Primary T-cell responses against SARS-CoV-2 in patients with hematological disorders

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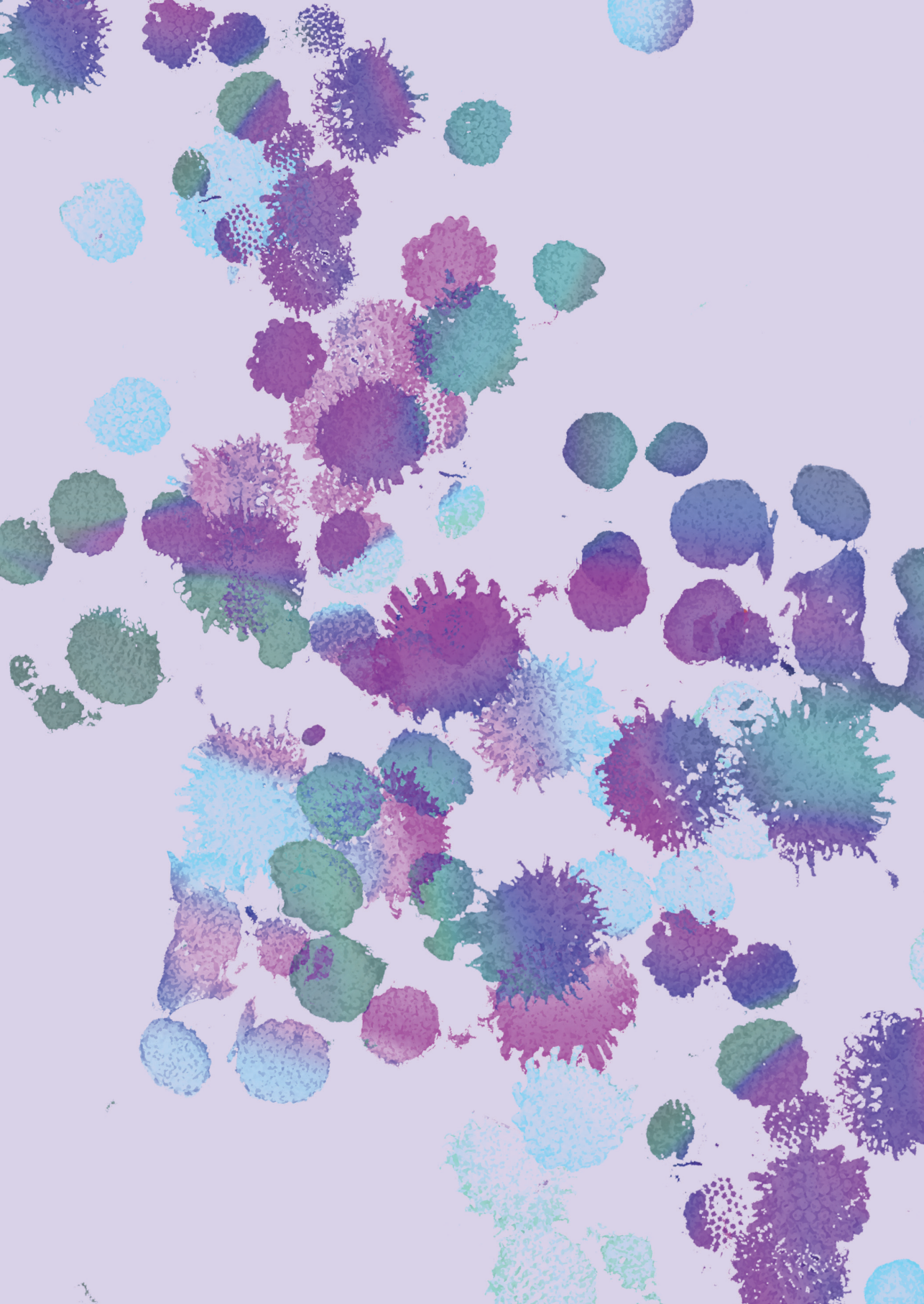

GENERAL INTRODUCTION

Chapter 1

1 SARS-COV-2

1.1 Pandemic

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was first identified in December 2019, leading to a pandemic in 2020.[1] The pandemic and the relative severity of symptoms, ranging from asymptomatic to severe coronavirus disease 2019 (COVID-19), induced by SARS-CoV-2 were largely caused by the fact that mankind had never been exposed to this virus and therefore did not possess an existing memory immune response. Since the emergence of SARS-CoV-2, efforts have been made to gain insight into the underlying mechanisms that might explain the large heterogeneity in disease severity. These efforts have been essential to determine which individuals are susceptible to severe disease and who should therefore be prioritized for enhanced protective measures against infection. Fortunately, vaccines against SARS-CoV-2 were quickly developed due to the rapid identification of the full SARS-CoV-2 sequence, availability of mRNA vaccine technology, and parallelization of developmental processes.[1,2] Since 2021, most individuals have developed a memory immune response against SARS-CoV-2 due to vaccination or infection, thereby ending the pandemic.[3] During the pandemic, massive efforts have been made by the scientific community to understand the virus or infection, resulting in the biobanking of samples from before and during the pandemic. These valuable samples offer a unique opportunity to investigate the development of the immune system after an encounter with a new virus.

1.2 Infection

SARS-CoV-2 is part of the beta coronaviruses, which also include the common coronaviruses OC43 and HKU1, as well as SARS-CoV-1 and MERS.[4] The virus comprises a lipid bilayer containing the spike protein, membrane protein, and envelope, which encapsulates the positive, single-stranded ribonucleic acid (RNA) and nucleocapsid protein. The spike protein is essential for viral cell entry as its receptor-binding domain (RBD) directly binds to angiotensin-converting enzyme 2 (ACE-2), which is expressed by, among others, human epithelial cells in the respiratory tract.[5] SARS-CoV-2 therefore predominantly targets the upper and lower respiratory systems. Virus RNA is released inside the cells and translated into polypeptides and enzymes that are needed for transcription and translation of the viral genome. New virus particles are generated and released from the human cell. This process results in rapid replication of new virus particles and severe damage to the cells, leading to pyroptosis.

