 13 – Correlations of anti-RBD antibody titers with cellular markers in the healthy cohort.
Graph a) shows anti-RBD antibody titers three to four weeks after the second vaccination (t1) and approximately six months after the second vaccination, i.e., before booster vaccination (t2), in HCs. Graph b) displays the correlations of initial absolute and naïve B-cell counts with anti-RBD antibody titers at t2. Graph c) shows the correlations between anti-RBD antibody titers at t2 and (left) the number of IFN-γ spot-forming cells (SFCs) measured by ELISpot after two COVID-19 vaccinations, (middle) the frequencies of IFN-γ^+ $CD4^+$ T cells, and (right) TNF-α^+ $CD4^+$ T cells determined by flow cytometry. ***$p < 0.001$. Adapted from Hodl et al., 2022 (78).

3.4. Analysis of T-cellular vaccination responses in patients with primary immunodeficiencies

3.4.1. Selected study population

Complementary to the prior analysis of T-cell vaccination responses in autoimmune patients after B-cell-depleting therapy, we aimed to investigate the ability of our patients with primary immunodeficiencies (PID) to mount T-cell responses after the first two COVID-19 vaccinations. Data from 24 PID patients and 24 age- and sex-matched healthy controls were included. These patients could be allocated to three different disease groups: common variable immunodeficiency (CVID), other antibody deficiencies than CVID (including IgG subclass deficiencies), or other PID (including cellular or combined immunodeficiencies). All patients received the Moderna vaccine, and the 20 of 24 patients (83.3%) were currently treated with regular immunoglobulin replacement therapy (IGRT). While all healthy controls mounted anti-RBD antibody responses, 87.5% of patients mounted at least a minimal vaccine-specific humoral immune response. Table 9 displays the baseline characteristics of the selected cohort.

Variable	Overall (n = 48)	Healthy (n = 24)	PID (n = 24)
Age, in years *	53.2 ± 13.8	52.6 ± 12.2	53.8 ± 15.4
Female gender (%)	26 (54.2)	13 (54.2)	13 (54.2)
Body mass index, in kg/m² °	24.8 [22.6–27.4]	24.0 [22.6–26.5]	25.2 [22.4–29.9]
Vaccine			
mRNA-1273 (Moderna; %)	47 (97.9)	23 (95.8)	24 (100)
BNT162b2 (BioNTech/Pfizer; %)	1 (2.1)	1 (4.2)	0
PID group			
CVID	11 (22.9)	-	11 (45.8)
Other antibody deficiency	11 (22.9)	-	11 (45.8)
Other PID	2 (4.2)	-	2 (8.3)
IGRT			
Active (%)	20 (41.7)	-	20 (83.3)
Paused (%)	2 (4.2)	-	2 (8.3)
None (%)	2 (4.2)	24 (100)	2 (8.3)
Antibody Response			
Anti-RBD antibodies, any response (%)	45 (93.8)	24 (100)	21 (87.5)

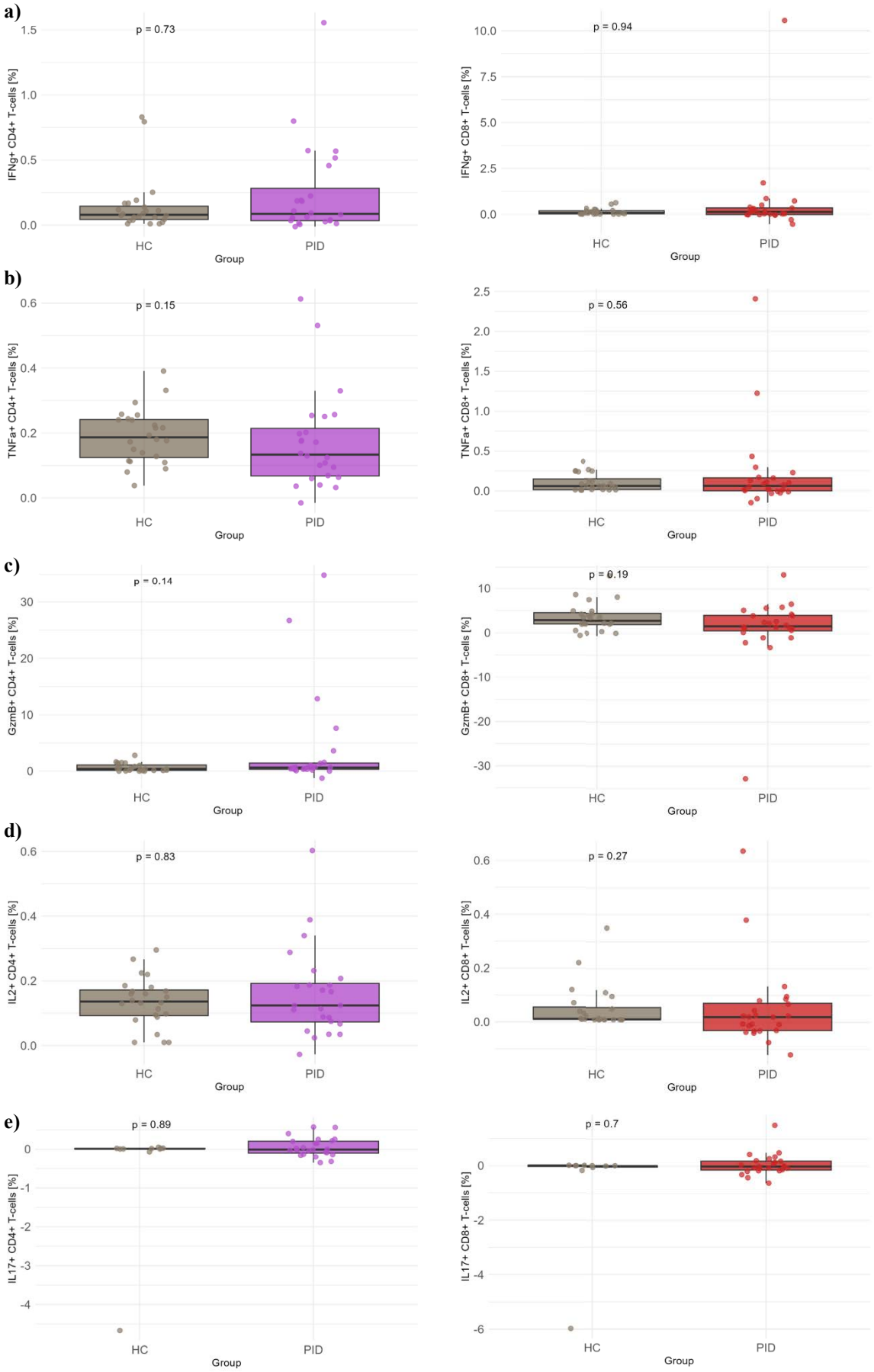
Table 9 – Baseline characteristics of the selected population of the 3rd interim analysis.

The last row shows the number of participants with a positive antibody response in the Roche assay. *Mean ± SD, normally distributed. °Median [25th–75th percentile], not normally distributed; BMI was only available for 23 of 24 HCs.

3.4.2. Comparison of T-cell responses to COVID-19 vaccination in PID patients and healthy controls

Given that our immunodeficient patients predominantly suffered from humoral immunodeficiencies, some were unlikely to mount adequate antibody responses following COVID-19 vaccination (see also 3.2.). Therefore, we intended to determine whether these patients could still elicit T-cell responses comparable to those observed in healthy individuals.

Data on Spike-protein-induced production of IFN-γ, TNF-α, IL-2, and Granzyme B, each by $CD4^+$ and $CD8^+$ T cells, three to four weeks after the second vaccination was available for all 48 patients and healthy controls (HCs). Moreover, we measured IL-17 production by $CD4^+$ and $CD8^+$ T cells in all patients and ten controls. Data on induction of the activation markers CD137 and CD154 was included for all patients and controls. Calculated by the Mann–Whitney U test, none of these T-cell responses was significantly different between PID patients and healthy controls ($p > 0.05$), indicating that our PID patients reached comparable T-cell response levels like HCs. The results are graphically illustrated in Figure 14.


a)
p = 0.73
IFNg+ CD4+ T-cells [%]
1.5
1.0
0.5
0.0
HC
PID
Group
p = 0.94
IFNg+ CD8+ T-cells [%]
10.0
7.5
5.0
2.5
0.0
HC
PID
Group
b)
p = 0.15
TNFa+ CD4+ T-cells [%]
0.6
0.4
0.2
0.0
HC
PID
Group
p = 0.56
TNFa+ CD8+ T-cells [%]
2.5
2.0
1.5
1.0
0.5
0.0
HC
PID
Group
c)
p = 0.14
GzmB+ CD4+ T-cells [%]
30
20
10
0
HC
PID
Group
p = 0.19
GzmB+ CD8+ T-cells [%]
10
0
-10
-20
-30
HC
PID
Group
d)
p = 0.83
IL2+ CD4+ T-cells [%]
0.6
0.4
0.2
0.0
HC
PID
Group
p = 0.27
IL2+ CD8+ T-cells [%]
0.6
0.4
0.2
0.0
HC
PID
Group
e)
p = 0.89
IL17+ CD4+ T-cells [%]
0
-1
-2
-3
-4
HC
PID
Group
p = 0.7
IL17+ CD8+ T-cells [%]
0
-2
-4
-6
HC
PID
Group

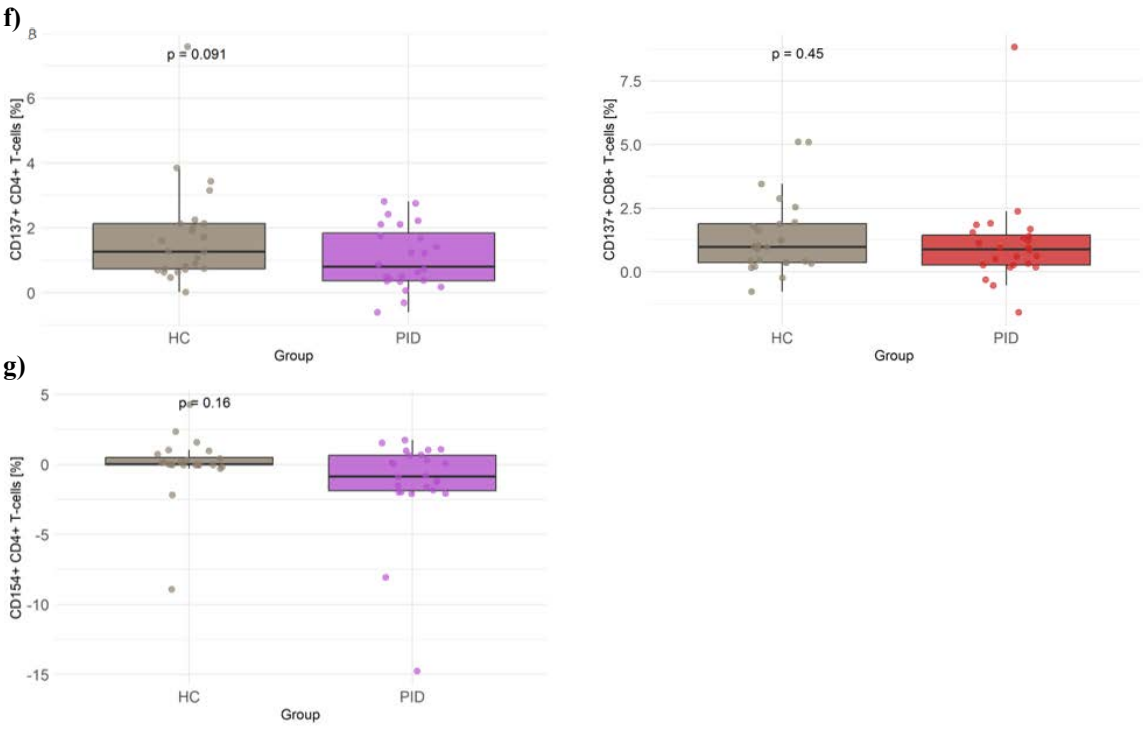


Figure 14 – T-cell responses to SARS-CoV-2 spike protein in healthy controls (HC) and PID patients after two COVID-19 vaccinations.

Graphs a), b), c), d), and e) depict the frequencies of IFN-γ-, TNF-α-, GzmB-, IL-2-, and IL-17-producing $CD4^+$ and $CD8^+$ T cells, respectively, in HCs and PID patients determined by flow cytometry. Graphs f) and g) display the frequencies of $CD4^+$ and $CD8^+$ T cells positive for activation markers CD137 and CD154, respectively. P-values are indicated for each analysis. IL-17 results were obtained from 10 of the HCs and all patients.

In order to further investigate whether patients and HCs exhibited a different cellular response pattern across cytokine responses, we applied MANOVA. For the multivariate tests, IFN-γ, TNF-α, IL-2, and Granzyme B responses by $CD4^+$ as well as $CD8^+$ T cells were included, as IL-17 results were incomplete for some participants. All multivariate tests displayed a trend towards group difference ($F = 2.116$) with a large effect size ($\eta^2_p = 0.303$) but did not reach significance by $p = 0.058$ (Table 10). Additionally, it should be noted that Box's test of equality of covariance matrices was significant ($p < 0.001$), and Levene's test of equality of error variances also indicated significant deviations, which both implies a diminished robustness of these MANOVA findings.

Overall, the findings might indicate a tendency towards group differences; however, due to statistical limitations, the data suggest mainly comparable cellular response patterns between PID patients and healthy controls.

Test Statistic	Value	*F*	Hypo-thesis *df*	Error *df*	p-value	Partial Eta Squared
Pillai's Trace	0.303	2.116	8.0	39.0	0.058	0.303
Wilks' Lambda	0.697	2.116	8.0	39.0	0.058	0.303
Hotelling's Trace	0.434	2.116	8.0	39.0	0.058	0.303
Roy's Largest Root	0.434	2.116	8.0	39.0	0.058	0.303

Table 10 - Multivariate test statistics assessing the effect of group (HC vs. PID) on combined T-cell outcomes.

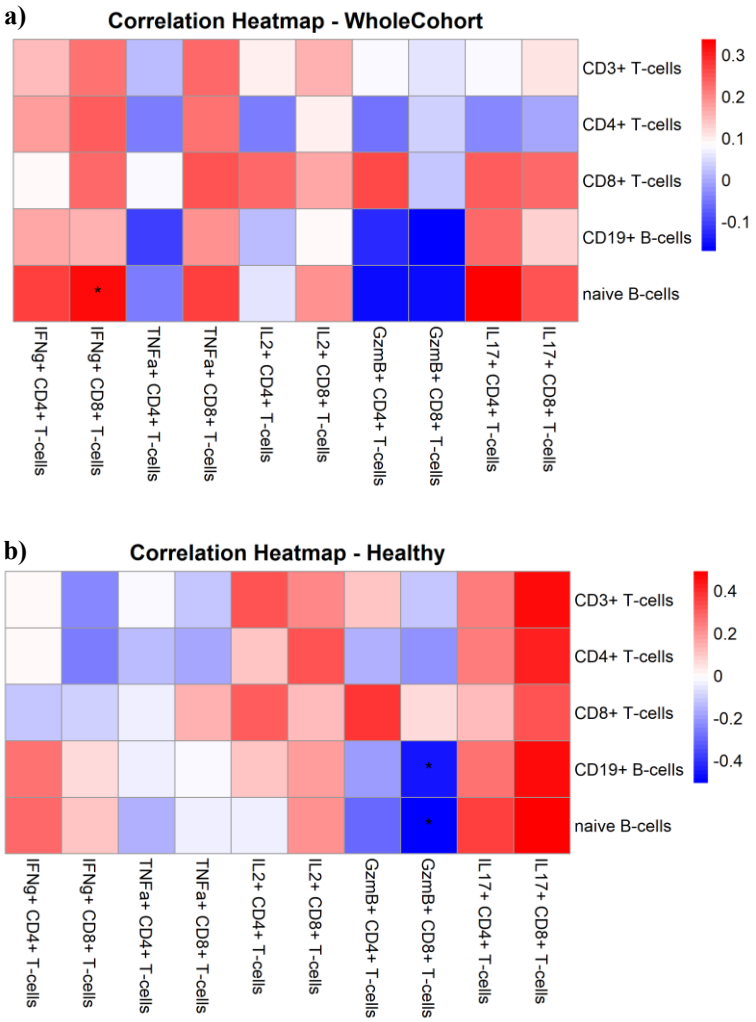
Values are shown for four multivariate tests, Pillai's Trace, Wilks' Lambda, Hotelling's Trace, and Roy's Largest Root, all yielding the same F-value that indicates the ratio of explained to unexplained variance by the group effect. The partial eta squared reflects the proportion of multivariate variance explained by the group effect.

