Mechanisms of vaccines against viral infections

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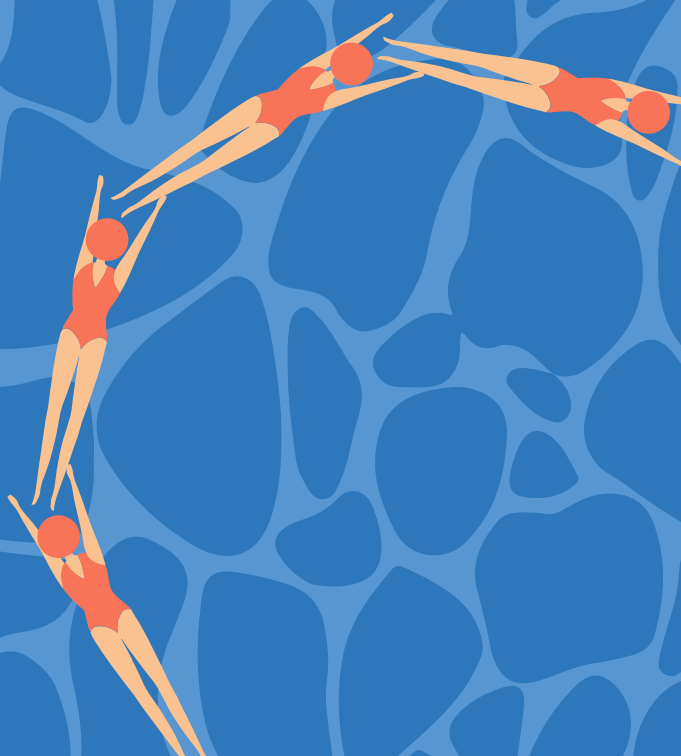
CHAPTER 1

General Introduction



THE IMMUNE SYSTEM

The immune system protects against infectious diseases such as viruses, parasites, fungi, bacteria, and malignant cells. The immune system consists of two different parts, the innate and the adaptive immune system. The innate immune system is activated by recognition of conserved pathogen-specific structures, which are common to many different types of pathogens, through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). The innate immune response is relatively fast. The response of the adaptive immune system, however takes days or weeks to develop, but is highly specific to the pathogen involved. This specificity is because of the presence of antigen-specific receptors on adaptive immune cells that recognize a unique part of a pathogen. Furthermore, the induction of the adaptive immune response results in long-lasting memory formation, enabling the immune system to respond better and faster upon re-infection. The type of response of the immune system is dependent on the specific infection (1). This thesis will focus on how the immune system shapes the responses to Cytomegalovirus (CMV) and Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) infections, and how these responses can be used to develop prophylactic vaccines against these viruses.

THE IMMUNE RESPONSE UPON VIRAL INFECTION

The physical and chemical barriers of our body are the first line of defense against viral infection. Upon breaking through these barriers, innate immune cells are activated by their TLRs that recognize different viral structures. On the cell surface, TLR1, TLR2, TLR4 and TLR6 recognize viral glycoproteins and nonstructural proteins (2, 3). In the endosomal compartment of the cells, TLR3 recognizes double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA and TLR9 is activated by unmethylated CpG-rich DNA (4). This activation leads to the production of type-I interferon molecules which induce apoptotic cell death of the infected cell, inhibit viral replication and induce antiviral responses in surrounding cells (5, 6).

Antigen-presenting cells (APCs), such as dendritic cells (DCs), respond particularly well to infections because of their high expression of TLRs, and are strategically located in the body. APCs present at the site of infection are not only activated, but also engulf viral particles and migrate to draining lymph nodes (LNs). Inside the APCs, the viral particles are processed and presented in major histocompatibility (MHC) class I or class II molecules on the surface of the APCs. Subsequently, pro-inflammatory cytokines are released by the APCs. In the LNs, the DCs further instruct the cellular

(T cell) and humoral (B cell) part of the adaptive immune response specific for the virus (1).