1.3 Host Immune Response

Infection by SARS-CoV-2 causes pyroptosis of the host cells, causing the release of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs).[6,7] These danger signals are recognized by pattern-recognition receptors (PRRs) expressed by innate immune cells (monocytes, macrophages, neutrophils, dendritic cells, and natural killer cells), which in turn release pro-inflammatory cytokines such as interferon-I/III (IFN-I/III), and chemokines. Pro-inflammatory cytokines and chemokines attract more immune cells including the adaptive immune system (T and B cells), further enhancing the pro-inflammatory environment. Cells of the innate immune system are fast responders and are important for local enhancement of a pro-inflammatory environment, clearing infected cells, and functioning as antigen-presenting cells (APCs) for the adaptive immune system. The adaptive immune system is initially slower but targets the virus specifically.[8] Helper CD4+ T cells have a wide array of functions that include supporting the function of innate cells, B cells, and CD8+ T cells by expressing stimulatory receptors and producing cytokines. Cytotoxic CD8+ T cells recognize cells that are infected by the virus specifically and kill the infected cells. B cells produce antibodies that can neutralize the virus itself, resulting in opsonization and blocking of viral entry. A fast and effective response of the innate and adaptive immune response limits viral spreading and reduces further damage to human cells.

1.4 COVID-19

Most healthy individuals clear SARS-CoV-2 infections with mild respiratory symptoms. However, in contrast to influenza infections, SARS-CoV-2 infections can also lead to gastrointestinal and neurological damage, and a higher percentage of SARS-CoV-2 infections lead to severe disease.[9] Patients with severe disease mainly suffer from pneumonia that can further develop into acute respiratory distress syndrome (ARDS). Autopsies revealed that most patients were indeed deceased due to ARDS (53%), but also multi-organ failure (18%) or heart complications (7%).[10] These complications are caused by tissue damage induced by the virus, but also by overactivation of the immune system. This is caused by delayed viral clearance due to immune-evasion mechanisms of the virus, combined with a delayed immune response and inability to limit overactivation.[8,11] Delayed viral clearance results in a high viral load and therefore an excessive release of PAMPs and DAMPs, which in turn results in highly activated and infiltration of innate immune cells into the lungs.[11] Ineffective initiation of the adaptive immune system further enhances the prolonged presence of the virus. Due to the high viral load, both the innate and adaptive immune responses become overactivated. Subsequently, these patients are unable to dampen this

excessive activation of the immune system. This is a clear hallmark of severe COVID-19 since hyperactivated cells and a cytokine storm can be detected in lung tissues and circulation.[11,12] Therefore, it is proposed that the timing and magnitude of the immune response are mismatched, resulting in excessive tissue damage.[11] Some individuals have trouble fully recovering and develop post-COVID-19 (also known as long COVID-19) with lingering symptoms such as persistent cough, fatigue, and neurological dysfunction.[13] Disease severity varies based on factors that include characteristics of the virus, host factors, and environmental factors.[14] The main host risk factors for severe COVID-19 include factors that enhance symptoms of COVID-19 such as respiratory diseases, cardiovascular diseases, and higher body mass index, or factors that disrupt normal immune function such as diabetes, primary immune deficiencies, or immunosuppressive treatment.[15-17] Another host factor that impacts disease outcome is vaccination, as vaccination results in the initiation of a fast and effective adaptive immune response, thereby limiting viral spread.

1.5 Vaccines

Vaccination is an effective method to induce an immune response against pathogens. Vaccines are designed to activate the adaptive immune system since these cells are pathogen-specific and can result in long-lasting immune memory. Different vaccine types exist for viral infections, which differ in the amount of antigens, protein delivery method, immunogenicity, and adjuvants, which can be roughly subdivided into: live attenuated, inactivated, vector, subunit, or DNA/RNA.[18] Live attenuated vaccines contain the live virus, but the virus is weakened to prevent disease, and initiate a strong immune response. For some vaccines, the virus particles are inactivated or killed, which is called an inactivated vaccine, and often require boosters since they are less effective compared to live attenuated vaccines. Vector vaccines typically consist of a different weak virus (vector) that is genetically modified to express proteins of the virus that the aim is to vaccinate against. Depending on the choice of vector virus, the vaccine might be less effective since individuals may already have developed immunity against the vector virus, or the vector virus is not sufficiently immunogenic. Subunit vaccines do not contain genetic material but are composed of purified proteins, peptides, polysaccharides, or a combination of two subunits (conjugate vaccines). DNA/RNA vaccines contain DNA or RNA that encodes for viral proteins, and are easy to develop but may be less effective. The most commonly administered SARS-CoV-2 vaccines are mRNA-based vaccines. Vaccines based on mRNA technology are not new, as the publication of the first pre-clinical trial dates back to 1993, and the first-in-human clinical trial was in 2017.[19,20] As the name suggests, messenger RNA (mRNA) vaccines consist of mRNA encoding the antigen of interest and are encapsulated by a (ionizable) lipid nanoparticle (LNP). The LNPs ensure efficient delivery of the mRNA

inside cells[21] but also act as an adjuvant.[22] The design of the mRNA and LNP can differ between different vaccines. In the case of the SARS-CoV-2 mRNA vaccines, the mRNA encodes for the full-length stabilized spike protein of the virus, flanked by the 5'- and 3'-untranslated regions. Currently, three companies have developed SARS-CoV-2 mRNA vaccines, but most individuals from the Dutch population have been vaccinated with the BNT162b2 (Pfizer/BioNTech) or mRNA-1273 (Moderna) vaccine. Although they are both LNP-mRNA vaccines, they differ in dosage, design of the LNP, and modifications to the mRNA such as capping method, codon optimizations, and choice of polyadenylation tail.[23]