3.4.3. Correlation of cellular baseline parameters with the cellular vaccination responses

In the following, similar to the preceding research question (see 3.3), we investigated whether selected lymphocyte subsets, that had shown promising correlations in prior analyses, were associated with the Spike-specific T-cell cytokine responses. For this aim, we calculated correlations between absolute counts of total T cells, T helper cells, cytotoxic T cells, total B cells, and naïve B cells with all ten cytokine results – for the entire cohort as well as separately for PID patients and healthy controls.

In the entire cohort, only the correlations between naïve B cells and IFN-γ-producing $CD8^+$ T cells ($\rho = 0.327$, $p = 0.026$) and between naïve B cells and IL-17-producing $CD4^+$ T cells ($\rho = 0.337$, $p = 0.051$) were significant or borderline-non-significant, respectively, both indicating a moderate positive correlation. However, after adjustment for multiple testing, these associations were no longer statistically significant ($p = 1.000$). To elucidate whether there are significant correlations in the subgroups, we analyzed them separately and observed additional significant correlations, which were also slightly stronger than in the calculation for the entire cohort. In the PID patient group, total T cells, T helper cells, cytotoxic T cells and, as in the entire cohort, naïve B cells each correlated moderately positive with IFN-γ-producing $CD8^+$ T cells. Furthermore, total T cells, T helper cells, and naïve B cells were moderately positively correlated with TNF-α-producing $CD8^+$ T cells, and, consistent with the entire cohort, naïve B

cells with IL-17-producing $CD4^+$ T cells. Interestingly, in the healthy cohort, we detected a moderate negative correlation between total and naïve B cells and GzmB-producing $CD8^+$ T cells. Nevertheless, after adjustment for multiple testing, these associations were no longer statistically significant either (all p-values between 0.731 and 1.000), indicating that the observed results represent only trends rather than definitive correlations. Figure 15 illustrates the results of the Spearman correlation analysis, including details on significance levels without adjustment, for the entire cohort and both cohorts separately as correlation heatmaps.



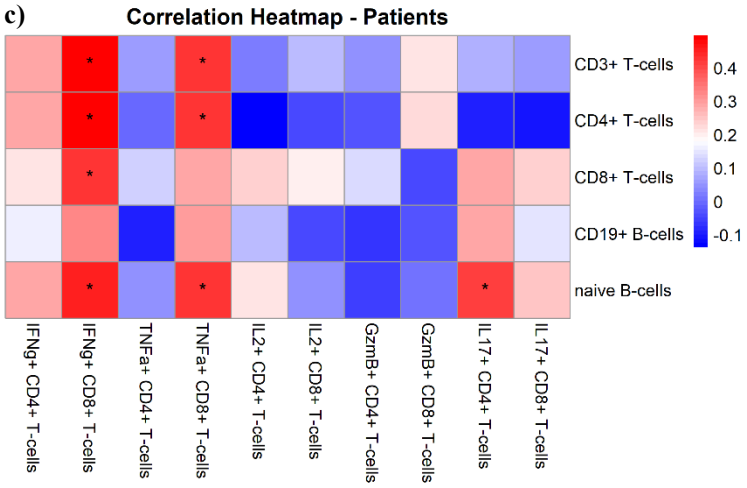


Figure 15 – Correlations of selected lymphocyte subsets with T-cell responses after vaccination.
Graphs are correlation heatmaps of five selected lymphocyte subsets (baseline, before vaccination) and all ten measured cytokine responses in $CD4^+$ and $CD8^+$ T cells after the second COVID-19 vaccination for a) the entire cohort, b) HCs, and c) PID patients separately. Note that asterisks correspond to significant values ($p < 0.05$) before adjustment for multiple testing. After adjustment, none of the correlations showed significance (all $p > 0.05$). IL-17 results were obtained from 10 of the HCs and all patients.

3.4.4. Comparison of the humoral vaccination response between PID patients and healthy controls

Next, to elucidate the potential link between humoral and cellular vaccination response as described in section 3.4.5., we first compared the humoral responses of PID patients and healthy controls (HCs) in detail, using the extended antibody titers obtained by serial dilution beyond the upper quantification range of the Roche assay (for a general overview, see also 3.2.2.). We detected a significant difference ($Z = 2.835$; $p = 0.005$, Mann–Whitney U test) between the two groups, with healthy controls exhibiting higher antibody titers, as expected (see Figure 16a). The median antibody concentration was 3866.5 U/ml in healthy participants and 1556.0 U/ml in PID patients. Calculation of the non-parametric effect size r ($r = \frac{Z}{\sqrt{N}} = \frac{2.835}{\sqrt{48}} \approx 0.41$) indicated a moderate effect size of this difference.

Furthermore, we investigated the influence of specific underlying diseases by comparing the three disease subgroups with each other and with the healthy controls. Although the Kruskal–Wallis test showed an overall significant difference between the subgroups (two-sided $p = 0.037$), subsequent pairwise comparisons revealed that significant differences were only

present between CVID patients and healthy controls ($p = 0.010$) and between patients with other antibody deficiency and healthy controls ($p = 0.038$), but not between the patient subgroups themselves (Table 11). The third comparison, between "other" PID patients and healthy controls, narrowly missed significance ($p = 0.514$), presumably due to the low group size of only two "other" PID patients. Moreover, after adjustment for multiple testing, even the described significant differences between the distinct disease subgroups and healthy controls could not be confirmed (adjusted $p = 0.058$ for CVID vs. HC; adjusted $p = 0.226$ for other antibody deficiency vs. HC), suggesting that there is an overall difference between our patients and healthy controls which cannot be clearly attributed to a specific PID subgroup – at least when applying strict Bonferroni correction. The results of the pairwise comparison are presented in Table 11, and Figure 16b illustrates the anti-RBD antibody titers across the subgroups.

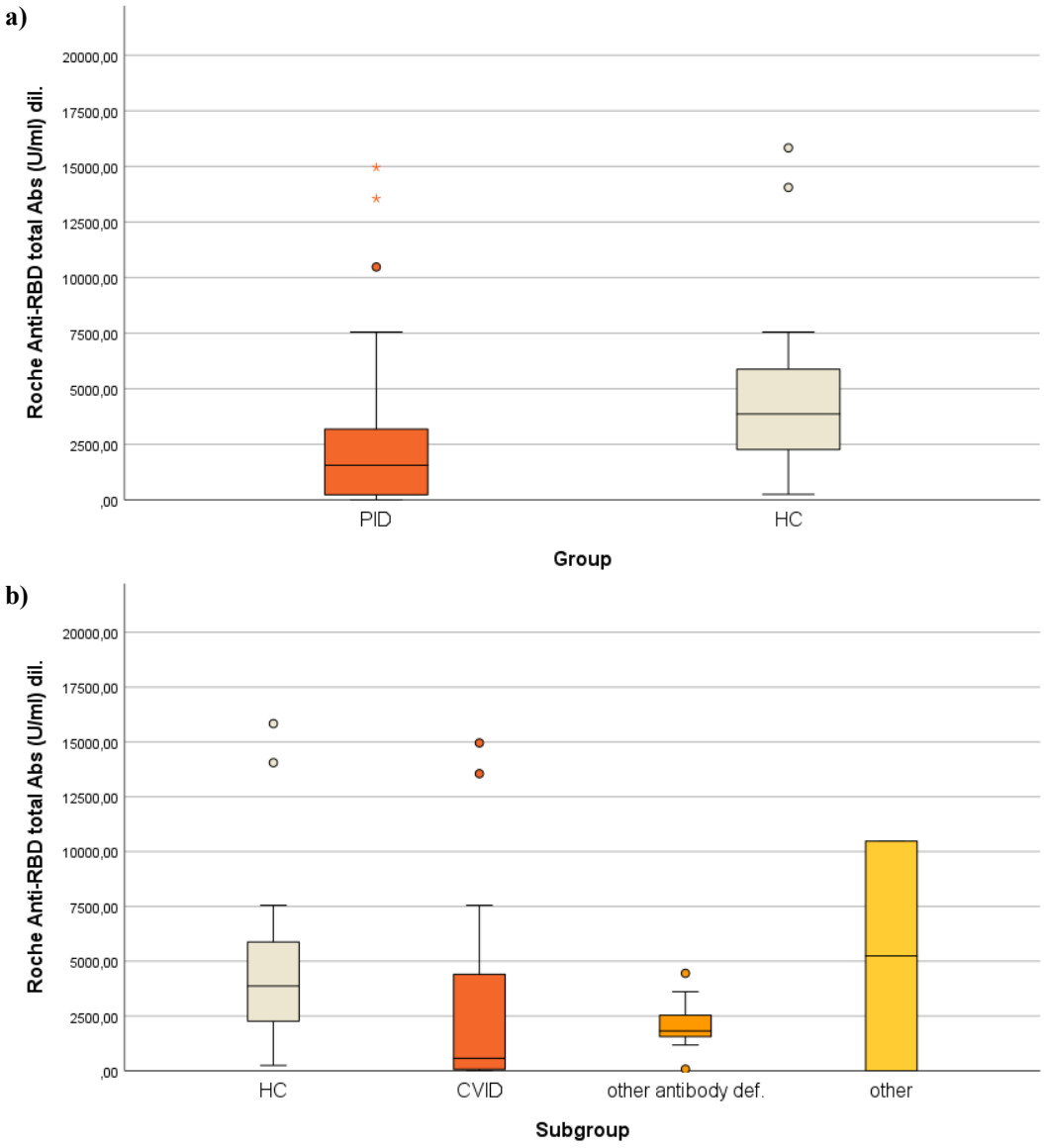


Figure 16 – Roche anti-RBD total antibody levels of HCs and PID patients.

Graph a) shows the results of the antibody dilution for PID patients and HCs, b) depicts the results for HCs and the three PID subgroups (CVID, other antibody deficiency, and other PID). Circles (°) indicate values that lie more than 1.5 times the IQR away from the box (i.e., beyond the 25th or 75th percentile). Asterisks (*) indicate extreme values that lie more than 3 times the IQR away from the box. Observed differences in a) are statistically significant (p = 0.005). Note that subgroup "other" consisted of only two patients. CVID, Common variable immunodeficiency; HC, healthy controls.

Pairwise comparison of subgroups

	Test statistic	Standard error	Std. test statistics	*p*	**Adj. Sig.[a]**
CVID – Other antibody def.	−2.591	5.969	−0.434	0.664	1.000
CVID – Other	−6.455	10.761	−0.600	0.549	1.000
CVID – HC	13.184	5.097	2.586	**0.010**	0.058
Other antibody def. – Other	−3.864	10.761	−0.359	0.720	1.000
Other antibody def. – HC	10.593	5.097	2.078	**0.038**	0.226
Other – HC	6.729	10.303	0.653	0.514	1.000

Table 11 – Comparison of mounted Anti-RBD antibody levels between the disease subgroups and healthy controls.

Every row shows the test statistics for the pairwise comparison of two subgroups. Calculated by Kruskal–Wallis test, asymptotic significance values (two-sided tests) are displayed. The significance level is 0.050, significant values are highlighted in bold type. Note that subgroup "Other" consisted of only two patients. CVID, Common variable immunodeficiency; HC, healthy controls. [a] Significance values are adjusted for multiple comparisons using the Bonferroni correction.

3.4.5. Correlation of humoral and cellular vaccination response

In the final step, we investigated whether the anti-RBD antibody responses correlated with components of the cellular vaccination response, again with focus on the cytokine responses. As the Spearman correlation analysis only provided approximate p-values due to tied ranks in the dataset, Kendall's tau was additionally calculated and the corresponding values are reported below.

In the entire cohort, we observed a weak but statistically significant correlation between anti-RBD antibodies and both IFN-γ-producing $CD8^+$ T cells ($\tau = 0.208$, $p = 0.037$) and IFN-γ-producing $CD4^+$ T cells ($\tau = 0.207$, $p = 0.038$). However, these associations did not remain statistically significant after adjustment for multiple testing (adjusted $p = 0.375$ and adjusted $p = 0.383$, respectively). When analyzing potential correlations separately in patients and healthy controls, these trends were not observed in either cohort. Moreover, none of the other tested Spike-specific cytokine responses demonstrated a significant correlation with the vaccination-induced humoral response. Figure 17 presents the Kendall's tau correlation coefficients for these three analyses before adjustment.

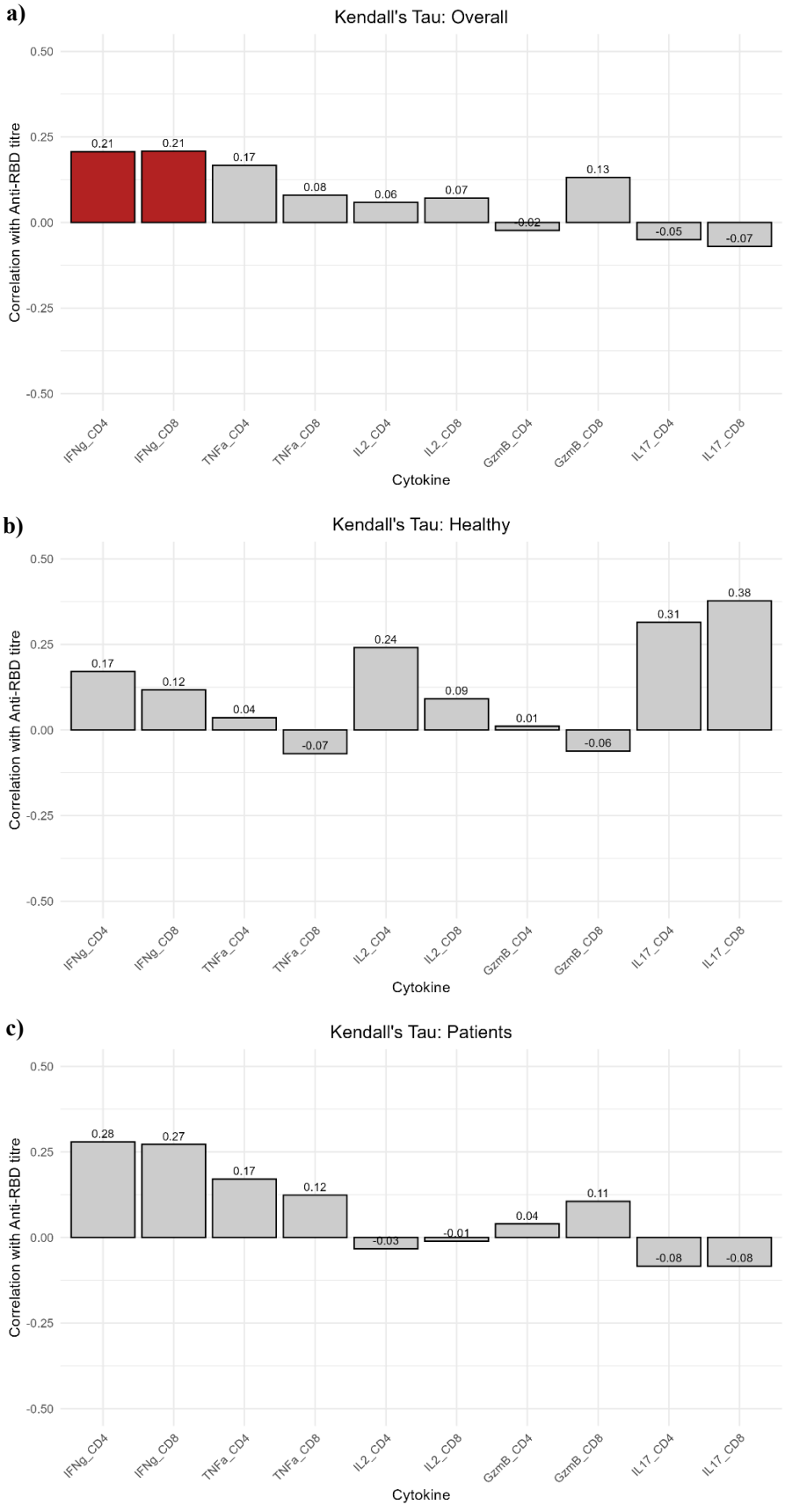


Figure 17 – Correlations of anti-RBD antibody titers and T-cell responses in HCs and PID patients.