DEVELOPMENT OF THE ANTIVIRAL T CELL RESPONSE

T cell precursors derive from hematopoietic stem cells in the bone marrow (BM). These precursors mature in the thymus, where they are selected for functional T cell receptor (TCR) expression. During T cell development, gene rearrangement results in millions of unique TCRs that recognize only a small part of the pathogen in class I or II MHC molecules. In the thymus, positive and negative selection ensures the correct maturation of T cells. By positive selection, immature $CD4^+CD8^+$ thymocytes expressing TCRs with intermediate affinity and/or avidity for peptide-MHC complexes are induced to differentiate into mature $CD4^+$ or $CD8^+$ thymocytes. Negative selection, also known as clonal deletion, eliminates thymocytes expressing TCRs with high affinity for self-antigens. When T cell development is finished in the thymus, $CD4^+$ and $CD8^+$ T cells migrate into the periphery (7, 8).

In secondary lymphoid organs (SLOs) like the LNs, the priming of T cells occurs. For the activation of $CD4^+$ and $CD8^+$ T cells, three different signals are required: (1) antigen recognition, (2) co-stimulation, and (3) cytokine-mediated differentiation and expansion. Viral antigen is presented by APCs to the TCRs of naïve $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells in class II and class I MHC molecules, respectively. Co-stimulation receptors colocalize with TCR molecules and together promote or inhibit T cell activation and function (9). Upon activation by the APCs, $CD4^+$ T cells can potentially differentiate into multiple subsets of T helper cells, such as T helper 1 (T_H1), T helper 2 (T_H2), T helper 17 (T_H17), follicular helper T cells (T_{FH}) and regulatory T helper cells (T_{Reg}). These subsets perform a variety of functions in the adaptive immune response (10). Distinct cytokine secretion by APCs and different receptor interactions determine the type of $CD4^+$ T helper subset that is induced. Production of interleukin 12 (IL-12) by a specific type of DC, conventional DC1 (cDC1), results in the differentiation of $CD4^+$ T helper cells into T_H1 cells (11). Next, $CD4^+$ T helper and $CD8^+$ T cells recognize their respective antigens on the same DC. The interaction of the cDC1 with $CD4^+$ T helper cells optimizes antigen presentation, cytokine production and delivery of costimulatory signals from the cDC1 to the $CD8^+$ T cell. In this way, the cDC1 further primes the $CD8^+$ T cell, and promotes its clonal expansion and differentiation into an effector or memory $CD8^+$ T cell (12).

Primed $CD8^+$ T cells lose expression of leukocyte adhesion molecule L-selectin (CD62L), a protein that ensures the homing of the cells in the SLOs (13). In addition, primed $CD8^+$ T cells express chemokine receptors such as CCR4, CCR6, CCR9, CCR10 and CXCR3, that allow their migration to the site of infection, where other cells produce the respective chemokines specific for these receptors (14). At the site of infection, these effector $CD8^+$ T cells induce apoptotic cell death of virus-infected cells by different mechanisms. Upon recognizing viral antigens on the MHC class I molecules of infected cells, virus-specific $CD8^+$ T cells release granzymes and perforin and secrete the cytokines interferon-γ (IFN-γ) and tumor necrosis factor (TNF). Granzymes and perforin create pores in the cell membrane of virus-infected cells, whereas IFN-γ increases MHC class I expression in infected cells and directly inhibits viral replication, as does TNF (15). When the viral infection is cleared, the $CD8^+$ T cell response contracts.

DEVELOPMENT OF THE ANTIVIRAL ANTIBODY RESPONSE

B cells develop in the bone marrow (BM) from hematopoietic precursor cells. In the early stages of B cell development, different gene segments (variable (V), diversity (D) and joining (J)) are recombined to assemble diverse unique antibody molecules that are also used as the B cell receptor (BCR) (16). In the BM, developing B cells undergo positive and negative selection based on BCR binding, resulting in non-autoreactive functional B cells that migrate to the periphery (17).

Upon antigen exposure in SLOs, B cells can become short-lived plasma cells (PCs) that have a high antibody production (18). Furthermore, together with T_{FH} cells and follicular dendritic cells (FDCs), B cells can form germinal centers (GCs) in SLOs. In these GCs, T_{FH} cells produce cytokines and interact via their CD40 ligand with the CD40 receptor on B cells. This interaction results in rapid proliferation, somatic hypermutation and antibody isotype switching of the GC B cells. The isotype of the antibody determines its function and in the antiviral response, IgG antibodies are the main isotype. After the GC reaction, B cells differentiate into short-lived PCs, memory B cells or long-lived PCs that migrate back to the bone marrow (19, 20).