1.6 mRNA vaccination

The vaccine is injected intramuscularly, causing a local transient infiltration of immune cells and uptake of the LNP-mRNAs mostly by muscle cells, monocytes, and dendritic cells through endocytosis.[24] The mRNA is released from the endosomes and translated into the spike protein. The spike protein is expressed on the cell surface as a protein and degraded into peptides in the cytosol. These peptides are subsequently loaded onto human leukocyte antigen (HLA) molecules and are presented as complexes on the cell surface. The transiently transfected dendritic cells migrate to the draining lymph nodes for presentation of the spike protein to B cells and T cells. Besides expression of the spike antigen, the LNP and mRNA themselves also induce a pro-inflammatory response, such as production of IFN-I by the transfected muscle and immune cells, further enhancing the immune response.[23] Multiple studies have shown that both mRNA vaccines induce long-lived B cells, spike-specific CD4+ and CD8+ T cells in healthy individuals.[25] Both the BNT162b2 and mRNA-1273 induce B-cell and T-cell responses that are detectable in circulation, but the mRNA-1273 induces a more durable humoral response and higher T-cell frequencies.[26] Booster vaccinations, usually meaning a prolonged interval between two vaccinations, enhance the durability of the adaptive immune response. Both vaccines were produced using the ancestral strain of SARS-CoV-2, which resulted in reduced efficacy against new variants of the virus.[27] This was mostly caused by mutations in the RBD, resulting in a reduced ability of neutralizing antibodies to prevent cell entry by binding to the virus.[28] To enhance the efficacy of neutralizing antibodies, both Moderna and BioNTech updated their vaccines to match newer viral variants, resulting in increased vaccine efficacy.[29-32] Interestingly, T-cell responses are less affected by the new variants because T cells typically recognize a wider range of epitopes, making it less likely that viruses can evade T-cell recognition by epitope mutation.[33,34] Furthermore, SARS-CoV-2 mRNA vaccine-induced T-cell responses can persist for a long period and are correlated with protection against severe disease.[34-38]

For these reasons, BioNTech developed a new vaccine (BNT162b4) that focuses on T-cell immunity by including multiple (non-spike) antigens.[39]

1.7 Monitoring

Monitoring of virus-specific immune responses is commonly done by measuring virus-specific antibodies, or immunoglobulins (Ig), titers in the circulation. SARS-CoV-2 infection can lead to antibodies binding to structural, non-structural, and accessory proteins.[40] The current SARS-CoV-2 mRNA vaccines only induce immune responses against the spike protein. For this reason, infection-induced immunity is measured by the presence of anti-nucleocapsid antibodies, whilst vaccine-induced immunity is measured through the presence of anti-spike antibodies in the circulation (in the absence of anti-nucleocapsid antibodies). Furthermore, spike-specific antibodies may be specified based on whether they bind to RBD, as this directly blocks the binding of the virus to human cells. There are five antibody isotypes: IgA, IgD, IgE, IgG, and IgM. For monitoring, IgG concentrations are usually measured because IgG can reach high levels in circulation, is quickly detectable after infection or vaccination, and is highly effective against viruses. Antibodies are measured using qualitative or (semi-)quantitative assays. Qualitative assays only indicate whether antibodies are present, whilst quantitative assays also give a concentration. Semi-quantitative tests give a concentration in a scaling that is specific for the test, whilst quantitative assays give the exact antibody concentration in plasma. The WHO developed International Units IU/ml) for neutralizing antibody levels and binding antibody units per milliliter (BAU/ml) for binding assays, so that the concentration levels are directly comparable between institutes.[41] The focus on antibody monitoring as a proxy for vaccine-induced immunity can be explained by the ease of antibody measurement and that the measurements are standardized between institutes. However, focusing on antibodies ignores the presence of T-cell-mediated immunity. Measuring T cells becomes especially important when realizing that antibodies wane in time, and some individuals might have trouble developing adequate antibody levels, but can induce strong T-cell responses.

2 T CELLS

2.1 Antigen

The antigens recognized by T cells are peptide-HLA complexes on the cell surface of other cells. These complexes consist of peptides presented in the context of HLA class I or II.[42] Intracellular proteins are degraded by the proteasome into peptides and are transported into the endoplasmic reticulum (ER). In the ER, the peptides

are loaded onto the peptide-binding groove of HLA class I and transported through the Golgi apparatus to the cell surface. For HLA class II, this process is slightly different. Exogenous proteins are encapsulated into endosomes, which contain enzymes that degrade the protein into peptides. These endosomes fuse with MHC class II compartments, which contain HLA class II molecules that are stabilized with class II-associated invariant chain peptide (CLIP).[43] The peptide replaces CLIP and the peptide-HLA complex is transported to the cell surface of APCs. Through these processes, peptides from intracellular proteins are presented in HLA class I, whilst peptides that are present extracellularly are typically presented in HLA class II. Which peptide is presented depends on the HLA type. HLA-A, HLA-B, and HLA-C are class I molecules that typically present 8-11 amino acid-long peptides to CD8+ T cells. HLA-DP, HLA-DQ, and HLA-DR are class II molecules that typically present 10-15 amino acid-long peptides to CD4+ T cells. These HLA loci are (highly) polymorphic and consist of different allotypes that differ in amino acids, mainly in the peptide-binding groove. This variation results in differences in which peptides have the highest binding affinity to which HLA isoform. For HLA class I, the peptide-binding motifs typically have an anchor residue at positions 2 and 9, which are essential for binding to the HLA. The peptide-binding motif is less strict for HLA class II, resulting in a larger variability in peptides that can bind to the same HLA. Furthermore, the pockets of HLA class II are open at the ends, resulting in a larger heterogeneity of peptide lengths. Therefore, which peptides are presented in a cell depends on the HLA type of the individual. The more diverse the two alleles for each HLA gene, the wider the diversity of peptides that are presented on a cell. It is therefore generally accepted that a more diverse HLA-type generates a wider T-cell response and therefore enhances protection against pathogens.