Graphs show the correlation τ of anti-RBD antibody titers and T-cell responses, both after 2nd COVID-19 vaccination, for a) the entire cohort, b) HCs, and c) PID patients. Significant values before adjustment for multiple testing are indicated by red bars ($p > 0.05$). After adjustment, none of the correlations was statistically significant. Note that IL-17 results were obtained from 10 of the HCs and all patients.

3.5. Summary of Results

In summary, immunocompromised patients achieved a significantly lower seroconversion rate after COVID-19 vaccination compared to healthy controls. However, the seroconversion rate varied depending on the underlying cause of immunodeficiency, with patients suffering from autoimmune diseases receiving B-cell-depleting therapy exhibiting the lowest anti-SARS-CoV-2 antibody amounts. In contrast, certain patients with hematologic malignancies, post-hematopoietic stem cell transplantation (HSCT), and with primary immunodeficiencies (PID) developed antibody responses comparable to those of healthy participants. The number of circulating naïve B cells was strongly associated with anti-SARS-CoV-2 antibody levels and the only independent predictor for achievement of high antibody levels comparable to healthy controls. A cut-off of ≥60 naïve B cells per µL was calculated to distinguish between patients with and without a robust (stringent) antibody response.

Regarding the analysis of T-cell responses following COVID-19 vaccination, patients with autoimmune diseases under B-cell-depleting therapy showed responses that were in part comparable to those of healthy controls. However, TNF-α production by $CD4^+$ T cells and Granzyme B production by $CD8^+$ T cells were significantly reduced in these patients. Moreover, while cytokines and activation markers correlated well with each other in healthy participants, this pattern was partially lost in patients, particularly concerning the correlation between activation markers and certain cytokine responses. Additionally, some lymphocyte subsets measured before vaccination showed a negative correlation with Spike-specific Granzyme B production by $CD8^+$ T cells after vaccination, specifically total and naïve B cells in healthy controls and total T cells and T helper cells in B-cell-depleted patients. The patients also exhibited a negative correlation between IFN-γ-producing $CD4^+$ T cells and total $CD8^+$ T-cell count.

In terms of T-cell responses of patients with primary immunodeficiencies, rates of cytokine responses were comparable to those of healthy controls. Contrary to the controls, PID patients displayed trends towards positive correlations between IFN-γ-producing $CD8^+$ T cells and total

T cells, $CD4^+$ and $CD8^+$ T cells, and naïve B cells; between TNF-α-producing $CD8^+$ T cells and total T cells, $CD4^+$ T cells, and naïve B cells; and between IL-17-producing $CD4^+$ T cells and naïve B cells. No distinct correlation of anti-RBD-antibody titers and the assessed cytokine responses after the second vaccination could be identified in either PID patient or HC cohort.

Taken together, these results highlight the complexity of humoral and cellular immune responses not only in healthy controls but also in immunodeficient patients, and their implications will be discussed in the following section.

4. Discussion

During the pandemic, several vulnerable groups in our society were at high risk of severe or even fatal COVID-19 infections. In 2021, the first SARS-CoV-2 specific vaccines became available, and it was uncertain, whether immunocompromised patients would benefit from this vaccination, even though their underlying immunodeficiencies might impair an adequate immune response.

This work comprehensively examines both humoral and cellular immune responses to COVID-19 vaccination in different groups of immunocompromised patients compared to healthy individuals, covering the period from before the initial vaccination up to the third (booster) dose. The particular focus of this thesis lies on patients suffering from autoimmune diseases (AID) receiving B-cell-depleting therapy and on patients with primary immunodeficiencies (PID). The aim was to investigate whether these patients could respond to the vaccination comparably to healthy controls, implying a potential protection against SARS-CoV-2 infection, and to identify potential markers predictive of protection.

Our main findings include the observation that immunodeficient patients exhibited reduced SARS-CoV-2 antibody responses compared to healthy controls, with naïve B-cell counts being a strong predictor for successful seroconversion. Nevertheless, patients showed distinct T-cell responses following vaccination, although the specific response patterns varied between healthy controls and patients, especially regarding patients post B-cell-depleting therapy.

In the following, the findings will be discussed in the context of existing literature and their current and future clinical implications.

4.1. Humoral vaccination response in immunodeficient patients

Antigen-specific antibodies are known to confer protection in many infectious diseases – both after natural infection and in response to vaccinations. However, a wide range of diseases or therapies, especially immunosuppressive medications, may impair the ability of the adaptive immune system to produce such antibodies. This was also evident in our study, in which immunocompromised patients showed significantly reduced SARS-CoV-2-specific antibody

responses following the first two SARS-CoV-2 mRNA vaccinations compared to healthy controls. Only 67% of patients exhibited any antibody response. In comparison to healthy controls, only 32% of patients achieved an antibody level that was at least as high as the weakest response of healthy controls, which we defined as “stringent response” (≥1000 U/ml).

Of note, depending on the underlying cause of immunodeficiency, the specific impairment in the vaccination response varied. While all healthy controls exhibited high anti-SARS-CoV-2 antibody titers with a median of 2500 U/ml, we observed that patients suffering from autoimmune diseases (AID) receiving B-cell-depleting therapy mounted the lowest antibody titers overall. They were followed by patients with hematologic malignancies with and without hematopoietic stem cell transplantation (HSCT). Interestingly, some of these hematologic patients still achieved high antibody titers and fulfilled our definition of a stringent response. This observation was even more pronounced in patients with primary immunodeficiencies (PID), who exhibited a wide range of antibody responses with a considerable median of more than 1500 U/ml.

When we started our study and performed this interim analysis in 2021, first comparable results from other research groups were also published. Mrak et al. analyzed 74 rheumatic patients who had undergone B-cell depletion with rituximab, and they also reported lower levels of antibodies against SARS-CoV-2 RBD in these patients compared to healthy individuals (75): Only 39% of patients developed any specific antibodies and 36 out of 37 patients without detectable peripheral B cells did not seroconvert at all (75). Another cohort of 264 patients with various inflammatory rheumatic diseases receiving different immunomodulatory therapies was examined by Braun-Moscovici et al. (79). After the second COVID-19 vaccination, 86% of their patients mounted anti-SARS-CoV-2 antibodies. Of the 37 patients who did not, the majority (22 patients) had received an anti-CD20 treatment, i.e., B-cell depletion (79). According to their study, other therapies that were associated with impaired seroconversion in multivariate logistic regression analysis included, besides rituximab, abatacept (a CTLA-4-Ig fusion protein) and mycophenolate mofetil (MMF) (79).

With regard to patients with hematological malignancies, Maneikis et al. also reported heterogeneous antibody responses in a cohort of 857 initially seronegative patients, depending on their specific therapy. Patients actively treated with Bruton’s tyrosine kinase inhibitors, ruxolitinib (a Janus kinase inhibitor), venetoclax (a Bcl-2 inhibitor), or anti-CD-20 therapies

exhibited particularly low anti-S1 IgG antibody responses, whereas patients after HSCT mounted the highest antibody responses within their cohort (80).

Early results of PID patients were reported by Hagin et al. In their heterogeneous cohort of 26 adult patients, 18 developed specific antibodies two weeks after the second COVID-19 mRNA vaccination (81). 13 of these 18 patients were CVID patients and therefore comparable to our cohort. Six of the eight patients who did not seroconvert suffered from X-linked agammaglobulinemia (XLA, n = 4, not included in our cohort), combined immunodeficiency (n = 1), or autoimmune lymphoproliferative syndrome (ALPS)-like disease (n = 1, also not included in our cohort); in addition, two CVID patients failed to mount an antibody response (81). Interestingly, the authors observed a trend towards higher antibody titers in younger CVID patients (81). In another study, Arroyo-Sánchez et al. investigated the immunogenicity of the first two doses of anti-SARS-CoV-2 vaccines specifically in CVID patients (82). Similarly, they reported S1-specific antibodies in 83% of patients. However, neutralizing antibodies were only detectable in 50% of all patients. Notably, two of the three patients without seroconversion had received rituximab within the previous two years, suggesting that B-cell-depleting therapy, rather than the underlying immunodeficiency itself, may have been the limiting factor in those cases (82).

In consideration of the heterogeneous humoral responses observed in our immunodeficient patient groups, we further explored immunological predictors of seroconversion. Notably, the number of circulating naïve B cells showed a strong association with antibody response. In our analysis, it was the only independent predictor for a stringent response, with a threshold of 60 cells per µL required to achieve antibody levels comparable to those of healthy individuals. This emphasizes their potentially essential role in generating a functional humoral immune response.

Although comparable studies have assessed the role of B cells and their subsets in the effectiveness of SARS-CoV-2 vaccination (83–86), publications quantifying cut-offs for naïve B cells remain scarce, if they exist at all – both for SARS-CoV-2 and other vaccines – thus making this a notable novelty of our study. Our general observation was supported a few months after our publication by Asplund Högelin et al., who reported a correlation between naïve and, additionally, memory IgG^+ B cells with SARS-CoV-2 vaccine-induced antibody levels in 94 anti-CD20-treated multiple sclerosis patients (84). However, they did not provide specific

thresholds. Moor et al. described in a study with 96 patients, who had also received B-cell-depleting therapy (rituximab or ocrelizumab) for autoimmune diseases, malignancies, or following transplantation, that peripheral B-cell count (>27 cells/µL), $CD4^+$ lymphocyte count (>653 cells/µL), and time since last anti-CD20 therapy (>7.6 months) were predictive of the humoral response to two doses of SARS-CoV-2 vaccine (83). Interestingly, this deviated from the observations in our cohort, which may be due to different statistical approaches or to our stricter definition of response, searching for correlations between lymphocyte subsets and stringent rather than any antibody response. Moreover, our results indicated that a shorter interval of at least 115 days (approximately 3.9 months) after the last B-cell-depleting therapy was already sufficient to mount any antibody response.

As naïve B cells are mature B cells circulating in the periphery that have not yet encountered their cognate antigen, they are a prerequisite for mounting a humoral immune response to novel antigens, such as those presented in vaccines. The importance of naïve B cells in generating efficient antibody responses had already been recognized before the COVID-19 pandemic. Turner et al. examined human germinal center responses after influenza vaccination by performing fine needle aspiration of draining lymph nodes (87). Comparing somatic hypermutation (SHM) levels of peripheral and germinal center (GC) B cells, they observed a population of vaccine-induced GC B cells that was absent in the early circulating peripheral B cells and expressed low SHM levels, indicating that they originated predominantly from naïve B cells (87). Remarkably, Turner et al. supported their findings in a later study analyzing GC B cells following SARS-CoV-2 mRNA vaccines (88). However, in this study, they additionally detected some cross-reactive GC B cells clones that bound conserved epitopes of betacoronavirus S proteins and showed significantly higher SHM rates, indicating an origin from memory B cells (88). This may imply that some individuals who do not develop (severe) COVID-19 may benefit from prior exposure to endemic coronaviruses and subsequent seroconversion – a hypothesis we are investigating in a follow-up study of the CoVVac study, CoVVacBoost.

Naïve B cells can be routinely measured in clinic settings and may therefore serve as a prognostic biomarker for the humoral vaccine response. Particularly in B-cell-depleted patients, it may be beneficial to first measure B-cell subsets and subsequently decide whether to administer a due vaccination immediately or at another time point. If B-cell subset analysis is not readily available, it might help to consider the time since last B-cell depletion. An interval

of at least four months prior to vaccination may enhance the likelihood of mounting an antibody response, at least in the context of COVID-19 mRNA vaccines, according to our analysis. Longer intervals further increase the probability of seroconversion due to rising (naïve) B-cell repopulation. Other authors have reported deviating minimal intervals, based on various statistical approaches, ranging from less than three months in a cohort of multiple sclerosis patients (89) to up to twelve months in a cohort of B-cell non-Hodgkin lymphoma patients (90). In a meta-analysis including 28 studies with a total of 1455 patients receiving anti-CD20 antibody therapy, Liu et al. calculated a cut-off of 5.5 months since the last B-cell-depleting therapy necessary for seroconversion (91).

However, it should also be acknowledged that immunocompromised patients, including those after B-cell-depleting therapy, may still benefit from T-cell responses that can emerge after some vaccinations, such as COVID-19 vaccinations, as discussed in the next paragraphs (see 4.2).

4.2. Cellular vaccination response in immunodeficient patients

T cells are key components of adaptive immunity against natural infections and certain vaccines – not only as T follicular helper cells (Tfh) that support B cells in producing specific antibodies, but also as cytotoxic T cells that can eliminate infected cells or cells presenting foreign surface antigens, such as the viral spike protein. Particularly in patients lacking (functional) B cells, as described in 4.1, these T-cell functions may become even more important for protection against infection. However, this ability may be impaired in immunocompromised patients as well, depending on the specific underlying cause of immunodeficiency.

In this thesis, T-cell responses specifically of patients with autoimmune diseases receiving B-cell-depleting therapies and of patients with primary immunodeficiencies following the first two doses of COVID-19 mRNA vaccines were examined. We found that both patient cohorts were able to mount spike-specific T-cell responses and thus might obtain a certain level of protection against SARS-CoV-2, but in detail, these responses differ from healthy controls, particularly in the B-cell-depleted patient cohort.

4.2.1. Cellular vaccination responses in B-cell-depleted autoimmune patients

Our autoimmune patients under B-cell-depleting therapy with rituximab or ocrelizumab were a heterogeneous cohort of patients with inflammatory rheumatic diseases and multiple sclerosis (MS). In comparison to healthy individuals, they exhibited a markedly reduced TNF-α production by $CD4^+$ T cells and GzmB production by $CD8^+$ T cells. Moreover, while the majority of tested cytokines and activation markers showed positive correlations in the healthy controls, this pattern was not observed in these patients: In contrast to healthy individuals, they showed neither positive correlations between activation markers and cytokines nor between GzmB and other cytokine responses. In addition, we observed altered correlation patterns between $CD4^+$ T cells producing IL-2, TNF-α, or IFN-γ and $CD8^+$ T cells producing TNF-α or IFN-γ. With regard to factors influencing the T-cell response, we found no major correlations with seroconversion status, concomitant immunosuppressive therapy, or underlying disease. However, we did observe a negative correlation between GzmB production by $CD8^+$ T cells and both total and $CD4^+$ T-cell counts in the patients. Furthermore, patients receiving ocrelizumab exhibited, for some cytokines, higher spike-specific T-cell responses than those treated with rituximab.

In contrast to our findings, Madelon et al. reported equal or even higher SARS-CoV-2-specific T-cell responses in patients than in healthy controls in a cohort of 37 patients with rheumatic diseases (n = 11) or multiple sclerosis (n = 26) treated with rituximab or ocrelizumab, respectively (92). Using flow cytometry, they described overall spike-specific response rates that were similar between patients and controls in $CD4^+$ T cells and even higher in $CD8^+$ T cells among patients (ocrelizumab 96.2%, rituximab 81.8%) than in controls (66.7%). Furthermore, spike-specific T cells from patients showed an increased expression of effector molecules (92). However, direct comparison with our results is limited due to differences in the methods: While Madelon et al. quantified general spike-specific $CD4^+$ and $CD8^+$ T-cell responses, our study focused on cytokine-specific T-cell subpopulations.

A different interesting approach was taken by Alfonso-Dunn et al., who investigated 43 multiple sclerosis patients under anti-CD20 therapy (93). They applied both a FluoroSpot assay – which is similar to our ELISpot but detects more than one secreted cytokine simultaneously at single-cell level – and a T-cell proliferation assay after spike protein stimulation. They report that even patients without seroconversion demonstrate significantly enhanced polyfunctional

IFN-γ^+ and IL-2^+ T-cell responses, as well as strong $CD4^+$ and $CD8^+$ T-cell proliferation in comparison to healthy controls (93). Based on these data, the authors concluded that B-cell-depleted MS patients mount a partial adaptive immune response after SARS-CoV-2 vaccination through functionally preserved T cells. In contrast, while we also observed detectable T-cell responses in B-cell-depleted patients, our findings suggest an altered rather than preserved response profile. This discrepancy may partly reflect differences in the analytical approach: Unlike Alfonso-Dunn et al., we did not assess polyfunctionality via ELISpot or perform T-cell proliferation assays, but instead conducted flow cytometric analyses of a broad panel of cytokines and activation markers at single-cell level, followed by a pattern analysis based on correlation analyses. Moreover, our cohort also included patients with rheumatic autoimmune diseases, who may exhibit other immunological features than MS patients.