Antibodies perform different antiviral functions. Antibodies produced by PCs can bind the virus, preventing it from infecting host cells, a process called antibody neutralization. In addition, there are non-neutralizing ways in which antibodies exert antiviral functions. Antibodies can coat the virus and target the antibody-virus complex for phagocytosis, via interaction with Fc receptors on phagocytic cells. This process is called antibody-dependent cellular phagocytosis (ADCP) (21). Furthermore, by antibody-dependent

cellular cytotoxicity (ADCC), antibodies can tag virus-infected cells for destruction by effector cells. Moreover, the complement pathway can be activated, also resulting in destruction of antibody-tagged virus-infected cells (22, 23).

MEMORY OF THE IMMUNE RESPONSE

The activation of the adaptive immune system does not only result in an effective response against the current viral infection. As briefly described above, antigen-specific memory B, $CD4^+$ and $CD8^+$ T cells are induced during the adaptive immune response. These memory cells remain present at a higher precursor frequency than their original naïve counterparts and can more rapidly differentiate into effector cells upon re-infection. In this way, memory cells can combat viral infection faster than the primary response (24).

Memory $CD8^+$ T cells are divided into tissue-resident memory (T_{RM}), effector memory (T_{EM}), central memory (T_{CM}) and memory stem (T_{SCM}) T cells. These subtypes are based on the T cell phenotype, localization, recall ability, and effector function. Most T_{RM} cells express type II C-lectin receptor CD69 and integrin CD103 and are located in tissues where they can provide immediate protection in the case of local secondary infection. T_{RM} cells act by direct effector functions and also recruit and reactivate other immune cells such as NK cells, DCs, T_{CM}, T_{EM} and B cells (25). T_{EM} cells express effector molecules (such as KLRG1) and lack expression of the homing molecules CD62L and CCR7, allowing them to migrate between SLOs and tissues. T_{CM} cells on the other hand, do express CD62L and CCR7, hence these cells reside primarily in SLOs where the ligands for CD62L and CCR7 are present (26). The T_{CM} memory subset and the T_{SCM} subset, have the largest proliferative capacity, and both can differentiate and expand into effector cells upon reinfection (15). Together, these cell subtypes provide overlapping layers of protection against re-infection (27).

In the case of the humoral response, antibodies secreted by long-lived plasma cells provide a first line of defense against re-infection. In addition, memory B cells become re-activated. This secondary B cell response is more rapid, of greater magnitude and produces antibodies that are already of high affinity and class switched (28).

CYTOMEGALOVIRUS

Human Cytomegalovirus (HCMV) is a member of the beta-herpesvirus family with a double-stranded (ds) DNA genome. Around 60% of the worldwide population is infected with this virus (29). The main mechanism of virus spread is thought to occur via the saliva and urine of young children (30). Other ways for the virus to spread are sexually, through blood transfusion or by organ transplantation (31, 32). Glycoprotein complexes on the outside of HCMV are used to enter host cells, and antibodies targeting these glycoproteins are generated by the humoral response to reduce virus infection (33, 34). Upon primary infection, HCMV develops into a latent infection, with some reactivations. Healthy individuals experience no symptoms upon infection. However, in individuals with a compromised or immature immune system, HCMV infection or reactivation can result in serious disease (35). Currently, HCMV infection or reactivation is treated with antiviral therapy (36), as no HCMV vaccine is licensed, but clinical trials with (synthetic) vaccines are ongoing (35).

HCMV infection triggers the activation of different PRRs in the innate immune response. TLR2, TLR3, TLR4 and TLR9 are activated, resulting in the production of antiviral cytokines (37). Furthermore, the CMV-specific T cell response has some remarkable features compared to other virus-specific T cell responses. This T cell response is extra-ordinarily large (around 10% of the total memory T-cell pool) and shows no contraction after primary infection, or even further increase over time while maintaining their effector function. This phenomenon is named memory inflation (38). The height of the CMV-specific T cell response is viral dose-dependent (39). In parallel to the T cell response, a strong CMV-specific antibody response is induced, of which the magnitude is also determined by the infectious dose (40). A simplistic overview of the adaptive immune response against CMV is shown in figure 1.