2.2 TCR

T cells recognize peptide-HLA complexes through their T-cell receptor (TCR). This interaction (signal 1), together with co-stimulation (signal 2) and cytokines (signal 3), triggers T-cell activation and therefore forms an essential part of T-cell-mediated immunity. Before T cells can exert these functions, they undergo a process of differentiation and selection. T cells originate from hematopoietic stem cells in the bone marrow, from where they travel as lymphoid progenitors to the thymus.[44] In the thymus, the gene encoding for the β-chain of the TCR undergoes rearrangement and pairs with a pre-TCR α-chain to ensure the functionality of the β-chain. After successful β-chain rearrangement, the α-chain starts its rearrangement. Germline *TRB* and *TRA* genes consist of multiple variable and joining segments, and variable segments are rearranged to a joining segment through somatic recombination. The rearrangement of the α-chain and β-chain is not completely the same. *TRA* contains only a single

constant region, whilst *TRB* has two constants. For the α-chain, both *TRA* alleles undergo rearrangement simultaneously, yielding two functional α-chains in ~20% of the cells. This process of rearrangement results in three complementary-determining loops (CDR1-3) in both chains, which are important for binding to the antigen. CDR1 and CDR2 are homologous among TCRs, whilst the CDR3 region is highly diverse due to genetic alterations during joining of segments and therefore is an important factor in dictating TCR specificity and diversity. After the formation of the TCR, the thymocytes undergo selection to eliminate thymocytes that are auto-reactive or lack reactivity at all. This is achieved by programmed apoptosis of thymocytes with TCRs that lack or have a too high binding affinity to peptide-HLA complexes expressed on cortical thymic epithelial cells. Part of the thymocytes with a higher affinity for self-peptide-HLA complexes may be selected to become regulatory T cells. Selected thymocytes are further matured, and cells that weakly recognize a self-peptide in HLA class I will remain single positive for CD8, whilst thymocytes that express a TCR that binds to HLA class II will only express the CD4 co-receptor. Due to the high sequence variability of TCRs, TCRs have a large diversity in binding affinity and specificity towards peptide-HLA combinations. TCRs are considered highly specific given the large diversity of potential peptide–HLA combinations and the limited number of peptide-HLA complexes a single TCR can recognize.[45-48] Recognition of more than one peptide-HLA complex by a single TCR is referred to as cross-reactivity, which can result from multiple mechanisms.[49-56] In most cases, cross-reactivity is caused by peptide-HLA complexes that share sequence or structural homology. Another mechanism is hotspot binding, a phenomenon in which the TCR strongly binds to only a few amino acids of the peptide. TCRs with hotspot binding are therefore more forgiving when amino acids change outside the hotspot. Apart from the reduced footprint of the TCR, cross-reactivity may also be caused by the plasticity of the TCR and peptide-HLA complex. The TCR and peptide-HLA complex may undergo conformational changes upon binding, potentially strengthening the binding. These forms of TCR and peptide-HLA cross-reactivity expand T-cell reactivity towards a wide range of pathogens, called heterologous immunity. However, cross-reactivity may also cause problems, as seen in auto-immune diseases and alloreactivity in allogeneic transplantation settings.

2.2 Differentiation

CD4+ or CD8+ T cells leave the thymus and start circulating in the blood and lymphoid organs. At this stage, they are called naïve T cells and express markers such as CCR7, CD45RA, and CD62L. Upon infection, the pathogen-derived peptides are presented to T cells by antigen-presenting cells (APCs) in lymphoid structures, which triggers T-cell differentiation and proliferation (expansion phase). The T cells leave the lymphoid

structures and recirculate in the blood and migrate to the site of infection.[57,58] After pathogen clearance, most T cells go into apoptosis (contraction phase) and the remaining cells differentiate into central memory (T_{CM}) or effector memory (T_{EM}) cells.[58] Typically, T_{CM} are less capable of producing cytokines but proliferate better compared to T_{EM}. They express lymph node-homing receptors such as CCR7 and CD62L. T_{EM} cells lack the expression of these receptors but express more tissue-homing receptors. Additionally, T_{EM} cells may re-express CD45RA which are then termed terminal effector memories (T_{EMRA}). Memory T cells are present throughout the human body and can respond quickly upon re-infection. They do this by quick upregulation of activation markers such as CD137, CD69, and CD154, and production of cytokines, all within a few hours.

2.3 Function

CD4+ T cells, or T-helper cells, are T cells with a wide range of functions with different phenotypes. Effector memory CD4+ T cells can be subdivided into seven subsets based on transcription factor expression and cytokine profile: Th1, Th2, Th9, Th17, Th22, regulatory T cells (Treg) and follicular helper T cells (Tfh).[58] Th1 cells are most important for protection against viruses and bacteria as they produce cytokines such as IFN-γ and TNF-α/β, and may also produce IL-2. These cytokines promote CD8+ T cell and macrophage function.[57] Th2, Th9, Th17, and Th22 typically play a role in other situations such as tissue repair, protection against parasites, fungi, and allergies. Treg express FoxP3 as well as high levels of CD25 and produce inhibitory cytokines IL-10, TGF-β, and IL-35. Compared to helper T cells, Tregs have a higher affinity for self-peptides and are important for dampening the immune response. Tfh cells express high levels of CXCR5 and PD-1, and are specialized in promoting B-cell isotype switching, affinity maturation, and differentiation through secretion of IL-21 and expression of CD40.[57,59] CD8+ T cells, or cytotoxic T cells, are T cells that are specialized in lysing cells that are infected. Upon antigen encounter, they produce cytokines including IFN-γ and TNF-α, and secrete perforin and granzyme B. Perforin induces cytolysis by forming pores in the cell membrane, and granzyme B induces apoptosis by disrupting essential cellular processes in the targeted cell. These cytokines and enzymes result in the effective clearance of diseased cells.

