Mechanisms of vaccines against viral infections

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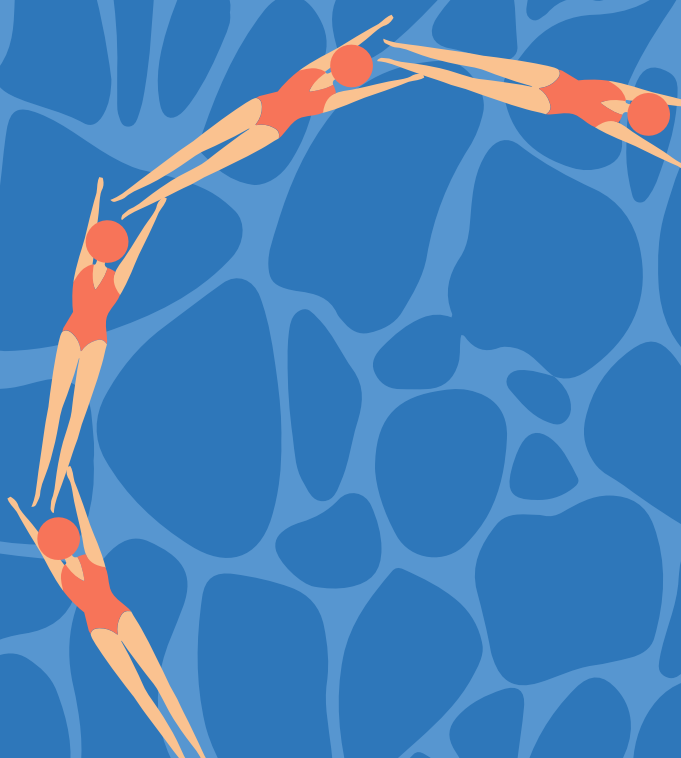
CHAPTER 9

Discussion



DISCUSSION

Viral infections impact society differently based on their infectivity and the severity of disease they induce. In the case of human cytomegalovirus (CMV), healthy individuals are generally not clinically affected by infection, but in immunocompromised individuals and neonates, HCMV can result in severe disease and even mortality (1). SARS-CoV-2 infection also results in a wide range of symptoms in individuals, ranging from non-clinical symptoms to lethality. Evidently, this virus caused a detrimental effect on society during the COVID-19 pandemic (2). Because of the effects virus infection can have on an individual to a societal basis, vaccine development is of high importance.

Different aspects need to be considered in vaccine development for infectious diseases caused by viruses. For instance, the natural immune response against each specific virus and which viral antigens induce this immune response need to be understood. Moreover, insight into the immunogenicity and protective capacity of different vaccine modalities, adjuvants and vaccination regimens is required. Knowledge on all these factors ensures the most rapid and optimal vaccine design in the case of a new virus outbreak.

Virus-specific immune response

In this doctoral thesis, two different viruses were studied: CMV and SARS-CoV-2. These two types of viruses result in different types of infection. SARS-CoV-2 is an enveloped positive strand RNA virus that is cleared by the immune system upon infection (2), whereas CMV is a double stranded DNA virus that results in chronic infection. Because of immune evasion mechanisms, CMV remains latently present in host cells and is not entirely cleared by the immune system (3). Moreover, these differences in genetic composition of SARS-CoV-2 and CMV and whether they are cleared or remain latently present upon infection also influence the immune response to these viruses.