SARS-COV-2

SARS-CoV-2 is a single-stranded positive-sense RNA virus that was discovered late 2019 in Wuhan, China and was the cause of the COVID-19 pandemic. The coronavirus is transmitted through respiratory droplets and aerosols and is able to infect the lower respiratory tract (41). Cellular infection occurs by binding of the Spike protein on the outside of SARS-CoV-2 to the ACE2 receptor on host cells (42). This infection can result in a wide range of symptoms. Some infected individuals show no symptoms, whereas for others the infection results in serious disease, or even death (43). As with CMV, SARS-CoV-2 infection is more devastating for the immunocompromised individuals.

Fortunately, many different vaccine modalities were rapidly developed and tested and resulted in protective vaccines against SARS-CoV-2 (44).

Based on experimental and bioinformatic studies, infection with SARS-CoV-2 is thought to activate different PRRs on innate immune cells such as TLR1-4 and TLR6. Activation of these PRRs results in antiviral cytokine production and consequently cell death (45). SARS-CoV-2 infection induces a strong humoral response (46). Important in the SARS-CoV-2 specific antibody response are dimeric mucosal IgA antibodies developed after the GC response that prevent SARS-CoV-2 transmission in the nasal tissues. Induction of neutralizing antibodies has been the main focus of vaccine development (46). Over time however, the SARS-CoV-2 specific antibody response wanes and with the emersion of new SARS-CoV-2 variants, the neutralizing capacity of the antibodies also declines (47).

Upon SARS-CoV-2 infection, $CD4^+$ and $CD8^+$ T cells are also activated and aid in virus clearance and protection upon re-infection. The T cell responses develop early after infection and the height of the response correlates with protection, as in severe disease, the T cell response has been found to be impaired (48). In contrast to the SARS-CoV-2 specific humoral response, the (memory) T cell response is not only directed against the Spike protein, but also against other SARS-CoV-2 proteins and is broad in antigen recognition. Therefore, the T cell response is less affected by the emergence of new viral variants (49). In addition, we have shown ourselves (**chapter 5 of this thesis**) that in an experimental mouse model, a high quantity of vaccine-induced epitope-specific $CD8^+$ T cells was able to protect against SARS-CoV-2 infection, further showing the importance of the SARS-CoV-2 specific T cell response (50). A simplistic overview of the adaptive immune response against SARS-CoV-2 is provided in **Figure 1**.