2.4 Monitoring

Monitoring of antigen-specific T cells is more laborious compared to antibodies because the measurement of living cells is more challenging than that of proteins. Similar to antibodies, assays can differentiate between infection-induced and vaccine-induced T-cell responses, depending on whether the antigen used in the monitoring assay is derived from spike (vaccination and infection) or non-spike

proteins (infection).[60] Antigen selection combined with sampling moment is essential to differentiate between pre-existing immunity, primary T-cell responses, and hybrid immunity in the monitoring assay. Samples frozen down before the pandemic, although SARS-CoV-2-unexposed, may contain SARS-CoV-2-specific T cells. These T cells must have been originally primed by another pathogen, developed into memory T cells, and are cross-reactive towards SARS-CoV-2 antigens. Primary T cell responses can typically be detected in samples frozen down in 2020 or 2021. This is because the virus was first described in December 2019, but most individuals in Europe were exposed to SARS-CoV-2 for the first time from March 2020 onwards. In 2021, the first SARS-CoV-2 vaccines were administered. Combined vaccine- and infection-induced immunity (hybrid immunity) occurs more often in later sampling moments since a large proportion of the population received SARS-CoV-2 vaccination, and time increases the chances of exposure to the virus itself. Apart from antigen selection and sampling moment, the method of detection is essential for the accurate interpretation of measured SARS-CoV-2-specific T cell responses.

2.5 Detection methods

Two distinct methods are being used for the detection of antigen-specific T cells: peptide-HLA tetramer staining which measures the presence of antigen-specific T cells, and peptide-stimulation assays which measure functional antigen-specific T cells.[61] Peptide-HLA tetramers consist of four biotinylated peptide-HLA complexes that are conjugated to a streptavidin-labeled fluorochrome. The peptide-HLA complexes used in these detection methods are composed of HLA prevalent alleles binding earlier identified epitopes from the pathogen of interest. Tetramers are incubated with peripheral blood mononuclear cells (PBMCs), T cells that recognize the peptide-HLA tetramers bind to the tetramer and become fluorochrome-labeled which is subsequently detected using flow cytometry. Setting up such staining is labor-intensive, since for each peptide-HLA combination, new tetramers need to be generated. Since HLA types in the population are diverse and various T-cells target multiple antigens, a large library of peptide-HLA tetramers is needed to cover most individuals and T cells. However, this method is generally highly specific, can detect low frequencies of antigen-specific T cells, and does not rely on cell functionality. Once a library of peptide-HLA tetramers is generated, this tool allows for relatively high-throughput analysis. For peptide-stimulation assays, PBMCs are incubated with peptide pools consisting of 15-amino-acid-long peptides with 11-amino-acid overlap translated from immunogenic proteins. These peptides bind to HLA molecules on the surface of APCs in the sample. T cells that recognize the peptide-HLA complexes become activated, express activation markers, and produce cytokines. These markers and cytokines are then used as a detection method for T cells that are specific for

the peptides that were added to the sample. The most common large-scale, high-throughput, methods are enzyme-linked immunosorbent spot (ELIspot) and the interferon-gamma release assay (IGRA). ELIspot captures the secretion of IFN-γ close to the source, resulting in spots that are counted as an estimate of the number of T cells that produce cytokines. IGRA measures a concentration of IFN-γ in supernatant. These assays are relatively standardized as companies offer specialized kits and protocols. Alternatively, the peptide-stimulated PBMCs are incubated with fluorochrome-labeled antibodies that target phenotypic markers combined with activation markers, and/or intracellular cytokines, and are measured using flow cytometry. This is more informative as it allows the measurement of activation markers, multiple cytokines, and which T-cell subset is the source of cytokine production. For peptide-stimulation assays, epitope prediction and HLA-typing are not necessary because hundreds of peptides are added in an HLA-independent manner. Combined with the large-scale measurement methods, peptide-stimulation assays can be used for high-throughput measurement of antigen-specific T cells. However, this method relies on optimal assay settings to ensure the functionality of the cells, and it can be labor-intensive when using flow cytometry. Therefore, depending on the research question, different tools are available. For high-throughput screening of antigen-specific T cells in a large cohort, IGRA or ELIspot assays are the most convenient. However, for more in-depth analysis (e.g., patient-specific) of epitope-specificity and phenotype, peptide-HLA tetramers are more suitable. To study functionality, peptide-stimulation assays combined with flow cytometry allow the most elaborate analysis of T-cell phenotype combined with functionality.

2.5 Immunity

Healthy individuals typically produce robust immune responses against SARS-CoV-2 after a 2-dose mRNA vaccination.[62] Memory responses are measured by detecting the three components of adaptive immunity: B cells, CD4+ T cells, and CD8+ T cells. Affinity-matured memory B-cell frequencies in circulation gradually increase until a peak moment around 3-6 months after vaccination. Neutralizing antibodies are less long-lived since they gradually wane from their peak concentration around two weeks after vaccination. This is thought to be caused by the induction of short-lived plasma cells instead of long-lived plasma cells derived from germinal center B cells. Due to the relatively short presence of neutralizing antibodies (and the emergence of new variants), booster vaccines are part of the vaccination schedule to enhance humoral and T-cell responses. Spike-specific CD4+ T cells are detected in nearly all individuals with peak frequencies within two weeks after vaccination, followed by a slow reduction. The T cells are of Th1 subtype as they produce IFN-γ and TNF-α, part of some cells produce IL-2, or are circulating follicular helper T cells. Spike-

specific CD8+ T cells are more challenging to measure, but more accurate methods show spike-specific CD8+ T cells in ~80% of healthy individuals. The kinetics of the frequencies in time are highly similar to CD4+ T cells. Both T cells and antibodies provide a layer of protection against disease. Antibodies can neutralize and clear viral particles, thereby preventing or reducing infection. T cells are important for the protection against severe disease by lysing virus-infected cells, and are more durable and more effective against new variants of concern.[63-65] Importantly, the antibody and T-cell responses induced by the mRNA vaccines result in a 94-95% efficacy against symptomatic infection with a good safety profile in healthy individuals.[66,67]

3 DISEASE

The SARS-CoV-2 mRNA vaccines can achieve high efficiencies in healthy individuals. However, the efficacy may be reduced when an individual is immunocompromised, as is often observed for patients with hematological malignancies. In specific cases, the disease itself can directly affect the immune system and result in reduced vaccine efficacy. In most cases, the treatment that these patients receive leaves them in an immunocompromised state. Patients may be treated with lymphocyte-depleting therapies, treated for a long period with immunosuppressive drugs, or treated with (high-dose) chemotherapy. Allogeneic stem cell transplantation is commonly offered to patients with high-risk hematological diseases as a curative option and has a large impact on the patient's immune system. Therefore, allogeneic stem cell transplantation will be discussed first, followed by disease- and treatment-specific characteristics of aplastic anemia and the most common hematological malignancies.