Comparable publications examining correlation patterns of T-cell responses and their association with other immune parameters remain scarce. In an early study by Apostolidis et al. (2021), the authors analyzed 20 patients with multiple sclerosis undergoing anti-CD20 monotherapy and conducted extensive immunophenotyping at three time points after SARS-CoV-2 mRNA vaccination (94). Their analysis included spike-specific IgG levels and activation-induced T-cell subsets, which were compared between patients and healthy controls. They reported a strong correlation between humoral responses and circulating T follicular helper (Tfh) cells in both groups, which emerged earlier in the patient cohort. In addition, they observed a strong negative correlation between induced $CD8^+$ T cells and humoral immunity exclusively in the patient group 25–30 days after the second vaccination. Moreover, in contrast to healthy controls, induced Th1 cells did not positively correlate with features of humoral immunity in B-cell-depleted patients (94). Although the authors mainly investigated different immunological markers than we did, their findings similarly suggest, in part, altered patterns of cellular coordination in B-cell-depleted patients – particularly demonstrated by a predominantly positive correlation pattern in the controls in contrast to the patients.

In another study by Stefanski, Rincon-Arevalo et al., involving 19 patients with rheumatoid arthritis or ANCA-associated vasculitis receiving rituximab, the authors analyzed correlations between anti-RBD antibody titers, RBD^+ B cells and their subsets, activated T-cell subsets, and spike-specific T-cell subsets (95). These relationships were visualized in an extensive correlation matrix. Interestingly, the study reported a significant and strong positive correlation between antigen-specific TNF- and IFN-γ-producing $CD4^+$ T cells, on the one hand, and RBD^+

plasmablasts, RBD^+ switched memory B cells, as well as neutralizing antibodies, on the other. In contrast, no such correlations were observed between antigen-specific $CD8^+$ T-cell responses and humoral immunity, RBD^+ B-cell subsets, or activated or spike-specific $CD4^+$ T-cell subsets (95). The authors interpreted this as indicative of a direct antigen-driven cellular immunity that is independent of the interaction of $CD4^+$ T cells and B cells typically needed for IgG production (95). However, the study did not include a healthy control group, which limits conclusions regarding the role of disease background or B-cell depletion in these associations. Furthermore, in contrast to our approach, no cytokines other than TNF and IFN-γ were analyzed as functional T-cell response, which further limits direct comparability with our findings as we focused on GzmB expression as a major $CD8^+$ T-cell effector molecule. Nevertheless, the absence of positive correlations between $CD8^+$ T-cell responses and B-cell-dependent parameters in both studies may indicate a more autonomous role of $CD8^+$ T cells in the vaccine-induced response of B-cell-depleted patients. In our study, however, this compartment was not functionally isolated but showed negative correlations with other lymphocyte subsets – suggesting a qualitative shift rather than a robust compensatory response.

Finally, although one might assume an identical outcome, patients receiving ocrelizumab exhibited higher spike-specific T-cell responses for some cytokines than patients treated with rituximab – a finding that was also reported by Madelon et al. (92), but not by Alfonso-Dunn et al. (93). This difference may be explained by several factors, including distinct effector mechanisms of B-cell depletion, differences in the underlying patient populations (MS patients versus rheumatologic patients), and variation in treatment intervals.

4.2.2. Cellular vaccination responses in patients with primary immunodeficiencies

In contrast to patients receiving B-cell-depleting therapies, individuals with primary immunodeficiencies (PID) exhibit congenital impairments of various components of the immune system, depending on the specific genetic defect. Given the heterogeneity of these conditions, immune responses are difficult to predict. Our cohort predominantly consisted of patients with humoral immunodeficiencies, namely patients with common variable immunodeficiency (CVID) and other antibody deficiencies. Thus, we hypothesized they might be able to exhibit a potentially protective T-cell response despite a reduced capacity to mount specific antibodies.

As expected, our PID patients mounted significantly lower spike-specific antibody levels than healthy controls. However, with a median of more than 1500 U/ml, many of these patients still achieved considerable levels – particularly when viewed in the context of our earlier analysis comparing humoral vaccination responses across various immunodeficient patient groups (see 3.2.2). Of note, patients and controls did not demonstrate different levels of spike-specific $CD4^+$ or $CD8^+$ T-cell responsiveness for any of the tested cytokines and activation markers. With regard to correlations of these cytokine responses and certain lymphocyte subsets, our PID patients showed positive trends of IFN-γ^+ and TNF-α^+ $CD8^+$ T cells with total T-cell, $CD4^+$ T-cell, and naïve B-cell counts; IFN-γ^+ $CD8^+$ T cells additionally with $CD8^+$ T-cell count. None of these trends were observed in the control group. Furthermore, our analysis revealed no correlation between these cytokine responses and anti-RBD antibody titers suggesting that the ability to mount humoral versus cellular anti-SARS-CoV-2 immune responses may be functionally independent in this cohort.

Concordant with our observations, Rosenthal et al. examined 12 patients with CVID, 7 with specific antibody deficiency (SAD), and 10 controls, and reported overall comparable antigen-specific $CD4^+$ and $CD8^+$ T-cell responses – measured by IFN-γ, TNF-α, and Granzyme B production – after two doses of COVID-19 mRNA vaccines (96). Interestingly, IFN-γ responses by $CD4^+$ T cells and TNF-α responses by both $CD4^+$ and $CD8^+$ T cells were even significantly higher in the patients than in healthy controls. The authors concluded that CVID patients are able to mount a normal T-cell response despite impaired antibody production (96).

Preserved cellular responses in CVID patients, in particular, have been described by several other authors, as summarized in a review article by Løken et al. (97). However, the extent of these responses compared to healthy individuals varies considerably across studies, as does the respective study design – including the complexity of T-cell assays, which in many cases is limited to interferon-γ release assays such as IFN-γ ELISpot (97). One of the more detailed analyses was conducted by Ainsua-Enrich et al., who investigated immune responses following the second and third COVID-19 vaccine doses in predominantly antibody-deficient patients, including 12 CVID and 9 unclassified primary antibody deficiency patients (unPAD) (98). The authors reported that IFN-γ responses were detected in only 67% of each CVID and unPAD patients, but in 100% of healthy controls after the second vaccination. Notably, the response rate in CVID patients declined to 33% over six months but was restored to prior results by the third vaccine dose, whereas it remained stable in most unPAD patients. Moreover, the

magnitude of responses was overall lower in these patient groups compared to controls (98). In their analysis of multiple T-cell activation markers, CVID and unPAD patients demonstrated a similar increase in spike-specific $CD69^+CD154^+CD4^+$, $CD69^+CD137^+CD4^+$, and $CD25^+OX40^+CD4^+$ T cells after the second vaccine dose as healthy controls. While the magnitude remained stable in unPAD patients, it declined in CVID patients over time and could only partially be restored by the booster vaccination. Activated $CD25^+CD8^+$ T cells were detected in all but one of these patients (a CVID patient), remained stable, and did not increase further after booster dose; however, their magnitude was again lower in CVID patients than in controls (98).

Taken together, the T-cell data reported in this and other studies – as well as our own – suggest that most patients with primary humoral immunodeficiencies are capable of eliciting substantial T-cell responses to COVID-19 vaccination, often comparable to those of healthy individuals. However, upon closer examination, the magnitude of these responses varies considerably, presumably due to differences in patient composition – even CVID represents a heterogeneous group of patients – and a considerable methodological variability across studies. Future studies with clearly defined diagnostic subgroups, larger patient numbers, and standardized, multiparametric assays beyond interferon-γ measurement are needed to reliably characterize T-cell responses in PID.

4.2.3. Pathophysiological consideration and clinical implications

From a pathophysiological point of view, one might assume that patients with numerically or functionally impaired B cells are still capable of mounting robust cytotoxic $CD8^+$ T-cell responses, as B cells are not essential for their priming. In contrast, the impact on $CD4^+$ T-cell responses may be more variable: Although their activation depends primarily on professional antigen-presenting cells such as dendritic cells, their functional maturation – particularly in the context of T follicular helper (Tfh) differentiation and cytokine production – can be modulated by B-cell interaction (99). This is in line with the observations of some of the previously mentioned studies, in which $CD8^+$ T-cell responses tended to be unaffected by failures in the humoral immune response, whereas $CD4^+$ T-cell responses tended to be influenced more strongly (95,98).

While we observed a reduced TNF-α production by $CD4^+$ T cells and GzmB production by $CD8^+$ T cells in autoimmune patients receiving B-cell-depleting therapies, our PID patients demonstrated comparable results to healthy individuals. This observation may reflect fundamental differences in the nature of immune dysregulation between these patient groups. Whereas B-cell depletion in autoimmune patients is caused iatrogenically in a system with immune dysregulation, but without inherent immune cell deficiencies, and leads to profound alterations (100,101) – potentially impairing T-cell function via disrupted antigen presentation and co-stimulatory signaling (99) – patients with humoral immunodeficiencies often exhibit a relatively intact or compensatorily strengthened T-cell compartment over time (102). The selective reduction of TNF-α and GzmB in our B-cell-depleted cohort may reflect an impaired T-cell maturation under suboptimal priming conditions, which can occur in the absence of B-cell-mediated co-stimulation and cytokine support. Evidence for altered T-cell activation or cytokine expression following anti-CD20 therapy (e.g., reduction of IL-2 and IFN-γ) has been reported by some authors (103,104) and has also been discussed by Van Meerhaeghe et al. in a review on B-cell-mediated regulation of $CD8^+$ T cells (105). However, comparable results particularly regarding TNF-α and GzmB remain lacking, highlighting the need for further investigation.

The – at least partially – preserved T-cell functionality in our immunocompromised patients underlines the complexity of immune interactions and the requirement for individualized immunological assessments and vaccination strategies. Although in the past many physicians were cautious in their vaccination recommendations for immunocompromised patients, there is rising evidence that even patients with profound primary or secondary humoral immunodeficiencies may benefit from vaccines by evoking T-cell responses – as discussed before for SARS-CoV-2 vaccines – and not only neutralizing antibodies. Therefore, these patients should actively be encouraged to receive T-cell-engaging inactivated vaccines, whenever necessary, as live-attenuated vaccines are contraindicated in many immunocompromised patients. To date, in Austria (22), the few available and widely used non-live vaccines known to elicit robust T-cell responses include mRNA-based, viral vector-based, and adjuvanted protein subunit-based SARS-CoV-2 vaccines, as well as the herpes zoster subunit vaccine Shingrix® and the adjuvanted protein subunit respiratory syncytial virus (RSV) vaccine Arexvy® (106–108). Hence, to protect patients with (humoral) immunodeficiencies, the development of vaccines engaging strong T-cell responses should be intensified, e.g., for

seasonal influenza or hepatitis B vaccines, where current vaccines primarily induce antibody responses (109,110). The same holds true for new vaccines against pathogens for which no sufficient vaccines are available so far, such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), Epstein–Barr virus (EBV), or *Mycobacterium tuberculosis*.

Moreover, the development and broad availability of post-vaccination functional T-cell assays in routine diagnostics would be favorable to assess the sufficiency of responses in patients. In order to reach this goal, correlates of protection not only for neutralizing antibodies, but also for T-cell markers such as cytokines, need to be defined in large healthy cohorts vaccine-specifically.

4.3. Strengths and limitations

Our study offers several notable strengths. First, the prospective study design, initiated early in the pandemic, allowed continuous data collection and patient follow-ups. Second, in our large cohort, multiple groups of immunodeficiency could be analyzed and thus both individual and broader analyses could be performed. Third, we introduced a novel concept of a "stringent antibody response", reflecting not merely any seroconversion but a response comparable to that of healthy controls. Fourth, we were the first to define naïve B cells as independent predictor of the humoral response to SARS-CoV-2 vaccinations and also delivered a threshold for baseline B-cell subset measurements. Fifth, our calculation of minimum time intervals between B-cell-depleting therapy and vaccination may further facilitate decision-making regarding optimal vaccination timing in the future. Sixth, our T-cell assays went beyond mere IFN-γ release measurements and allowed extensive correlation analyses in different patient groups, which have not been published by others in this form before. Seventh, through these analyses, we could demonstrate that immunodeficient patients in our cohorts were able to mount notable T-cell responses that were in many aspects comparable to those of healthy controls – underlining the importance of protective vaccinations even in these vulnerable patient groups.

Nevertheless, our study had several limitations that need to be mentioned. First, although the entire cohort was relatively large compared to other studies, certain subgroup analyses were limited by smaller patient numbers, affecting the statistical power. Second, we measured total binding antibodies, which correlated well with neutralizing antibodies according to publications

available in 2021, but a diminished level of validity as a correlate of protection cannot be excluded. Third, no additional functional tests, such as cytotoxicity assays or polyfunctional T-cell assays, were performed. Fourth, heterogeneous features of the patients, such as concomitant diseases, prior medications, or differing intervals since B-cell depletion, might have influenced certain analyses. Since in the first T-cell analysis only autoimmune patients under B-cell-depleting therapy were included, the individual effects of the underlying disease and the B-cell depletion could not be dissected. Fifth, although the CoVVac study lasted about two years and included a booster vaccination, the results presented in this thesis do not contain long-term data following the booster dose; therefore, conclusions about the persistence of vaccination responses cannot be drawn. Sixth, we exclusively investigated the effect of SARS-CoV-2 mRNA vaccinations, so the results cannot be generalized to other vaccine platforms or pathogens.

4.4. Clinical implications

Our results underline the importance of individualized vaccination strategies in immunocompromised patients, demonstrated by SARS-CoV-2 mRNA vaccinations. Measuring B cells, and particularly B-cell subsets including naïve B cells, prior to vaccination can facilitate decision-making regarding the optimal time point for vaccination. According to our analysis, at least 60 naïve B cells per µL are required for an optimal seroconversion. If such measurements are not feasible in daily clinical routine, for patients receiving B-cell-depleting therapy the interval since last treatment can be considered: In general, vaccination should be delayed by at least four months after the last treatment. However, we were able to demonstrate that our immunodeficient patients may still benefit from vaccination by mounting robust T-cell responses. Therefore, vaccinating these patients should be encouraged even if insufficient humoral responses are expected and (naïve) B-cell numbers are low. For a reliable evaluation of patients, however, a broader implementation of pathogen-specific functional T-cell assays in routine diagnostics is needed. Moreover, the development of further vaccines capable of eliciting strong T-cell responses is of high importance to enable protection of patients with humoral immunodeficiencies against a broader range of pathogens. Finally, awareness of hygiene measures, such as hand hygiene, wearing masks, and avoiding crowded environments,

should continue to be promoted in these vulnerable patients, as their overall level of protection is likely lower than that of healthy individuals.

4.5. Conclusion and outlook

Immunodeficient patients were particularly vulnerable during the COVID-19 pandemic and were prioritized for vaccination in 2021, although it remained unclear to which extent they could mount specific immune responses. This work highlights that even in the presence of severe humoral immunodeficiency, SARS-CoV-2 mRNA vaccinations can elicit notable T-cell responses and should therefore be encouraged. Furthermore, we found that certain clinical parameters, predominantly the naïve B-cell count, can be utilized to predict the probability of seroconversion.

Future studies with larger cohorts are needed to assess the fine differences between disease entities as well as the durability und clinical effectiveness of vaccine-induced T-cell responses. By this, the definition of distinct correlates of protections – for serum antibody levels as well as T-cell parameters – could be realized and ultimately improve vaccination strategies and clinical outcomes for patients worldwide.