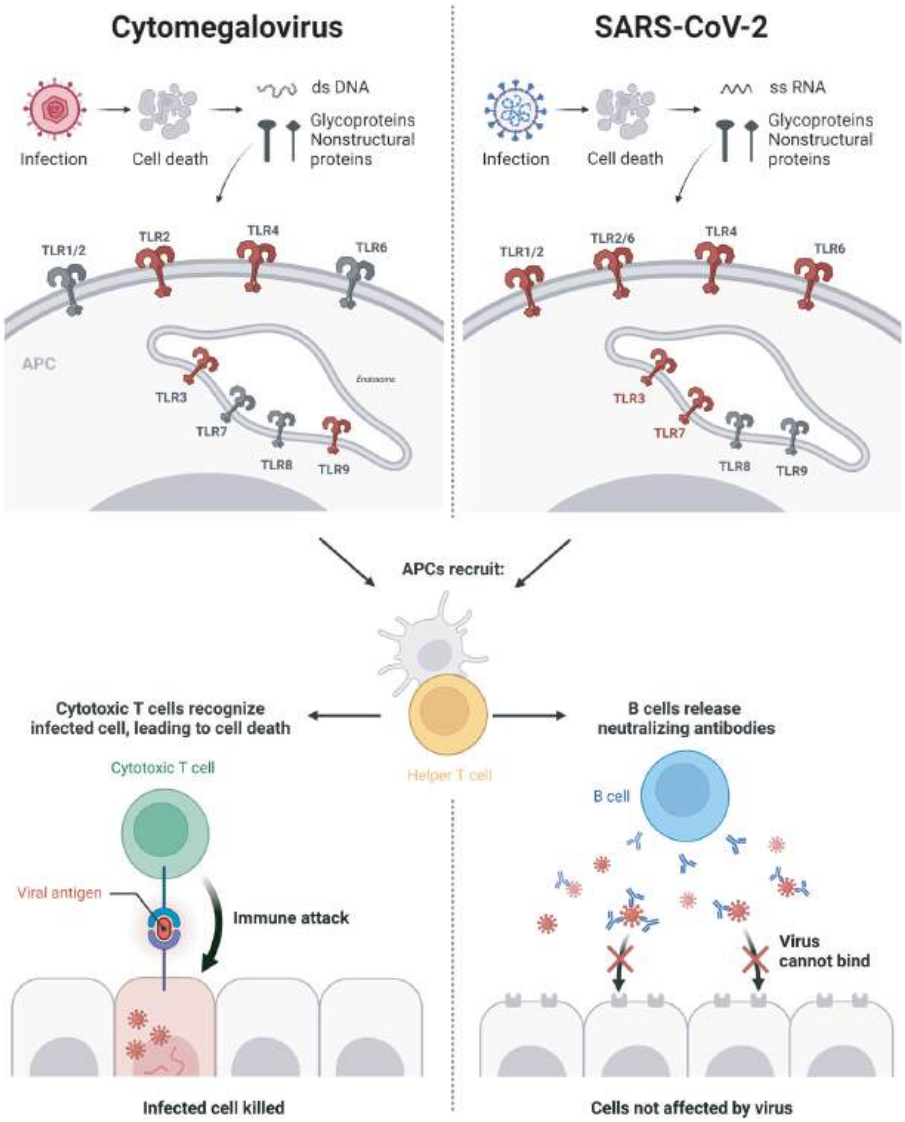


Figure 1. A simplistic overview of the adaptive immune response against CMV and SARS-CoV-2. CMV and SARS-Cov-2 infection, resulting in cell death gives release of viral genetic material and proteins that bind TLR receptors in or on APCs (involved TLRs are depicted in red). With the help of helper T cells, cytotoxic T cells and B cells are activated, resulting in the killing of infected cells or neutralization of the virus, or other antibody-dependent antiviral mechanisms (not shown here).

VACCINATION

The increasing understanding of the above described memory immune response provides the basis for vaccine development. This is visualized in **Figure 2**. So far, the main focus of most vaccines has been the induction of long-lasting antibodies. However, there is currently an increasing interest in the induction of long-lasting T cell memory as well.

Many vaccine modalities require the addition of adjuvants. Adjuvants enhance the virus-specific immune response by directly triggering the innate immune response, thereby inducing increased help for the humoral and cellular immune responses. Adjuvants used in this thesis are CpG, agonistic OX40 antibody and Incomplete Freunds Adjuvant (IFA). CpG improves (memory) B cell and $CD8^+$ T cell responses, by inducing TLR9-mediated activation of APCs (50, 51). The agonistic OX40 stimulates the OX40 costimulatory receptor on T cells (52, 53). IFA forms a depot that supports the slow release of antigen, induces inflammation and recruits immune cells (54). In addition, the use of adjuvants can reduce the antigen dose and the amount of booster vaccinations required for optimal viral protection (55).

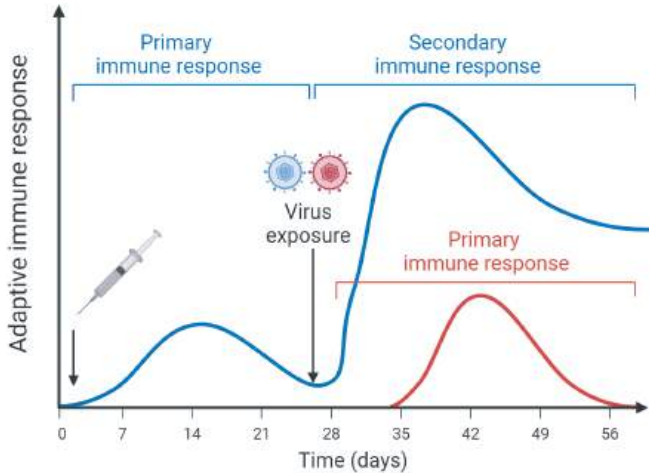


Figure 2. Prophylactic vaccination rationale. Upon vaccination with a new antigen (day 0), the primary immune response is induced, resulting in a relative lower and slower developing adaptive immune response that leads to memory formation. Upon virus encounter (day 27), expressing the same antigen, the secondary adaptive immune response peaks faster and higher and therefore results in better protection against the virus infection than a primary immune response (induced at day 27) against a virus would do.