3.1 Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation is an option for patients whose initial treatment failed or for patients who are at high risk for relapse.[68] Allogeneic stem cell transplantation is used as a therapy in which the patient's stem cells are replaced by stem cells from a healthy donor, allowing an immune response to take place of donor T cells targeting the (malignant) hematopoietic cells from the patient. The patient is first conditioned to eradicate malignant cells and to suppress the patient's immune system to ensure engraftment of the donor stem cells. Pre-conditioning is typically done using chemotherapy with or without radiation or T cell-depleting therapy, anti-thymocyte globulin (ATG) or alemtuzumab (anti-CD52). Pre-conditioning chemotherapies mostly include melphalan, cyclophosphamide, fludarabine, or busulfan. After transplantation, the patients are immunocompromised due to delayed reconstitution of the immune system. Since transplantation depletes

a large part of the pre-existing immune system, patients are often revaccinated. Revaccination is commonly done approximately six months post-transplant to ensure reconstitution of the immune system before vaccination. It remains unclear whether allogeneic transplantation depletes the complete memory immune responses and whether vaccination shortly after transplantation can induce an immune response. This is important since the immunocompromised state of these patients leaves them vulnerable to a severe course after infection. Although potentially only partially effective, revaccination may still provide a layer of protection. Apart from pre-conditioning, patients can become immunocompromised due to immunosuppressive treatments after transplantation. Allogeneic transplantation can induce (severe) graft-versus-host disease due to alloreactive T cells, which can be prevented or treated using post-transplantation immunosuppressive therapies. However, ongoing treatment with immunosuppressive drugs can hamper the induction of vaccine responses.

3.2 Aplastic Anemia

Aplastic Anemia is a rare and severe disease that is characterized by bone marrow hypocellularity.[69] As a result, these patients develop pancytopenia, which affects the normal function of the immune system. The consensus is that aplastic anemia is caused by auto-reactive immune cells targeting hematopoietic stem and progenitor cells. The majority of aplastic anemia patients are treated with immune-depleting strategies consisting of a short course of anti-thymocyte globulin (ATG), combined with long-term use of cyclosporin.[70] Younger patients may also receive allogeneic stem cell transplantation with cyclophosphamide and ATG pre-conditioning, followed by immunosuppression. The lymphodepleting effect of ATG can result in the depletion of existing memory responses. Vaccination during or shortly before ATG treatment might therefore be ineffective. Furthermore, cyclosporin is immunosuppressive, and long-term use can therefore result in an immunocompromised state of the patient.

3.3 Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is a group of syndromes that is caused by somatic mutations in hematopoietic stem cells, resulting in bone marrow hypercellularity or hypocellularity and pancytopenia due to ineffective hematopoiesis.[71] MDS is linked to multiple genetic abnormalities and the abnormalities may be present in one or multiple lineages. A subclone in MDS may also develop into AML. Patients with low-grade MDS are often monitored but not treated, whereas patients with high-grade MDS may be treated with chemotherapy, including hypomethylating agents (azacytidine). Hypomethylating agents are immune-modulatory drugs, but are not necessarily known as immunosuppressive.[72,73] Some studies show enhanced efficacy due to hypomethylating agents when vaccinating for anti-leukemic effects.

In contrast, hypomethylating agents preferentially target proliferating cells and could thereby target activated cellular responses during vaccination.[74] Therefore, whether these patients reach an immunocompromised state due to long-term use of hypomethylating agents is unclear.

3.4 Myeloproliferative neoplasm

Myeloproliferative neoplasm (MPN) is a separate group of hematopoietic stem cell disorders that includes primary myelofibrosis, polycythemia vera, and essential thrombocytopenia.[75] MPNs are characterized by somatic mutations in JAK2, CALR, and/or MPL, which are important genes in signal-transduction pathways. These mutations consequently disrupt normal hematopoiesis. Patients may be treated with chemotherapy, ruxolitinib, interferon, or allogeneic stem cell transplantation. Ruxolitinib is a JAK2 inhibitor that inhibits the proliferation of hematopoietic stem cells and cytokine signaling. Ruxolitinib is effectively used as a treatment for graft-versus-host disease to dampen mostly the T-cell responses, which strongly suggests that active ruxolitinib treatment can hamper vaccination-induced T-cell responses. This is supported by the fact that ruxolitinib inhibits the signaling of cytokine receptors, which are essential for effective immune cell function.

3.5 CML

Chronic myeloid leukemia (CML) is an MPN that is characterized by malignant BCR-ABL1-positive stem cells.[68] *BCR-ABL1* is a fusion gene that is caused by the chromosome 9 and 22 translocation in a pluripotent hematopoietic stem cell.[76,77] This fusion gene results in the expression of an active tyrosine kinase that drives the formation of CML cells. CML cells gradually displace healthy hematopoiesis, resulting in bone marrow hypercellularity, anemia, and leukocytosis of immature to mature granulocytes. CML can be effectively treated using tyrosine kinase inhibitors (TKI), resulting in complete responses or minimal disease in most patients.[78] In rare cases, CML may eventually develop into acute leukemia. Since the patients are treated with tyrosine kinase inhibitors that mostly target the BCR::ABL1 protein, the healthy immune cells are thought to be minimally affected.

3.6 AML

Acute myeloid leukemia (AML) is a heterogeneous malignancy of myeloid precursor cells and is diagnosed based on the presence of myeloid blast cells in bone marrow, genetic abnormalities, and differentiation state.[79] Genetic abnormalities are variable but most mutations are in genes involved in signaling, DNA methylation, chromatin modification, and more.[80] Genes *FLT3, NMP1,* and *DNMT3A* are mutated in at least 50% of the AML cases.[80] AML cells accumulate in bone marrow, resulting in bone

marrow hypercellularity, leukocytosis, anemia, and thrombocytopenia. Patients with AML are preferentially treated with remission-induction therapy consisting of high-dose chemotherapy (cytarabine), with or without targeted inhibitors (such as FLT3 or IDH1/2 inhibitors) or hypomethylating agents (azacytidine or decitabine), and venetoclax. Patients are usually treated with several courses of chemotherapy followed by allogeneic stem cell transplantation for high-risk AML. Due to the combination and dosage of these therapies, vaccination during active treatment may result in a hampered immune response.