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Appendix

CoVVac study protocol v4.0

Humoral and cellular immune response to COVID-19 vaccines in immunocompromised and healthy individuals – The CoVVac study

A prospective monocentric cohort study

Version 4.0, 29.09.2021

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Sponsor: **Medical University of Graz**

Sponsor's study protocol code	CoVVac
EudraCT number	2021-001040-10

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Contents

List of abbreviations

ACE2	**Angiotensin converting enzyme 2**
AE	**Adverse event**
AMG	**Arzneimittelgesetz (Austrian Act on Pharmaceutical Products)**
AR	**Adverse reaction**
BASG	**Bundesamt für Sicherheit im Gesundheitswesen (Federal Agency for Safety in Healthcare)**
BCIP	**5-bromo-4-chloro-3′-indolyl phosphate**
BMI	**Body mass index**
cDNA	**Complementary deoxyribonucleic acid**
CLL	**Chronic lymphocytic leukemia**
CoV	**Coronavirus**
COVID-19	**Coronavirus disease 2019**
CRF	**Case report forms**
CVID	**Common variable immunodeficiency**
DII	**Dietary inflammation inde**
DMSO	**Dimethyl sulfoxide**
E	**Envelope protein**
ECIL	**European Conference on Infections in Leukemia**
EFSA	**European Food Safety Authority**
ELISpot	**Enzyme-linked immunosorbent spot**
EMA	**European Medicines Agency**

FACS	**Fluorescence-activated cell sorting**
FPFV	**First Patient First Visit**
GCP	**Good clinical practice**
HSCT	**Hematopoietic stem-cell transplantation**
IB	**Investigator brochure**
IBD	**Inflammatory bowel disease**
ICH	**International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use**
IFNγ	**Interferon γ**
IgA/IgG/IgM	**Immunoglobulin A/G/M**
IMPD	**Investigator Medicinal Product Dossier**
International Society for the Advancement of Kinanthropometry	**ISAK**
LPLV	**Last Patient First Visit**
M	**Membrane protein**
MACS	**Magnetic-activated cell sorting**
MERS-CoV	**Middle East respiratory syndrome-related coronavirus**
MPN	**Myeloproliferative neoplasm**
mRNA	**Messenger RNA**
N	**Nucleocapsid protein**
NBT	**Nitro blue tetrazolium**
PBMCs	**Peripheral blood mononuclear cells**
PCR	**Polymerase chain reaction**
PHM	**Patients with hematological malignancies**

RBD	**Receptor-binding domain**
RDA	**Research, Documentation & Analysis**
RNA	**Ribonucleic acid**
S	**Spike protein**
SAE	**Serious adverse event**
SAR	**Serious adverse reaction**
SARS-CoV (-2)	**severe acute respiratory syndrome coronavirus (type 2)**
SDV	**Source data verification**
SLE	**Systemic lupus erythematosus**
SOP	**Standard operating procedure**
SUSAR	**Suspected unexpected serious adverse reaction**
TCR	**T cell receptor**
TREC	**T cell receptor excision circles**
UAR	**Unexpected adverse reaction**
VFR	**Vienna Food Record**

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Synopsis

Sponsor	Medical University of Graz
Name	Humoral and cellular immune response to COVID-19 vaccines in immunocompromised and healthy individuals.
Running head	CoVVac study
Target population (or indication)	Patients with primary or secondary immunodeficiency planning on vaccination against SARS CoV-2
Study design and phase	A prospective monocentric phase IV cohort study
Version of protocol	4.0
Total duration of the trial	24 months
Aims of the clinical trial	<u>Primary aim of the trial</u> Characterization of the anti-SARS-CoV-2 humoral immune response after vaccination with a focus on the anti-spike protein response: an analysis in immunocompromised patients compared to healthy controls. <u>Secondary aims of the trial</u> To answer the following research questions: 1. Is there a difference in the T cell response between healthy subjects and immunodeficient patients? 2. Is there a difference in seroconversion or T cell response between different vaccines? 3. How does aging of the immune system impact the humoral and cellular immune response? 4. What is the impact of the various forms and severities of immunosuppression/immunodeficiency due to malignancy and/or immunosuppressive therapies on the vaccine-induced immune response?

	5. What is the impact of a previous SARS-CoV-2 infection and its severity on the immune response after vaccination? e.g. the recognized epitopes/antigens, the distribution of immunoglobulin classes, the occurrence of neutralizing antibodies and the quantity and persistence of the vaccine response? 6. What is the role of previous asymptomatic (undiagnosed) SARS-CoV-2 infection (proven through specific antibodies) on the vaccine induced response? 7. How does the immune response differ between the various vaccines in immunocompromised individuals, COVID-19 recovered individuals and age-matched healthy controls? 8. Is there any difference in the induction of secretory IgA and serum IgA in comparison to the serum IgG and IgM response in immunocompromised individuals and in COVID-19 recovered individuals compared to controls? 9. What is the influence of previous infections caused by endemic CoV (proven through cross-reactive antibodies) on the vaccine response in immunocompromised individuals and in COVID-19 recovered individuals compared to controls? 10. Is there any difference in the induction of neutralizing antibodies in subsets of immunocompromised individuals and in COVID-19 recovered individuals compared to COVID-19 naïve controls? 11. Is there any difference in the neutralizing capacity of antibodies after vaccination towards the emerging SARS-CoV-2 variants in immunocompromised individuals and in COVID-19 recovered individuals compared to controls? 12. Do diet and body fat have an impact on the extent of seroconversion after vaccination? 13. What is the humoral and cellular effect of a third/booster vaccination?
Outcome measures (endpoints) of the	<u>Primary outcome measure</u>

clinical trial	The levels of anti-SARS-CoV-2 spike protein humoral immune response at day 21-28 after the second vaccination measured by SARS-CoV-2 antigen-binding Ig assay, comparing immunocompromised patients to healthy controls. Secondary outcome measures • Seroconversion measured by SARS-CoV-2 antigen-binding Ig assay 6 and 12 months after second, 3-5 weeks and 6 months after third vaccination • Concentrations of recombinant S protein-binding IgG after third vaccination in comparison to response after second vaccination. • Concentrations of recombinant S protein-binding IgG depending on prior exposure to SARS-CoV-2. • Concentrations of secretory and serum IgA in comparison to IgG and IgM after second and third vaccination in immunocompromised, in recovered individuals and in healthy controls. • Cytokine production of T cells after SARS-CoV-2 antigen exposure, measured by FACS and ELISpot. • Identification of parameters predicting the response to COVID-19 vaccinations: prior CoV infection (cross-reactive antibodies), quantitative immunoglobulins, B cell subsets, T cell subsets; T cell aging (TCR diversity, telomere length, TREC levels). • Neutralizing capacity of antibodies in respect of different SARS-CoV-2 variants. • Correlation of dietary inflammation index and subcutaneous fat thickness sum with seroconversion after second vaccination.
Number of patients	195 immunodeficient patients and 195 healthy controls
Time schedule	With reference to the trial Recruitment period: March 2021 - August 2021 Planned start (FPFV): March 2021

	Planned end (LPLV): May 2023 <u>With reference to patients</u> Individual duration of Study: 13 to 15 months
Inclusion criteria	1. Noninfectious immunocompetent participants (healthy participants) as determined by medical history and clinical judgement. or 2. Patients with primary immunodeficiencies or 3. Patients with B-cell depleting therapy due autoimmune disease or 4. Patients with benign and malignant hematological diseases receiving specific treatments with known immunosuppressive effects including cytotoxic agents, systemic corticosteroids, monoclonal antibodies and targeted therapies. or 5. Patients with active hematological diseases and secondary immunoglobulin deficiency (e.g. CLL, MM) currently not receiving specific treatment. or 6. Patients >3 months but <12 months after autologous HSCT. or 7. Patients >3 months but <12 months after allogeneic HSCT. or 8. Recipients of HSCT >12 months after allogeneic HSCT but under immunosuppressive therapy. or 9. Patients with chronic GvHD and persistent immunodeficiency.
Exclusion criteria	*Healthy participants* 1. Presence of diseases or therapies that are likely to interfere with the immune response to vaccination. 2. Presence of a disease requiring change in therapy during 4 weeks before enrollment.

	3. Any contraindications to the vaccine planned to receive as listed in the product characteristics. 4. Lack of willingness to undergo serial blood draws and attend follow-up appointments. 5. Women who are pregnant or breastfeeding. 6. Previous vaccination with any coronavirus vaccine. *7.* Persons who are not willing to sign the informed consents (biobank informed consent and study specific informed consent) *Immunodeficient participants* 1. Patients with hematological diseases within three months from B-cell-depleting immunotherapy (rituximab, ofatumumab, obinutuzumab, blinatumomab, CAR-T cells). 2. Patients with hematological malignancies in remission and >12 months after end of specific therapy. 3. Patients within three months from HSCT. 4. Any contraindications to the vaccine planned to receive as listed in the product characteristics. 5. Lack of willingness to undergo serial blood draws and attend follow-up appointments. 6. Women who are pregnant or breastfeeding. 7. Previous vaccination with any coronavirus vaccine (exception: if serum prior to vaccination is available from the biobank). 8. Patients who are not willing to sign the informed consents (biobank informed consent and study specific informed consent).
Product characteristics	Comirnaty: https://www.ema.europa.eu/en/documents/product-information/comirnaty-epar-product-information_de.pdf Moderna Covid-19 vaccine: https://www.ema.europa.eu/en/documents/product-information/covid-19-vaccine-moderna-epar-product-information_de.pdf AstraZeneca COVID-19 vaccine:

	https://www.ema.europa.eu/en/documents/product-information/covid-19-vaccine-astrazeneca-epar-product-information_de.pdf

1. Scientific background

SARS-CoV-2 immunity and vaccines

The enveloped RNA virus SARS-CoV-2 and causative agent of the COVID-19 pandemic belongs to the family of *Coronaviridae*, which comprises a number of viruses that can cause disease in animals and humans. Among them are SARS-CoV and MERS-CoV and four endemic coronaviruses (HCoV-229E, -NL63, -OC43, and -HKU1) that cause relatively mild upper and lower respiratory tract infections in humans (1,2).

The induction of protective anti-SARS-CoV-2 immunity is a key strategy in the containment of the COVID-19 pandemic. Vaccine data from infection models with non-human primates indicate a correlation between the induction of neutralizing antibodies and reduced viral loads (3,4). The successful treatment of COVID-19 patients with convalescent plasma (5) and the recent emergency use authorization for neutralizing monoclonal antibodies by the United States Food and Drug Administration strongly support an important role for antibodies in anti-SARS-CoV-2 immunity (6). Furthermore, SARS-CoV-2 –specific CD4+ and CD8+ T cell responses in patients correlated with less severe disease suggesting a protective role in COVID-19 (7,8). Therefore, both, strong B cell and T cell mediated responses are likely to be necessary for an effective immunity.

Most studies have focused on the spike protein (S), containing the S1 and S2 subunits, as a vaccine target (9,10). The S1 subunit recognizes the host receptor human angiotensin converting enzyme 2 (ACE2) via its receptor-binding domain (RBD), whereas the S2 subunit is involved in membrane fusion and required for viral entry (11,12). The RBD is located at the C-terminal domain of the S1 subunit and is the major target for neutralizing antibodies, although the N-terminal domain is also recognized by neutralizing antibodies (13). The S2 subunit can also be recognized by antibodies with neutralizing capacity through interfering with membrane fusion (14). The SARS-CoV-2 structural proteins M (membrane) and E (envelope) seem to be less immunogenic but have a higher sequence homology with other corona viruses and exhibit cross-reactive T cell epitopes (15). The structural protein N (nucleocapsid) elicits a strong antibody response and also contains T cell epitopes (15), but has not been selected as a sole vaccine target against SARS-CoV-2 so far.

The S protein, either in full length with or without amino acid substitutions or as the S protein RBD, is the target of the two mRNA-based BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) and the vector-based vaccine ChAdOx1 nCoV-19 (AstraZeneca), which have recently been approved by the European Medicines Agency (EMA). These vaccines have been shown to induce humoral as well as cell mediated immune responses in the study populations (9,16,17). The efficacy of BNT162b2 to prevent COVID-19 was 95% (95% CI 90.3 to 97.6) with a similar efficacy in secondary analyses including age groups, ethnicity and body-mass index. Prevention of severe disease was also highly efficient (18). The overall efficacy of the mRNA-1273 vaccine was 94.1% (95% CI, 89.3 to 96.8). Again, secondary analyses revealed similar efficacies in subgroups stratified by age, sex, ethnicity and risk factors for severe COVID-19 (9). This vaccine was also shown to significantly reduce severe COVID-19. The efficacy of ChAdOx1 nCoV-19 reached 81.3% (95% CI 60·3–91·2) in an ongoing trial when the second dose was given after an interval of 12 or more weeks. More than 14 days after vaccination with the second dose, 15 COVID-19 hospitalizations occurred in the control group, but no hospitalization in the ChAdOx1 nCoV-19 group (19). A number of other vaccine candidates have entered phase III clinical trials and are expected to be available in the coming months (20).
The currently licensed vaccines induce a strong anti-S protein IgG response with virus neutralizing capacity (21–23). Available data on the anti-S protein T cell response of the mRNA-based vaccines demonstrate a predominant Th1-profile of SARS-CoV-2 specific CD4+ and CD8+ cells (22,23).

Since the vaccine efficacy trials did not include a comprehensive antibody status prior to vaccination (9,16,17), the impact of previous COVID-19 on the vaccine induced immune response, is unclear. Another significant gap of knowledge concerns the vaccine efficacy and immune response in severely immunocompromised individuals. Patients with acquired or innate immunodeficiencies were not included in the vaccine efficacy trials (9,16,17). Therefore, neither the type of induced immune response nor the efficacy of available vaccines in these patient cohorts have been thoroughly investigated.

So far, all available vaccine candidates are administered intramuscular or intradermal and the analysis of the corresponding antibody response has focused on serum antibodies. A gap of knowledge is the role of mucosal immunity in the defense against SARS-CoV-2, in particular the role of secretory immunoglobulins such as secretory IgA (SIgA). Although recent reports demonstrate that SARS-CoV-2-specific immunoglobulins can be detected in saliva after infection

(24) their persistence, diagnostic usefulness and protective capacity remains to be determined. In this context, it is unclear, if SARS-CoV-2-specific antibodies can be detected in mucosal secretions of the respiratory tract after immunization with the currently licensed vaccines and if a mucosal vaccine via the respiratory tract might further enhance immunity in this compartment.

The fact that mutations in the spike protein occur which not only alter the function of the protein but also the recognition by the immune system is of concern. Recently emerging SARS-CoV-2 variant strains from the UK, South Africa (SA) and Brazil appear to be more transmissible and accumulated a number of mutations/deletions in the spike protein that deserve close attention regarding the possible occurrence of vaccine escape variants (25). Currently most prevalent is the UK variant B.1.1.7, which is characterized by multiple changes in the spike protein including a deletion at position 69-70 and a mutation in the RBD at position 501. The latter resulting in a higher affinity to ACE2. The SA variant B.1.351 harbors multiple changes in the spike protein, with mutations K417N, E484K, and N501Y within the RBD, but no deletion 69-70. The Brazil variant P.1 accumulated also multiple amino acid changes in the spike protein, including K417T, E484K and N501Y in the RBD.

The efficacy of the vector-based vaccine ChAdOx1 nCoV-19 against the UK variant B.1.1.7 seems to be similar to the efficacy of the vaccine against other UK lineages (26). However, studies have shown that sera from either BNT162b2- or mRNA-1273-vaccinated individuals or convalescent plasma had a reduced neutralizing capacity towards engineered SARS CoV-2 variants with key spike mutations from the UK and SA variant (27,28). The neutralizing potency of a number of monoclonal antibodies recognizing different epitopes in the RBD were reduced or even abolished by either K417N, E484K, or N501Y mutations.

Taken together these results indicate the potential of SARS CoV-2 immune escape mechanisms after vaccination. It seems likely, that a continuous monitoring of the immunity of vaccinated individuals towards SARS CoV-2 variants will guide possible vaccine updates. This might be of particular relevance in patients with immunodeficiencies.

SARS-CoV-2 and vaccination in patients with primary immunodeficiencies

Immunodeficiency can be primary due to underlying genetic cause, like common variable immunodeficiency (CVID), or acquired (secondary immunodeficiencies), i.e., patients requiring immunosuppressive therapies due to autoimmune diseases (e.g. rheumatoid arthritis, SLE,

vasculitis, IBD, multiple sclerosis), hematologic malignancies, after solid organ transplant or hematopoietic stem cell transplant. The ability to secrete protecting virus-specific antibodies and/or to create sufficient cellular immune responses is often reduced in these patients. Therefore, the question arises if they benefit from COVID-19 vaccines at all.

CVID is characterized by reduced immunoglobulin secretion and increased susceptibility to infection. In 2017, a review (29) already highlighted the immunization of CVID patients by influenza vaccination. It was demonstrated that the majority of tested CVID patients produced influenza-specific activated CD4+ T cells secreting IFN-γ, TNF-α and IL-2 on exposure to influenza antigens and, therefore, benefited from the vaccination despite their humoral immunodeficiency.