Different vaccine modalities have been developed over the years, all aiming to induce a strong memory immune response. These vaccine modalities have various advantages and disadvantages. Both inactivated and live-attenuated viruses are used in vaccine design. An advantage of these vaccine modalities is that the induced immune response resembles the normal virus-specific immune response as such vaccines already contain natural TLR ligands, and therefore do not require the addition of adjuvants. However, there are safety concerns with these types of vaccines for immunocompromised patient groups. In addition, developing inactivated and live-attenuated vaccines can be time-consuming (56).

Synthetic vaccines are designed and produced synthetically to represent the antigenic components of the virus of interest. Examples of synthetic vaccines are synthetic long peptides (SLPs), plasmid-based DNA vaccines, and RNA-based vaccines, or virus-like particles. Both SLP vaccines and DNA vaccines are studied in this thesis. These vaccines have a good safety profile and a cost- and time-effective production. However, SLP vaccines only contain the antigen and therefore require the deliberate addition of adjuvants, while DNA vaccines do not require adjuvants, as DNA itself can be recognized by PRRs (55).

Overall, different aspects are important for successful vaccine development. Insight into the mechanisms of the immunogenicity and protective capacity of different vaccine modalities, adjuvants and vaccination regimens is important for optimal vaccine design. Continuously broadening our knowledge on these factors, ensures the most rapid and optimal vaccine design in the case of a new virus outbreak.

SCOPE OF THIS THESIS

The main scope of this thesis, is the development of synthetic vaccines against CMV and SARS-CoV-2 together with studying the immune correlates of protection of these vaccines. In the first part of this thesis, the chapters focus on CMV infection, the CMV-specific T cell response and synthetic prophylactic vaccination. Both **chapter 2** and **chapter 3** review the differentiation of $CD8^+$ T cells upon CMV infection. These chapters describe that the CMV-specific $CD8^+$ T cells have a distinctive phenotype and are characterized by an advanced differentiation state based on expression of markers KLRG1, CD27, CD44 and downregulated expression of CD62L. In **chapter 2**, the CMV-specific T cell differentiation is also compared to other (chronic) infections and **chapter 3** shows a detailed visualization of the progressive differentiation of MCMV-specific $CD8^+$ T cells upon infection.

In **chapter 4**, the design for an SLP-based HCMV vaccine is proposed in which both CD4 and CD8 T cell epitopes are combined in one SLP. The vaccine's proof of concept is studied in both murine and human studies. **Chapter 5** shows the importance of inducing both the humoral and cellular parts of the adaptive immune response with CMV vaccines for optimal protection. First modifications of a DNA vaccine, administration routes and vaccination time points are studied to optimize the CMV-specific antibody response. Next, this DNA vaccine is combined with a MCMV-specific T cell response inducing SLP vaccine, to show the importance of the combination of the antibody and T cell response for MCMV protection.

In the second part of this thesis, the studies are focused on SARS-CoV-2. These studies are based on the results and knowledge obtained in **chapters 2-5**, but translated into the SARS-CoV-2 setting. In **chapter 6**, we study the different roles of the adaptive immune response in vaccine-induced SARS-CoV-2 protection. First, we optimize a DNA vaccine encoding the Spike protein to produce a strong immunogenic response based on (neutralizing) antibody responses and virus-specific $CD4^+$ and $CD8^+$ T cell (memory) responses. Next, we confirm that this immune response is protective against SARS-CoV-2 infection, and by depleting parts of the adaptive immune response, we decipher the roles of the different arms of the adaptive immune response in DNA vaccine-induced protection.

The importance of the vaccine-induced SARS-CoV-2 specific T cell response is shown in **chapter 7**. First, the vaccination strategy of a T cell SLP vaccine, containing both $CD4^+$ and $CD8^+$ epitopes was optimized. Next, this SLP vaccination proved to be effective in protecting against SARS-CoV-2 infection. In **chapter 8**, we further demonstrate the importance of the $CD8^+$ T cell response in SARS-CoV-2 vaccine-induced protection, through vaccination with an SLP vaccine containing a single $CD8^+$ T cell epitope. We show that three vaccinations result in complete protection against SARS-CoV-2, without the aid of a SARS-CoV-2-specific antibody response. Furthermore, the epitope-specific $CD8^+$ T cells were characterized in depth after different numbers of vaccinations.

This thesis ends with a general discussion of all described studies in **chapter 9**.

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