3.7 ALL

Acute lymphocytic leukemia (ALL) is a malignancy of lymphoid precursor cells (mostly from the B cell lineage). Cells transform into ALL due to multiple different genetic alterations of genes that are involved in self-renewal, proliferation, and survival pathways, from which some of them appear more frequently than others. These alterations include hyperdiploidy, chromosomal translocations resulting in altered expression of tyrosine kinases and transcriptional factors, mutations or deletions, and epigenetic changes. Patients often suffer from anemia, neutropenia, and thrombocytopenia as a result of bone marrow failure due to leukemic blast hypercellularity. Furthermore, symptoms may also be related to infiltration into other organs such as the central nervous system. Patients with ALL are often treated with multiple courses of chemotherapy, tyrosine kinase inhibitors, corticosteroids, and/or immunotherapy. Corticosteroids are known immunosuppressants that can dampen active immune responses and can have a lingering effect. Vaccination during or shortly after corticosteroid therapy may result in reduced efficacy of cellular responses. Immunotherapy for ALL commonly includes rituximab, which binds to CD20 on malignant cells, resulting in depletion of rituximab-bound cells. Healthy B cells also express CD20 and are thereby targeted by rituximab as well, resulting in the depletion of vaccine-induced B-cells during treatment. Similarly, bispecific antibody therapy, such as blinatumomab, which redirects T cells towards CD19-expressing malignant and healthy cells, results in depletion of malignant and healthy B cells, including vaccine-induced B-cells during this therapy. Furthermore, ALL patients who enter remission frequently undergo allogeneic stem cell transplantation as a consolidation treatment, which induces as mentioned above an immune-compromised state.

3.8 CLL

Chronic lymphocytic leukemia (CLL) is an indolent B-cell malignancy that causes lymphocytosis of mature B cells.[81,82] B-cell transformation occurs due to genetic alterations that affect B-cell receptor (BCR) signaling and increased expression of proteins that reduce apoptosis in these cells. CLL cells are present in circulation,

bone marrow, secondary lymphoid organs, and other tissues, where they can affect the normal function of immune cells. Untreated patients often have hypogammaglobulinemia due to weakened function of immune cells in circulation and tissues. CLL treatment includes cytostatic chemotherapy (*e.g.,* fludarabine, cyclophosphamide, bendamustine, or chlorambucil), anti-CD20 monoclonal antibody therapy (rituximab or obinutuzumab), Bruton's tyrosine kinase inhibitors (BTKi), and/or venetoclax. Vaccination responses are likely hampered during active treatment of some of these therapies. For example, the chemotherapies given are known to deplete lymphocytes, thereby hampering existing or the induction of both B and T-cell immune responses. Furthermore, BTKi blocks BCR signaling, and rituximab depletes B cells, thereby hampering humoral immune responses.

3.9 DLBCL

Diffuse large B-cell lymphoma (DLBCL) is the most prevalent aggressive mature B-cell malignancy. Genetic alterations are heterogeneous in DLBCL, which has resulted in subclassifications of DLBCL based on genetic abnormalities that could give a more accurate prognosis such as rearrangements in *MYC, BCL2,* and/or *BCL6*. Most patients have enlarged lymph nodes due to the accumulation of malignant cells. Patients are often treated with a combination of rituximab (anti-CD20 antibody), cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). Treatment may include radiotherapy or the use of alternative chemotherapies or corticosteroids. R-CHOP compromises the immune system, as rituximab results in short-term depletion of both malignant and healthy B cells, leading to hypogammaglobulinemia. Furthermore, short-term treatment with cyclophosphamide results in depletion of lymphocytes, and long-term corticosteroids result in hampering of immune cell function. In the case of relapsed or refractory DLBCL, cellular therapies can be offered. Patients may be eligible for autologous or allogeneic stem cell transplantation with BEAM (carmustine, etoposide, cytarabine, and melphalan) or carmustine/thiotepa conditioning. A recent advancement in the field is chimeric antigen receptor (CAR) therapy, which includes genetic modification of T cells with a CAR that recognizes surface proteins found on tumor cells.[83] The conditioning regimen consists of fludarabine combined with cyclophosphamide resulting in lymphodepletion. Pre-conditioning regimens for cellular therapies can weaken existing immune responses, and the induction of immune responses during or shortly after treatment may also be hampered. Furthermore, anti-CD19 CAR T-cell therapy leads to hypogammaglobulinemia, since CD19 is expressed on both healthy and malignant B cells.

3.10 Multiple Myeloma

Multiple Myeloma is a malignancy of the post-germinal center plasma cells, which typically includes the accumulation of malignant cells in the bone marrow.[68,84] The malignant cells are monoclonal and therefore produce the same immunoglobulin, referred to as M-protein. Genetic alterations include hyperdiploidy, increased expression of cyclin D1-D3, secondary translocations, mutations, and epigenetic changes. Symptomatic myeloma is characterized by organ or tissue damage: hypercalcemia, renal impairment, anemia, and bone disease (CRAB). Malignant cells can affect normal immune function by repressing healthy immune cells in bone marrow or lymph nodes, or hampering immune cell function by creating an immunosuppressive environment, resulting in hampered cellular immunity and reduced antibody production by non-malignant cells. Patients with symptomatic myeloma who are eligible for autologous transplantation are first treated with intensive induction therapy, which typically includes daratumumab (anti-CD38 antibody), bortezomib/velcade, lenalidomide, and dexamethasone (dara-VRd). Lenalidomide and the other thalidomide analogs are usually referred to as immune modulatory drugs (IMiD) and bind to cereblon, altering protein degradation by the proteasome. This results in alterations in apoptotic pathways, stromal cell and malignant cell interactions, and promotion of T-cell activation by increasing T-cell priming and inhibiting regulatory T cells.[85] Alternatively, patients may be treated with variations on VRd. After Dara-VRd treatment, patients receive high-dose cytostatic melphalan (HDM) followed by autologous stem cell transplantation. Patients ineligible for autologous stem cell transplantation are treated with VRd. Patients with multiple myeloma can become immunocompromised during treatment. Daratumumab can result in short-term partial elimination of the immune system by depleting CD38-expressing plasma cells, activated conventional T cells, and regulatory T cells. Treatment with immune modulatory drugs and corticosteroids is usually long-term, therefore, prior long-term use of the drugs can affect vaccine-induced immune responses during treatment. However, the mode of action of immune modulatory drugs is diverse, and the effect might therefore depend on multiple factors.