The immunogenicity of different vaccines has been examined in patients with secondary immunodeficiency due to rituximab and other antirheumatic therapies, as well. In a review from 2017, it was pointed out that influenza and pneumococcal vaccinations administered to patients taking B-cell depleting rituximab are significantly less immunogenic, i.e. an impaired humoral response occurs (30). In this context, however, dosing intervals played an important role: vaccinations given at least 6 months after last rituximab dose resulted in better humoral responses than short intervals of 1-2 months. A recent review about considerations of COVID-19 vaccines in patients with autoimmune diseases receiving anti-CD20-depleting therapies summarized that these patients do not necessarily suffer from severe SARS-CoV-2 infection because of the assumed central role of effective T cell responses in COVID-19 (31). Nevertheless, establishment of a protective immunity in terms of neutralizing antibodies following vaccination might be impaired until repopulation of naïve B cells (31), indicating that either intervals should be prolonged or that vaccination-induced cellular immunity could suffice, which needs further investigation now.

The potentially protective long-term effect of T cell responses to SARS-CoV-2 has recently been investigated in convalescents of the SARS outbreak in 2003 (32). Although 17 years ago, there still was a CD4+ and CD8+ T cell response to the N protein of SARS-CoV, which proved to be robustly cross-reactive against the N protein of SARS-CoV-2, as well. This could imply a lower susceptibility to COVID-19.

The special importance of cellular immune responses in primary immunodeficiencies was demonstrated in several cases of patients with X-linked agammaglobulinemia (XLA) and CVID who got infected with SARS-CoV-2 (33–35). Despite the defects in B cell differentiation and immunoglobulin production in these patients, they were able to recover – some even without need of oxygen ventilation, intensive care or treatment like COVID-19 convalescent plasma (34). These cases corroborate the important role of T cell responses in fighting COVID-19 (34).

Vaccination and obesity

An association between obesity and lower vaccine-induced H1N1-specific antibodies and obese individuals with double risk of developing influenza have been reported and the same is supposed for COVID-19 (36). However, it is still unclear whether obese adipose tissue may also alter vaccine response and vaccination side effects in patients with autoimmune deficiencies.

Diet and nutrient profile are crucial for the maintenance of health and appropriate immune function. Certain dietary components have the ability to shift the metabolic response and may trigger pro- and anti-inflammatory conditions (37). Thus, the evaluation of food and nutrient intake and the diet's inflammatory potential is indicated to assess the progression of the immune response to the vaccine.

COVID-19 vaccination of patients with hematological malignancies

Secondary immunodeficiencies in hematologic diseases can be caused by the malignant disease itself as in chronic lymphocytic leukemia (CLL), multiple myeloma (MM) and malignant lymphomas (NHL), its therapy (immunosuppression due to conventional chemotherapy, radiotherapy, targeted therapies, biological agents and immunotherapies) and/or associated metabolic dysfunctions (e.g. protein loss due to enteropathy, nephrotic syndrome with hypogammaglobulinemia, hepatic dysfunction, malnutrition with protein and vitamin deficiency). Furthermore, infections such as herpes viral infections can have a negative impact on patients' immune system. Secondary immunodeficiencies can affect both the innate as well as the adaptive immune system and increase patients' susceptibility for opportunistic infections that are one of the main causes of death of patients with hematologic malignancies (PHM). Immunodeficiency can also lead to reduced immune response towards leukemic or tumor cells. Patients with MM are uniquely susceptible to viral and bacterial diseases with a 7-to-10-fold higher risk of infection (38). MM and its treatments lead to humoral immunodeficiency, hypogammaglobulinemia and impaired B-and T-cell responses (39,40). MM usually affects the elderly population, a more vulnerable group of patients due to immunosenescence together with other comorbidities. Younger MM patients are frequently treated with high-dose chemotherapy followed by autologous hematopoietic stem cell transplantation (HSCT) with high infection susceptibility during the 3-month period following transplant (41). CLL is the most common form of leukemia and is associated with impaired immune responses to common pathogens in a context of profound immune dysregulation reflected by hypogammaglobulinemia, qualitative and quantitative B- and T-cell defects, including impaired response to vaccination, and CD4+ lymphopenia as well as innate immune dysfunction and

neutropenia (42,43). In other diseases such as T-cell and Hodgkin lymphomas the clinically observed immunodeficiency has, so far, not been analyzed in more detail. Treatment of B-cell malignancies with anti-CD20 monoclonal antibodies such as rituximab causes profound B-cell depletion lasting in the majority of patients for a median of 6 to 9 months (44). The prevalence of hypogammaglobulinemia after rituximab therapy varies dependent on malignant disease and concomitant therapies and reportedly was 38% in a cohort of B-cell lymphoma patients including 6% with need for immunoglobulin substitution.

PHM have a high risk of developing severe COVID-19 disease course after SARS-CoV-2 infection. The first case study specifically examining PHM who became infected at the onset of the COVID-19 pandemic in Wuhan showed a high mortality of 38.5% (45). Further retrospective studies in other European centers confirmed this high fatality rate for PHM. A case series of 59 patients from four hospitals in Italy, Spain and the Netherlands showed an age dependent mortality rate of 34% (46). In Italy, data from 66 hospitals on 536 PHM with COVID-19 disease demonstrated a worse overall survival (OS) compared to PHM without COVID-19. Older age (hazard ratio [HR] 1.03, 95% CI 1.01–1.05), progression of malignant disease (HR 2.10, 95% CI 1.41–3.12), diagnosis of acute myeloid leukemia (HR 3,49, 95% CI 1,56–7,81), indolent NHL (HR 2.19, 95% CI 1.07–4.48), aggressive NHL (HR 2.56, 95% CI 1.34–4.89), or plasma cell neoplasms (HR 2.48, 95% CI 1.31–4.69) were associated with worse OS (47). A recent meta-analysis of 3377 PHM with COVID-19 demonstrated a case fatality rate of 34% (48). The pooled risk of death for several diseases was as listed: Acquired bone marrow failure syndromes (14 studies, 231 patients), 53% (95% CI, 34-72); acute leukemias (18 studies, 289 patients), 41% (95% CI, 30-52); plasma cell dyscrasias (23 studies, 412 patients), 33% (95% CI, 25-41); NHL, including CLL (20 studies, 1324 patients), 32% (95% CI, 24-40); NHL, excluding CLL (14 studies, 485 patients), 32% (95% CI, 18-48); CLL specifically (15 studies, 517 patients), 31% (95% CI, 23-40); myeloproliferative neoplasms (MPNs) (12 studies, 293 patients), 34% (95% CI, 19-51) (48). It must be noted that the majority (77%) of patients analyzed had been hospitalized.

PHM who received a hematopoietic stem-cell transplantation (HSCT) constitute an important group of immunodeficient individuals. After allogeneic HSCT patients reconstitute their immune system from donor-derived immune precursor cells in the stem cell graft. Complete immune regeneration can take 1 to 3 years depending on duration of immunosuppression for graft-versus-host disease (GvHD) prophylaxis and therapy. Several factors significantly influence immunologic reconstitution after allogeneic HSCT including the restoration of protective, anti-infective host

immunity and the reestablishment of expanded T cell and B cell repertoires as well as functional tolerance with preservation of graft-versus-leukemia (GvL) effects. These factors include conditioning regimen intensity, graft source, donor type, HLA-identity, donor and recipient age (49). Immune dysregulation and alloreactivity drive the development of chronic GvHD resulting from the predominance of donor-derived effector mechanisms that cannot be controlled by donor- or host-derived regulatory immune responses. GvHD can attack the thymus resulting in the generation of donor T cells with antihost reactivity due to defective negative selection (50). The pathophysiology of chronic GvHD is complex and involves multiple distinct interactions among alloreactive and dysregulated T and B cells and innate immune cell populations (49). Patients with chronic GvHD can present with autoimmune phenomena, autoantibody production, severe immunodeficiency due to lack of T and B cells and functional asplenia.

The Center for International Blood and Marrow Transplant Research (CIBMTR) analyzed the outcome of 318 HSCT recipients who developed COVID-19. The estimated OS at 30 days after COVID-19 diagnosis was 68% (95% CI 58–77) for recipients of allogeneic HSCT and 67% (55–78) for autograft recipients (51). Male sex, age older than 50 years and being within a year from allogeneic HSCT were factors strongly associated with worse OS (51). These reported outcomes are comparable to results from other studies from two North American cohorts (52,53).

Vaccination is important in PHM because they have an increased risk of infection related morbidity and mortality due to common pathogens like influenza, pneumococci and varicella zoster virus. The new COVID-19 vaccines have not been tested in PHM or recipients of HSCT. It is unknown what level of protection and type of immune response will result from COVID-19 vaccination.
Most data on serologic response to COVID-19 infection have come from immunocompetent adults (54). In a small case series, nine of ten patients with acute leukemia on systemic anticancer therapies were able to induce anti-SARS-CoV-2 IgG antibody responses but only 67% of 30 patients with CLL (55,56).
Since the method of vaccination differs according to nature of malignancy, treatment, infection, and vaccine, the European Conference on Infections in Leukemia (ECIL 7) developed guidelines for PHM including HSCT recipients in 2017 (57,58). Most of these recommendations are not based on clinical efficacy but on serological responses because hematological malignancies are rare and the overall incidence of infections is low. Studies whose results were used to establish the ECIL7 guidelines show lower overall effectiveness of most vaccines (summarized by (57,58)). Of note, immunotherapy with rituximab at the time of vaccination (and probably other B-cell depleting

antibodies), strongly impairs vaccination response for at least 6 months. Since PHM have a high risk of poor outcome after COVID-19, the German Society of Hematology and Oncology recommends COVID-19 vaccination at the earliest 3 months after B-cell depleting therapy (59).

With the increasing spread of the SARS CoV-2 delta variant, the U.S. Food and Drug Administration and the Austrian national vaccination board recommend a third (booster) vaccination for immunocompromised patients, health care workers and those over 65 years of age (https://www.sozialministerium.at/dam/jcr:6cce0185-da10-41c9-ac64-4950c07b84fe/COVID-19-Impfungen_Anwendungsempfehlung_des_Nationalen_Impfgremiums_Version_5.1_(Stand%2029.09.2021).pdf). This vaccination should be given 6-9 month after the first series of vaccination using an mRNA vaccine, preferentially BNT162b2.

2. Aim of the trial

Currently, the efficacy of COVID-19 vaccination in immunodeficient patients is unknown. Here we aim to evaluate the efficacy of COVID-19 vaccines in immunodeficient patients compared to healthy controls. We will assess the humoral and cellular response to COVID-19 vaccination in these subjects in detail. Furthermore, we will identify factors associated with good response to vaccination. The results of our study will help to guide future recommendations on COVID-19 vaccination in this population.

Primary objective

Characterization of the anti-SARS-CoV-2 humoral immune response after vaccination with a focus on the anti-spike protein response: an analysis in immunocompromised patients compared to healthy controls.

Secondary objectives

To answer the following research questions:

1. Is there a difference in the T cell response between healthy subjects and immunodeficient patients?
2. Is there a difference in seroconversion or T cell response between different vaccines?
3. How does aging of the immune system impact the humoral and cellular immune response?

4. What is the impact of the various forms and severities of immunosuppression/immunodeficiency due to malignancy and/or immunosuppressive therapies on the vaccine-induced immune response?
5. What is the impact of a previous SARS-CoV-2 infection on the immune response after vaccination? e.g. the recognized epitopes/antigens, the distribution of immunoglobulin classes, the occurrence of neutralizing antibodies and the quantity and persistence of the vaccine response?
6. What is the role of previous asymptomatic (undiagnosed) SARS-CoV-2 infection (proven through specific antibodies) on the vaccine induced response?
7. How does the immune response differ between the various vaccines in immunocompromised individuals, COVID-19 recovered individuals and age-matched healthy controls?
8. Is there any difference in the induction of secretory IgA and serum IgA in comparison to the serum IgG and IgM response in immunocompromised individuals and in COVID-19 recovered individuals compared to controls?
9. What is the influence of previous infections caused by endemic CoV (proven through cross-reactive antibodies) on the vaccine response in immunocompromised individuals and in COVID-19 recovered individuals compared to controls?
10. Is there any difference in the induction of neutralizing antibodies in subsets of immunocompromised individuals and in COVID-19 recovered individuals compared to COVID-19 naïve controls?
11. Is there any difference in the neutralizing capacity of antibodies after vaccination towards the emerging SARS-CoV-2 variants in immunocompromised individuals and in COVID-19 recovered individuals compared to controls?
12. Do diet and body fat have an impact on the extent of seroconversion after vaccination?
13. What is the humoral and cellular effect of a third/booster vaccination?

2.1. Study design

We will conduct a prospective cohort study including 195 adult participants with primary or secondary immunodeficiencies and 195 healthy control subjects at a single center. The study will take place at the Medical University of Graz. Blood and saliva samples will be obtained before COVID-19 vaccination (study visit 1) and after the second vaccination (study visits 3-6, see Table

1). A third vaccination/booster will be optional between visit 4 and 5 in accordance with Austrian national vaccination board recommendations.

2.1.1 Time schedule

The total duration of the study is 24 months, starting in March 2021 with a recruiting period until 31. August 2021 and finish of all scheduled visits until 30. September 2022. Recruitment of patients and healthy controls will be organized as described under 3.1. Five on-site visits and one telephone visit are scheduled within an individual study period of 13 to 15 months. A detailed description of the time schedule and the individual visits is provided under 4.

2.2 Outcome measures

2.2.1 Primary outcome measures

Levels of anti-SARS-CoV-2 spike protein antibodies at day 21-28 after the second vaccination measured by SARS-CoV-2 antigen-binding Ig assay.

2.2.2 Secondary outcome measures

- Seroconversion measured by SARS-CoV-2 antigen-binding Ig assay 6 and 12 months after second, 3-5 weeks and 6 months after third vaccination
- Concentrations of recombinant S protein-binding IgG after third vaccination in comparison to response after second vaccination.
- Concentrations of recombinant S protein-binding IgG depending on prior exposure to SARS-CoV-2.
- Concentrations of secretory and serum IgA in comparison to IgG and IgM after second and third vaccination in immunocompromised, in recovered individuals and in healthy controls.
- Cytokine production of T cells after SARS-CoV-2 antigen exposure, measured by FACS and ELISpot.
- Identification of parameters predicting the response to COVID-19 vaccinations: prior CoV infection (cross-reactive antibodies), quantitative immunoglobulins, B cell subsets, T cell subsets; T cell aging (TCR diversity, telomere length, TREC levels, epigenetic changes).

- Neutralizing capacity of antibodies in respect of different SARS-CoV-2 variants.
- Correlation of dietary inflammation index and subcutaneous fat thickness sum with seroconversion after second vaccination.

3. Recruitment of probands

3.1 Number of probands and the duration of the trial

Male and female subjects older than 18 years will be recruited before undergoing COVID-19 vaccination according to the Austrian vaccination plan. 195 immunodeficient patients will be recruited at the Medical University of Graz (Division of Rheumatology and Immunology, Division of Hematology). 195 healthy controls will be recruited from 1.) already existing COVID-19 convalescent cohort (Department of Internal Medicine, Section of Infectious Diseases and Tropical Medicine, Department of Blood Group Serology and Transfusion Medicine, Institute of Hygiene, Microbiology and Environmental Medicine); 2.) healthy controls such as whole blood and platelet donors (Department of Blood Group Serology and Transfusion Medicine) or other volunteers recruited by adequate advertisement. Vaccination of immunodeficient patients will occur prior to healthy controls according to the Austrian vaccination plan. Therefore, healthy controls will be selected to match immunodeficient patients' age, sex and type of vaccine and will be recruited as soon as the immunodeficient group is characterized and according to the Austrian vaccination plan, i.e. prospectively from June 2021 on. From September 2021 on, a third (booster) vaccination will be offered in accordance with the recommendations of the Austrian national vaccination board. The individual duration of the study will be 13 to 15 months depending on the type of vaccine.

3.2 Inclusion criteria

In order to be enrolled, participants must be 18 years or older, able to understand study procedures, provide written informed consent (Biobank informed consent and study specific informed consent), and meet one of the following inclusion criteria:

1. Noninfectious immunocompetent participants (i.e., healthy participants) as determined by medical history and clinical judgement.
   or

2. Patients with primary immunodeficiencies
   or
3. Patients with B-cell depleting therapy due autoimmune disease
   or
4. Patients with benign and malignant hematological diseases receiving specific treatments with known immunosuppressive effects including cytotoxic agents, systemic corticosteroids, monoclonal antibodies and targeted therapies.
   or
5. Patients with active hematological diseases and secondary immunoglobulin deficiency (e.g. CLL, MM) currently not receiving specific treatment.
   or
6. Patients >3 months but <12 months after autologous HSCT.
   or
7. Patients >3 months but <12 months after allogeneic HSCT.
   or
8. Recipients of HSCT >12 months after allogeneic HSCT but under immunosuppressive therapy.
   or
9. Patients with chronic GvHD and persistent immunodeficiency.

3.3 Exclusion criteria

Subjects meeting any of the following criteria cannot be enrolled into the trial:

Healthy participants

1. Presence of diseases or therapies that are likely to interfere with the immune response to vaccination.
2. Presence of a disease requiring change in therapy during 4 weeks before enrollment.
3. Any contraindications to the vaccine planned to receive as listed in the product characteristics.
4. Lack of willingness to undergo serial blood draws and attend follow-up appointments.
5. Women who are pregnant or breastfeeding.
6. Previous vaccination with any coronavirus vaccine.

7. Persons who are not willing to sign the informed consents (biobank informed consent and study specific informed consent).

Immunodeficient participants

1. Patients with hematological diseases within three months from B-cell-depleting immunotherapy (rituximab, ofatumumab, obinutuzumab, blinatumomab, CAR-T cells).
2. Patients with hematological malignancies in remission and >12 months after end of specific therapy.
3. Patients within three months from HSCT.
4. Any contraindications to the vaccine planned to receive as listed in the product characteristics.
5. Lack of willingness to undergo serial blood draws and attend follow-up appointments.
6. Women who are pregnant or breastfeeding.
7. Previous vaccination with any coronavirus vaccine (exception: if serum prior to vaccination is available from the biobank).
8. Patients who are not willing to sign the informed consents (biobank informed consent and study specific informed consent).

3.4 Clinical history

Date and type of COVID-19 vaccinations, medical history (including immunosuppressive therapies, chronic GvHD, severe opportunistic infections, prior vaccination history (types, titers if available), current and past disease including COVID-19), current medication, BMI, smoking habits, and nutritive assessment will be obtained in all participants.

3.5 Parameters, Laboratory parameters

In order to assess parameters predicting the vaccination response, quantitative immunoglobulins and B cell subsets will be measured at the Diagnostic Immunology Lab of the Div. of Rheumatology and Immunology at baseline (visit 1) and at visit 4. Pregnancy tests will be performed in the urine of female participants by detecting Human chorionic gonadotropin beta (once before each vaccination).

4. Implementation of the trial / Course of the trial

The total duration of the study is 24 months, starting in March 2021 with a recruiting period until 31. August 2021 and finish of all scheduled visits until 30. September 2022. Recruitment of patients and healthy controls will be organized as described under 3.1. Individuals having consented and fulfilling the inclusion and exclusion criteria are included in the trial. A baseline visit will take place up to 60 days before the planned date of vaccination according to the Austrian vaccination plan. Scheduled vaccination with any COVID-19 vaccine approved in Austria will allow recruitment. If appropriate pre-vaccine samples from study participants exist in the biobank of the Medical University of Graz, patients may also be included in the study after vaccination starting with visit 3. In this case the biomaterial available at the biobank will be used for the analyses planned on visit 1. The investigators will not influence the date of vaccination or the type of the vaccine used. After first vaccination a telephone visit (visit 2) will assess adverse events and schedule visit 3 at the appropriate time after the second vaccination. At visit 3 the patient`s vaccination certificate will be checked to verify correct vaccination and document the type of vaccine received. Further visits (4-6) will be performed for up to one year after the second vaccination. After visit 4, participants may be vaccinated for a third time. The follow-up period will allow an assessment of the duration of the immune response. A detailed description of the individual visits is provided under 4.1 and in table 1. COVID-19 vaccination, COVID-19 infection, Vaccination history, Pregnancy test, Adverse Events, BMI, and Laboratory Specimen Collection are recorded directly on the paper or electronic CRF and therefore are considered to be source data. Data will be merged in an electronic database (RDA Research, Documentation & Analysis; Medical University of Graz, version 07.03.2019). CRFs will be inspected in respect of their accuracy and completeness and compared to original data by the monitor.

4.1 Description of the individual visits

Visit 1:

After informed consents, inclusion and exclusion criteria will be checked to include or exclude the subject in the trial. Clinical history (see 3.4) will be assessed and blood (107ml – Serology, immune status, T cell immunity, and T cell aging) and saliva (1.5ml) will be taken. Urine from female participants will be collected to perform a pregnancy test. The subjects will be provided with the Vienna Food Record questionnaire and one further pregnancy test for use before 2nd vaccination.

Visit 2:

Visit 2 (0-14 days after first vaccination) is a telephone visit assessing safety and scheduling visit 3 at the appropriate time after the second vaccination. Female participants are reminded to perform a pregnancy test in urine before receiving the 2nd vaccination.

Visit 3:

21-28 days after 2nd vaccination, vaccination reactions and clinical history will be assessed. Blood (68ml – serology, T cell immunity) and saliva (1.5ml) will be taken. Safety monitoring will be performed. The Vienna Food Record questionnaire will be collected and body fat composition will be measured by ultra sound.

Visit 4:

6 Months (±4 weeks) after 2nd vaccination, clinical history will be assessed. Blood (12ml – serology, blood group) will be taken. Subjects with an anti-SARS CoV-2 antibody titer of <1000 U/ml IgG at visit 3 will donate a total of 71ml of blood for serology, blood group, T-cell immunity and immune status; Saliva (1.5ml) will be taken. Safety monitoring will be performed. Participants eligible to receive a 3rd vaccination in accordance with the Austrian national vaccination board recommendations, may be vaccinated up to 4 weeks after visit 4. Female participants will perform a pregnancy test prior to vaccination.

Visit 5:

3-6 weeks after third (booster) vaccination or (for those not having received a third vaccination) after visit 4, clinical history will be assessed. Blood (68ml – serology, T cell immunity) and saliva (1.5ml) will be taken. Safety monitoring will be performed. The subjects will be provided with the Vienna Food Record questionnaire.

Visit 6:

6 Months (±4 weeks) after Visit 4, clinical history will be assessed. Blood (68ml – serology, T cell immunity) and saliva (1.5ml) will be taken. Safety monitoring will be performed. The Vienna Food Record questionnaire will be collected and body fat composition will be measured by ultra sound.

Visits	*1*	*2*	*3*	*4*	*5*	*6*
Time points	*60-0 days before vaccination*	*0-14 days after first vaccination*	*+ 21-28 days after 2nd vaccination*	*+6 months after 2nd vaccination*	*+3-6 weeks after V4 or 3rd vaccination*	*+6 months after Visit 4*
Informed consent	X		(x)			
Inclusion/Exclusion criteria	X					
Medical history incl. COVID-19	X		X	X	X	X
Telephone visit		X				
Serology + saliva	X		X	X	X	X
T cell immunity	X		X	(X)	X	X
T cell aging	X					
Immune status	X			(X)		
Nutritive assessment			X		_	X
Body fat composition			X		_	X
Pregnancy test	X	(X)		(X)		
Safety monitoring		X	X	X	X	X

Table 1. Visits and assessment schedule

4.2 Methods

Sampling

For serology, 8 ml of blood and 1.5 ml saliva samples will be taken at every visit.

For testing cellular immunity, additional 60 ml of blood will be drawn at visits 1, 3, 5, 6, and for patients without seroconversion also at visit 4. Assessment of T cell aging requires 20 ml of blood at visit 1. In order to assess parameters predicting the vaccination response (immune status) 19ml of blood will be drawn at visit 1 and visit 4 (- only subjects with an anti-SARS CoV-2 antibody titer of <1000 U/ml IgG at Visit 3). Blood group antigens (3 ml) will be determined at visit 4. Pregnancy tests will be performed in the urine of female participants by detecting Human chorionic gonadotropin beta.

Storage

All samples will be stored at the Biobank of the Medical University of Graz for further analysis. Samples that are not consumed by analyses of the current study will be made available for further serologic, immunologic, genetic, or metabolomics analyses. For further analyses, not covered by this protocol, a separate ethical vote has to be obtained.

Serum samples will be stored at -80°C. Peripheral blood mononuclear cells will be frozen in DMSO-based media and stored in liquid nitrogen. Saliva will be collected by using the “passive drool“ method. Alternatively, specimens will be collected using a device which is placed into the oral cavity or by a mouth wash. Details of the procedures are provided in the respective SOPs. Handling and storage of saliva samples will take place at the Institute of Hygiene, Microbiology and Environmental Medicine. In order to remove cell debris, the samples will be centrifuged for 15 min with 10.000g at 4°C and the supernatant will be stored at -80°C with constant temperature monitoring.

Antibody tests

Antibody tests with serum and saliva specimens will be conducted at the R&D Institute of Hygiene, Microbiology and Environmental Medicine. Anti-SARS-CoV-2 Ig isotypes against various forms of the S and N protein as well as the Ig-mediated immune response against other viral and bacterial antigens will be determined by using immunoassays currently applied in routine diagnostics. SARS-CoV-2 Live virus neutralization assays will be conducted in our biosafety level 3 laboratory. Either commercially available recombinant SARS-CoV-2 wild type proteins, homologous proteins of related viruses and SARS-CoV-2 variant proteins or recombinant SARS-CoV-2 proteins produced in our laboratory will be used in multiplex immunoassays for an in-depth analysis of the humoral immune response.

T cell assays

As part of the clinical studies for approval of the COVID-19 vaccines, virus-specific CD4+ and CD8+ T cell responses were investigated by IFNγ enzyme-linked immunosorbent spot (ELISpot) (22). In this study, we will perform ELISpot and other methods for research on T cell responses, such as fluorescence-activated cell sorting (FACS), before and after COVID-19 vaccination. Moreover, we will examine T cell aging in our patients as possible pre-existing factor for diminished immune responses to vaccinations.

Isolation of PBMCs & T cell subsets

As a precondition for all further T cell analyses explained below, peripheral blood mononuclear cells (PBMCs) will be isolated by Ficoll density gradient centrifugation. Total or naïve CD4+ and CD8+ T cells will be obtained using MACS technology according to manufacturer's instructions.

Immunophenotyping and flow cytometry

In order to detect and quantify SARS-CoV-2-specific T cells by stimulation of their cytokine secretion, flow cytometry and ELISpot will be performed.

Immunophenotyping will be executed on PBMCs directly ex vivo. Cytokine-producing T cells will be identified by intracellular cytokine staining. For that reason, PBMCs will be restimulated with a peptide pool representing the vaccine-encoded SARS-CoV-2 RBD (2 µg/ml/peptide; JPT Peptide Technologies) in the presence of GolgiPlug (BD Biosciences, San Jose, CA, USA) for 18 h at 37 °C. For both approaches, surface staining will be performed for 20 min at 4 °C. Afterwards, samples will be fixed and permeabilized using the Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Biosciences). Intracellular staining will be performed in Perm/Wash buffer for 30 min at 4 °C. Samples will be acquired on a FACSLyric instrument (BD Biosciences) analyzed with FlowJo software version 10.5.3 (FlowJo LLC).

ELISpot

IFNγ ELISpot analysis will be performed using 3.3 × 105 isolated CD4+ and CD8+ T cells per well according to the manufactorer's instructions. In brief, cells will be stimulated for 16–20 h with an overlapping peptide pool representing the vaccine-encoded RBD. Bound IFNγ will be visualized using a secondary anti-IFNγ antibody directly conjugated with alkaline phosphatase (1:250; ELISpotPro kit, Mabtech AB, Nacka Strand, Sweden) followed by incubation with a 5-bromo-4-chloro-3′-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate (ELISpotPro kit, Mabtech). Plates will be scanned using an AID Classic Robot ELISPOT Reader and analyzed by AID ELISPOT 7.0 software (AID Autoimmun Diagnostika, Straßberg, Germany).

Epigenetic determination of the immune response

Naïve B and T cells will be sorted from frozen visit 1 samples using FACS Sorting technology (FACS Aria). DNA will be PCR-amplified to generate libraries, which will be analyzed by Assay for transposase-accessible chromatin (ATAC) sequencing.

Cytokine measurement

To detect a variety of different cytokines produced by T cells after exposure to the S protein, additional Cytokine measurements will be done. PBMCs will be restimulated for 48 h with SARS-CoV-2 RBD peptide pool (2 µg/ml). Concentrations of various cytokines in supernatants will be determined using a magnetic bead-based Multiplex technology (MagPix, Merc, Darmstadt, Germany) according to the manufacturer's instructions.

Methods on T cell aging

TCR diversity

RNA will be extracted using RNeasy Protect Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer instructions. RNA will be used for reverse transcription with First Strand cDNA Synthesis Kit for RT-PCR AMV (Roche) according to the manufacturer regulations. cDNA will be diluted 1:5 for PCR using AmpliTaq Gold™ DNA Polymerase (Applied Biosystems, Waltham, MA, USA), 1× PCR Gold Buffer (Applied Biosystems), 2.5mM MgCl2 (Applied Biosystems), 0.4 mM dNTP Polymerization Mix (GE Healthcare), 0.5 µM TCR C β 5′FAM labeled primer (Ingenetix, Vienna, Austria), and 0.5 µM unlabeled TCR V β primers (Ingenetix). Cycle conditions will be a denaturation step at 94°C for 6 min, 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, a final annealing step at 72°C for 7 min. After amplification, the PCR product will be supplemented with GeneSCan™-500 TAMRA™ Size Standard (Applied Biosystems) and HI-DI Formamide (Applied Biosystems). Electrophoresis will be performed with ABI Prism 310 Genetic Analyzer (Applied Biosystems), and 310 Data Collection Software. Analysis will be done by GeneScan® Software (Applied Biosystems). Calculations will include peak count (Complexity score) and single peak area in percent of whole peak area.

Telomere length analysis

We will measure telomere lengths using DNA from isolated naïve CD4+ and CD8+ T cell subsets by quantitative real-time PCR analysis and LightCycler FastStart DNA Master SYBR Green I (Roche) according to manufacturer's instructions. The following primers will be used: 5´-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3´ and 5´GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3´. A single copy gene (36B4) (fw primer: 5´CCCATTCTATCATCAACGGGTACAA-3´; rev primer: 5´CAGCAAGTGGGAAGGTGTAATCC-3´) served for normalization. Ct-values of test samples and control single copy genes will be calculated and 2ΔCt value will be determined.

TREC measurement

T cell receptor excision circles (TRECs) from naïve CD4+ and CD8+ T cells will be analyzed. In brief, DNA will be extracted using QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA, USA). TREC concentrations will be determined by quantitative real-time PCR based on the coding TREC sequence using LightCycler 480 II (Roche) and primers to amplify a DNA fragment 108 bp across the remaining recombination sequence δrec/ψalpha (5'-CACATCCCTTTCAACCATGCT-3' and 5'-GGTGCAGGTGCCTATGC-3'). For quantification, a synthetic internal standard (Ingenetix, Vienna, Austria) will be used. PCR reaction will run with 0.25 µg DNA, primers and hybridisation-probe labelled with FAM-TAMRA (5'-ACACCTCTGGTTTTTGTA AAGGTGCCCAC-3'). To avoid bias by different numbers of naïve T cells, TRECs will be calculated in relation to numbers of T cells.

Nutritive assessment

Evaluation of Nutrient and Energy Intake:

To assess the nutrient and energy intake as well as the participants' dietary habits an expanded version of the Vienna Food Record (VFR) will be used (60). The VFR has been developed in accordance with Austrian eating preferences and habits, considers commonly available food and is weighted for consumption frequencies in Austria based on data of the European Food Safety Authority (EFSA). It is a prospective food frequency questionnaire that enables insides into the individual's dietary patterns and nutrient and energy intake. The participants will be asked to report their food intake of four days including one weekend day prior the second vaccination.

The diet's nutrient and energy composition will be analysed by a national specific nutritional software (dato Denkwerkzeuge 2021) that comprises an Austrian specific food and nutrient database.

Calculation of the Dietary Inflammation Index:

Since diet plays an important role in the regulation of chronic inflammation, the dietary inflammation index (DII) will be applied to the food records. The DII is a tool that categorizes the individual's diet on a continuum from maximally anti-inflammatory to maximally pro-inflammatory. The DII can be used as a categorical variable, and predicts the overall inflammatory potential of the diet in accordance with a set of global norms (37,61). The DII will be calculated based on the VFR data.