4 AIMS

SARS-CoV-2 has circulated in the human population since December 2019 and subsequently caused a pandemic in 2020. Fortunately, vaccines were quickly developed and were effective in limiting the viral spread and hospitalization, thereby ending the pandemic. Due to the urgency of the pandemic, blood samples were bio-banked before and during the vaccination rollout. These precious materials allow us

to study the immune system during the encounter with a new virus or vaccine. First encounter with a virus or vaccine typically results in the induction of T-cell responses from the naïve repertoire, called primary T-cell responses. However, individuals may also exhibit pre-existing immunity: induction of T cells from the memory repertoire that were originally primed by another pathogen. In most healthy individuals, the virus is effectively cleared by the innate and adaptive immune system without causing severe disease. Individuals with a hampered immune system have an increased chance of developing severe COVID-19. Patients with hematological malignancies are often immunocompromised due to the disease itself or the treatment that they receive, causing them to be more susceptible to infections. Typically, vaccine-induced immunity is often measured by the presence of humoral immune responses only, but some patients are unable to produce a humoral response and T cells are important for effective viral clearance as well. As a result, the ability of patients with hematological malignancies to develop effective T-cell responses is unclear. Therefore, this thesis aims to investigate the mRNA vaccine-induced T-cell response in patients who are immunocompromised due to disease or treatment.

The aim of **chapter 2** is to investigate how the T cells of SARS-CoV-2-naïve individuals respond to the virus to get insight into pre-existing T-cell responses. T cells can be cross-reactive towards different viruses, which is usually caused by sequence homology. Therefore, studies investigating T-cell cross-reactivity towards SARS-CoV-2 focus on other common coronaviruses. However, we hypothesize that these T cells could also originate from T cells that recognize cytomegalovirus (CMV). This is because previous reports showed that cross-reactive T cells are present in a large group of individuals, independent of geographical location, and cross-reactive T cells are present in a relatively high percentage in the blood. Both are typical characteristics of CMV. Additionally, CMV-seropositivity has been associated with cross-reactive T cells, and T cells can be cross-reactive between two dissimilar viruses. We will investigate this by randomly selecting PBMCs from healthy individuals that were frozen down before May 2019, to ensure that they are SARS-CoV-2-naïve. We will measure SARS-CoV-2-specific CD4+ and CD8+ T cells and separate the individuals based on CMV serology. SARS-CoV-2-specific T cells will be isolated, and we will aim to identify their peptide-HLA specificity. If successful, we will further aim to understand these T-cell responses by investigating the peptide affinity, T-cell phenotype, and the efficacy of cross-reactive T cells against SARS-CoV-2.

The aim of **chapter 3** is to investigate the SARS-CoV-2 spike-specific humoral and T-cell response following mRNA vaccination in patients with aplastic anemia. Patients with aplastic anemia are often immunocompromised due to therapy,

making them more susceptible to a severe course after infection. However, current guidelines recommend caution with vaccinating against SARS-CoV-2 due to the risk of potential disease relapse and due to the speculation that the vaccine might be ineffective in these patients. These guidelines are given irrespective of whether immunosuppressive treatment is completed. We will investigate whether previous treatment with immunosuppressive therapy can have a lingering effect on the humoral and T-cell responses and whether vaccination may cause aplastic anemia relapse. We will therefore collect blood from patients with aplastic anemia who have been previously treated with immunosuppressive treatment. Spike-specific antibodies and T cells will be measured before and during vaccination, as well as the ability of the T cells to produce IFN-γ, TNF-α, and IL-2. Furthermore, symptoms of aplastic anemia relapse will be monitored.

The aim of **chapter 4** is to investigate the spike-specific humoral and T-cell responses following mRNA vaccination in a selected group of patients with hematological malignancies. Antibody responses are often measured as a proxy for the presence of developed immunity. However, immunity also includes the development of an effective T-cell response. This becomes especially important for patients with hematological malignancies who have a hampered B-cell compartment due to disease or treatment. To investigate this, patients with CLL, lymphoma, or multiple myeloma will be included, and the ability of these patients to develop spike-specific antibodies, CD4+ T cells, or CD8+ T cells will be measured. The developed immune responses will be shown of the patients stratified based on malignancy, but also stratified based on seroconversion. This will be done to investigate whether patients who are unable to seroconvert also lack the ability to mount T-cell responses.

The aim of **chapter 5** is to further extend the patients with hematological malignancies to investigate the spike-specific humoral and T-cell responses in a large cohort of patients, stratified based on disease, but also therapy. Several studies have investigated spike-specific antibodies in a large cohort of patients, but measurement of T cells is more labor-intensive and therefore typically restricted to a smaller group of patients with a specific disease or treatment. As a result, it is challenging to pinpoint whether reduced immune responses are due to disease or therapy and whether these patients have a combined deficient B-cell and T-cell response. Therefore, spike-specific antibodies and CD4+ and CD8+ T cells will be measured during vaccination, and the patients will be stratified based and disease and therapy. Apart from frequencies of spike-specific T cells, the ability to produce cytokines will be measured. Furthermore, these patients often have lymphopenia, and

therefore, we aim to investigate whether reduced T-cell counts or a reduced naïve T-cell pool are associated with a poor T-cell response.

In **chapter 6** the results of the studies will be discussed.

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