Measurement of Body Fat and Fat Patterning:
For the determination of the patient's body fat and the fat patterning the ultrasound method will be applied (62,63). This method reaches an extremely high accuracy and is capable of detecting subcutaneous adipose tissue thicknesses on a fine scale in people of various body composition (64). The thickness of subcutaneous fat will be measured at eight precisely defined body sites and the thickness sum will be calculated (D_{incl}). Anthropometric measurements of body mass (m), body height (h), sitting height (s) and the circumferences of waist and hip will be performed in accordance to the International Society for the Advancement of Kinanthropometry (ISAK) (65).

4.3 Risk-benefit assessment and precautionary measures

Risk from blood draws and collection of biomaterial and data is minimal. Participants of the study will benefit by knowing the levels of neutralizing antibodies to SARS CoV-2, the main factor for sterilizing immunity. Furthermore, our study will add to the knowledge on vaccination in immunodeficient patients and will inform future guidelines. The trial is carried out according to GCP and legal principles as outlined under 8.

4.3.1 Pregnancy tests

Pregnancy tests will be conducted before each vaccination. In the inactive phase of the study (visits 3,5,6), no increased study-related risk can be expected for pregnant women or unborn children. Therefore, no further pregnancy tests are planned in this phase.

4.4 Adverse events

Scheduled vaccination with any COVID-19 vaccine approved in Austria will not take place in the course of the trial but will happen according to the Austrian vaccination schedule. The investigators will not influence the date of vaccination or the type of the vaccine used. Adverse Events related to COVID-19 vaccines might occur.
Furthermore, events related to study procedures blood draw and salvia collection might occur.
Based on these facts, safety monitoring will be performed as follows:

- Any Adverse Event related to COVID-19 vaccines,
- Any Adverse Event related to blood draw and salvia collection,

- Any Serious Adverse Event.

Safety monitoring will be performed at every visit.

4.4.1 Definitions

An **adverse event (AE)** is defined as any harmful incident experienced by a study participant, not necessarily causally related to the clinical trial.
An **adverse reaction (AR)** is any harmful unintentional reaction to a study medication.
An **unexpected adverse reaction (UAR)** is an adverse effect which, in terms of its type and severity, is not anticipated on the basis of the existing product information.
A **serious adverse event (SAE) or a serious adverse reaction (SAR)** is an adverse event or a serious adverse effect that is fatal or life-threatening (independent of the dose), necessitates in-hospital treatment or its prolongation, leads to permanent or serious disability or incapacity or a congenital abnormality or birth defect.
A **suspected unexpected serious adverse reaction (SUSAR)** is designated as such according to Guideline 2001/20/EG. A serious adverse reaction is deemed unexpected when it is not listed in the corresponding basic document (Summary of Product Characteristics, IB, IMPD).

4.4.2 Reporting obligations

Principal Investigator:
Reporting AE as specified above, SAE and SUSAR to the sponsor according to AMG (Austrian Act on Pharmaceutical Products) § 41d.

Sponsor:
The sponsor must report all important information about suspected serious adverse to the Federal Agency for Safety in Healthcare as well as the competent ethics committee according to AMG §41e (Austrian Act on Pharmaceutical Products §41e).

4.5 Interim analyses

An interim analysis will be done when at least 30% of the subjects have completed visit 3. At that point, we will check if the described methods have worked properly and provided valid, comparable results. Interim analysis will predominantly include the primary endpoint and also the secondary endpoints of T cell responses. Depending on the results, the protocol may be amended. A change in sample size based on the results of the interim analysis will also lead to an amendment of the protocol. Amendments will be reported to the ethics committee and authorities as detailed under 7.

4.6 Termination of the trial

4.6.1 Termination of the trial for a subject (drop-out)

Participants may discontinue study procedures and withdraw from the study at his/her own request at any time. Definitive discontinuation is also possible in case of adverse events after the first dose of vaccination, request of the investigator or diagnosis of pregnancy. Violation of the protocol or incorrect vaccination, as documented in the vaccination certificate, will lead to termination of the trial for the subject. In case of discontinuation, participants will be followed for safety, and already collected data as well as biomaterial will still be analyzed according to study protocol. After withdrawal of consent, participants may demand that collected subject data have to be deleted and biomaterial must be destroyed. The investigator must document the reason of discontinuation or withdrawal and any request made by participants.

4.6.2 Termination of the entire trial

Premature termination of the clinical trial will be considered when the sponsor believes it is necessary to terminate the clinical trial for safety reasons, or when the clinical trial proves to be impracticable.
For the benefit of, and in the interest of the probands, the Principal Investigator may terminate the trial at any time when serious adverse effects or other unforeseen circumstances occur.
Reporting obligation in case of termination: In case of termination of the trial, the responsible ethics committee and the authorities (BASG) must be informed within 15 days; the reasons for termination must be clearly stated herein.

5. Monitoring and Audit – Assurance of data quality

Monitoring and audits shall be performed during the clinical trial for the purpose of quality assurance.

5.1 Monitoring

The investigator consents to data evaluation being performed by the person in charge of monitoring in order to ensure satisfactory data collection and adherence to the study protocol.
Furthermore, the investigator states that he/she is willing to cooperate with this person and shall provide this person with all required information whenever necessary. This includes access to all documents associated with the trial, including study-relevant medical files of patients in original form. The tasks of the investigator include maintenance of these patients' medical files as comprehensively as possible; this includes information concerning medical history, accompanying diseases, inclusion in the trial, data about visits, results of investigations, dispensing medication, and adverse events. The monitor will also be permitted to perform data evaluation and draw comparisons with the relevant medical files in accordance with the SOPs and ICH-GCP guidelines at pre-determined intervals, in order to ensure adherence to the study protocol and continuous registration of data. All original medical reports required as sources for the information given in the CRF or the database shall be inspected. The study participants will have given their consent to such inspection by signing the consent form.
The person in charge of monitoring is obliged to treat all information as confidential, and to preserve the basic claims of the study participants in respect of integrity and protection of their privacy.

The purpose of these visits specifically includes the following:

- Inspection of informed consent forms,
- Inspection of patient safety (occurrence and documentation / reporting of AEs and SAEs),
- Inspection of CRFs in respect of their accuracy and completeness,
- Validation of CRFs compared to original data (source data verification, SDV),
- Evaluation of the progress of the trial,
- Control of compliance with the study protocol,

- Ensuring that the trial is being performed at the study centre in conformity with GCP
- Discussion with the principal investigator about the implementation of the trial and any deficiencies that may have been noted

A monitoring report shall be issued about every visit. The report will document the progress of the clinical trial and will provide information about any difficulties that may have occurred.

5.2 Audit

The sponsor is authorised to perform audits at the study centre and other facilities that may be involved in the trial, as part of quality assurance. The purpose of the audit is to check the validity, verifiability, and completeness of the data and the credibility of the clinical trial as well as inspect the maintenance of patients' rights and patient safety. The sponsor may authorise persons not otherwise involved in the clinical trial for this task (auditors). These persons shall be permitted to inspect all study-related documents (especially the study protocol, survey forms, medical files, documentation of the study medication, study-related correspondence).
The sponsor and all participating study centres are obliged to support auditors and inspections of appropriate authorities, and to provide authorised persons access to original documents for this purpose.
All persons conducting audits are obliged to treat personal data and other data as confidential.

6. Biometry

Statistical evaluation of the data will be performed by Dr. Florian Posch.
For the primary endpoint, the number of individuals with positive seroconversion to anti-SARS-CoV-2 spike protein at day 21-28 after the second vaccination measured by SARS-CoV-2 antigen-binding Ig assay will be assessed comparing immunocompromised patients to healthy controls.

Null and alternative hypotheses:

H_0: There is no statistical difference between the seroconversion rate of immunodeficient patients and healthy controls.

H_1: There is statistical difference between the seroconversion rate of immunodeficient patients and healthy controls.

6. 1. Biometric design

Group comparison, i.e. immunodeficient patients to healthy controls, will be performed for the primary as well as secondary endpoints according to the statistical methods described in 6.4. Depending on the respective secondary endpoint, statistical analyses may be performed after visit 3, 4, 5 and/or 6.

6. 2. Sampling plan

To estimate the sample size for the primary endpoint, we considered levels of anti-SARS-CoV-2 IgG antibodies after 2nd vaccination. Therefore, we used nQuery, version 8.6.1 (Statistical Solutions Inc.), based on previous studies investigating the seroconversion of immunodeficient patients vaccinated against influenza (66). Mann–Whitney U test with a significance level of 0.01 and a power of 95% resulted in 175 subjects per group. Assuming 10% drop out rate, we calculated a sample size of 195 subjects per group.
At the interim analysis in August 2021 the primary endpoint was met. Therefore, recruitment was stopped at this point. The actual sample size was 195 immunodeficient and 173 healthy subjects.

6. 3. Data registration and evaluation

Statistical analysis of results will be done with IBM SPSS Statistics, version 26 or higher and R software (https://www.r-project.org/). Data quality will be assured by comparing random samples from processed data with the appropriate source data.

6. 4. Statistical methods

Primary endpoint:
The difference between healthy and immunodeficient individuals in the levels of anti-SARS-CoV-2 spike protein antibodies at day 21-28 after the second vaccination measured by SARS-CoV-2 antigen-binding Ig assay. Statistical significance will be calculated by comparison of

immunocompromised patients to healthy controls via rank-sum tests. Patients developing SARS-CoV-2 infection or COVID-19 until primary endpoint evaluation will be omitted from the per-protocol analysis. P-values < 0.05 will be considered as statistically significant.

Secondary endpoints:

The levels of anti-SARS-CoV-2 spike protein antibodies at 6 and 12 months after vaccination will be compared between immunocompromised patients and healthy controls by parametric or non-parametric tests (t-test, Mann-Whitney-U test, Kruskal-Wallis test), as appropriate. P-values < 0.05 will be considered as statistically significant.

Change of recombinant S protein-binding IgG levels after second vaccination to recombinant S protein-binding IgG levels after first vaccination will be compared between both time points using the one-sample t-test or Wilcoxon test, as appropriate. P-values < 0.05 will be considered as statistically significant.

Concentrations of secretory (saliva) and serum IgA as well as IgG and IgM after second vaccination will be treated as continuous variable and compared between immunocompromised patients and healthy controls by parametric or non-parametric tests (t-test, Mann-Whitney-U test, Kruskal-Wallis test), as appropriate. P-values < 0.05 will be considered as statistically significant.

IFNγ production of T cells after SARS-CoV-2 antigen exposure, measured by FACS and ELISpot, will be treated as continuous variable and compared between immunocompromised patients and healthy controls by parametric or non-parametric tests (t-test, Mann-Whitney-U test, Kruskal-Wallis test), as appropriate. P-values < 0.05 will be considered as statistically significant.

Baseline characteristics between immunocompromised patients and healthy controls will be compared with rank-sum tests, χ2-tests, and Fisher's exact tests, as appropriate. Associations of variables with high levels of anti-SARS-CoV-2 Ig will be studied in descriptive statistics. Although this is a hypothesis generating step, for further adequately power studies a correction for multiple testing will not be applied. P-values < 0.05 will be considered as statistically significant.

Neutralizing capacity of produced anti-SARS-CoV-2 IgG will be assessed in a descriptive manner.

For evaluation of dietary and/or body fat composition effects on anti-SARS-CoV-2 IgG levels, we will use Spearman's rank correlation or Pearson's correlation, as appropriate. P-values < 0.05 will be considered as statistically significant. Moreover, possible changes in body fat, ACE2 expression and dietary quality from visit 3 to 6 will be assessed in the studied groups via descriptive and explorative statistics.

Median follow-up time will be estimated with the reverse Kaplan-Meier method.

Risks of developing COVID-19, death-related-to-COVID-19 and death-from-other-causes will be calculated with competing risk cumulative incidence estimators Gray's tests, and Fine & Gray proportional subdistribution hazards models, respectively.

Missing data will not be replaced or estimated; only available data of participants will be considered in the analysis.

7. Modification of the study protocol

The vote of the ethics committee applies solely to the information contained in the application; it does not include extensions or modifications of the research project undertaken at a later point in time. In case of any modification, an amendment of the study protocol signed by the Principal Investigator is required. Any modification of the study protocol must be attached as an amendment to all study protocols in circulation. The ethics committee must be informed of all modifications in the study protocol. In case of modifications in the study protocol that are not merely of a formal nature but contain changes pertinent to the study participants, a renewed vote of the ethics committee must be obtained. If applicable, the patients/probands must be informed in the information and consent form about changes in the terms and conditions of the study.

Substantial amendments must also be reported to the authorities (BASG). This is subject to non-prohibition in a period of 35 days. The ethics committee in charge must provide its statement within a period of 35 days.

8. Ethical and legal matters

8.1 Legal principles

During the implementation of the trial, the (current versions of) following guidelines and laws must be followed in addition to the Declaration of Helsinki (such as):

- Current version of AMG (Austrian Act on Pharmaceutical Products)
- ICH-GCP guideline
- EU directives 2001/20/EC and 2005/28/EC, etc.

8.2 Vote of the ethics committee;

In accordance with § 40 AMG (Austrian Act on Pharmaceutical Products), the clinical trial may be started only after the competent ethics committee has issued its statement of approval and the appropriate authorities (BASG) have provided their non-prohibition/approval.

8.3 Insurance

During the clinical trial, a no-fault insurance (personal injury protection insurance in accordance with § 32 of the Austrian Act on Pharmaceutical Products) shall be concluded for the patient on behalf of the sponsor.
Exact contact data of the insurance company and the policy number is mentioned in the patient information form.

8.4 Principal Investigator

By signing the study protocol the Principal Investigator confirms that he/she has read and understood the study protocol, and will work in accordance with the protocol.
The Principal Investigator guarantees the confidentiality of all information.

8.5 Storage and Data protection

The registration, transfer, storage and evaluation of personal data in this clinical trial shall be subject to legal regulations (AMG - Austrian Act on Pharmaceutical Products - and data protection law). A prerequisite for this purpose is the participant's voluntary consent in the consent form, given prior to his/her participation in the clinical trial. The participants shall be informed of the following as part of the information about this clinical trial:

1. Data obtained in the course of this clinical trial shall be recorded on paper forms or electronic data storage devices, treated as strictly confidential, and passed on exclusively to the following persons without mentioning names (pseudonymised):

- the sponsor of the trial for scientific evaluation and assessment of adverse events.

2. If required for the inspection of the clinical trial, persons authorised by the sponsor and committed to secrecy (monitoring, auditing), authorised persons in national and foreign health authorities, and authorised persons of the competent ethics committee may inspect personal data at the study centre.

3. The participant shall be informed that he/she may terminate his/her participation in the clinical trial at any time without stating reasons and with no subsequent disadvantages. However, due to the legal obligation of documentation (AMG; Austrian Act on Pharmaceutical Products), authorised persons committed to secrecy may be permitted to inspect personal data for test purposes, for a period of time specified by law.

9. Publication

Publication of data derived from this study requires the consent of the principal investigator and any co-principal investigator involved. Publications with the main focus on humoral immunity shall be coordinated by Ivo Steinmetz. Publications with the main focus on T cell immunity, CVID or rituximab shall be coordinated by Martin Stradner. Publications with the main focus on vaccination in hematologic diseases shall be coordinated by Hildegard Greinix. The coordinating authors will appoint junior authors. Coordinating and junior authors are responsible for the preparation of the

manuscripts. Authorship will be granted according the Uniform Requirements for Manuscripts Submitted to Biomedical Journals.

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11. Signatures

11.1 Sponsor

By signing this document I confirm that the trial shall be performed in accordance with ICH-GCP, the Declaration of Helsinki, national laws, and the current study protocol.

Sponsor or his representative

_____	_____
Name, First name (in block letters)	**Date, Signature**

11.2 Principal Investigator

I confirm herewith that I have read and understood the present study protocol, and acknowledge all parts of it. I promise to ensure that the persons introduced in the trial at my centre shall be treated, observed and documented in accordance with the terms and conditions of this study protocol.

_____ _____

Name, First name (in block letters) **Date, Signature**

Appendix

Product characteristics (in German)

Comirnaty: https://www.ema.europa.eu/en/documents/product-information/comirnaty-epar-product-information_de.pdf

Moderna Covid-19 vaccine: https://www.ema.europa.eu/en/documents/product-information/covid-19-vaccine-moderna-epar-product-information_de.pdf

AstraZeneca COVID-19 vaccine: https://www.ema.europa.eu/en/documents/product-information/covid-19-vaccine-astrazeneca-epar-product-information_de.pdf