Upon primary CMV infection there is a robust natural killer response, followed by the production of neutralizing antibodies and the generation of CMV-specific $CD4^+$ and $CD8^+$ T cells (4). In this thesis, we specifically reviewed the memory $CD8^+$ T cell response upon CMV infection in **chapters 2 and 3**. Large T cell responses are elicited against CMV that remain high or even increase over time, a phenomenon named memory T cell inflation (5, 6). The degree of memory T cell inflation is strongly determined by the infectious dose (7). Furthermore, these memory T cells have an unique distinctive phenotype, characterized by an advanced differentiation state. This phenotype is based on the expression of costimulatory receptors, natural killer receptors, inhibitory receptors, homing receptors, cytokine receptors and the expression of cytokines and transcription

factors, which was reviewed in detail in **chapter 2**. This described phenotype is unique in comparison to memory CD8$^+$ T cells induced by other chronic virus infections such as Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV). In **chapter 3**, we have shown with computational tools that the inflationary mouse CMV (MCMV)-specific CD8$^+$ T cells undergo progressive differentiation, which is most clear upon high-dose infection. In addition, our group previously studied the antibody response to MCMV. The humoral response to MCMV is also inflationary, with no waning of the response observed over time (8).

In this thesis, we did not review the SARS-CoV-2-specific immune response, as many others did during the COVID-19 pandemic (9-11). The SARS-CoV-2 specific humoral response results in neutralizing antibodies that prevent SARS-CoV-2 infection. Over time, 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 (10). CD4$^+$ and CD8$^+$ T cells are also activated upon SARS-CoV-2 infection and aid in virus clearance and protection upon re-infection. These responses develop early after infection and the height of the T cell response correlates with protection against SARS-CoV-2 (11). Overall, the rough comparison of SARS-CoV-2 and CMV infections shows that different types of virus infections, and even different doses of virus infections result in distinct immune responses.

Vaccine modalities

The choice of vaccine modality is of great importance in vaccine design (12). In this thesis, two different types of synthetic vaccines were studied: synthetic long peptide (SLP) and DNA vaccines. These types of vaccines have a good safety profile (13, 14). In addition, these vaccine modalities can be easily adjusted to new emerging viruses or virus variants and be used to induce both humoral and cellular responses, as also shown in this thesis. The knowledge obtained in the studies with CMV-specific DNA and SLP vaccines in the first part of this thesis, was used to design the SARS-CoV-2 specific synthetic vaccines at the start of the SARS-CoV-2 pandemic. Furthermore, during the pandemic new variants of SARS-CoV-2 emerged. In **chapter 6**, we showed that an optimized DNA vaccine based on the original Wuhan sequence could be easily adjusted to the Omicron variant with intact immunogenicity .

Antigen optimization

Furthermore, this thesis shows that the choice of antigen or adjustments to the antigen affect the immunogenicity of vaccines. In **chapter 5**, we showed that an adjustment to the MCMV glycoprotein B (gB) affected the height of the antibody response induced by the DNA vaccine. The gB variant lacking the transmembrane and cytoplasmic domain

of the protein (secreted variant) induced a higher MCMV-specific antibody response compared to the variant containing the protein's transmembrane and cytoplasmic domain. This effect was also seen with DNA vaccines encoding the SARS-CoV-2 Spike protein (**chapter 6**). The SARS-CoV-2 specific antibody response was improved by having a secreted variant (sS) compared to a non-secreted variant. However, in a MERS-CoV mRNA vaccine setting, the transmembrane-anchored Spike protein elicited a more potent pseudovirus neutralizing antibody response than the secreted variant (15). This contrasting outcome can be possibly explained by the use of a different vaccine modality, and therefore it is crucial to test different antigen optimizations per vaccine design.

Stabilization of an antigen can too result in improved immunogenicity. In **chapter 6**, different adjustments to the wild-type Spike protein were made based on previous studies, in which these adjustments stabilized the protein and improved (neutralizing) antibody responses (15-20). The furin cleavage site between the S1+S2 domain was mutated and two prolines were substituted in the S2 domain of the SARS-CoV-2 Spike protein. As seen in other studies (15-20), these adjustments to the Spike protein improved the Spike-specific antibody response.

The number of epitopes present in a vaccine can also affect vaccine efficiency. In **chapter 6**, we compared two different DNA vaccines with different amounts of epitopes. The DNA vaccine encoding the receptor binding domain (RBD; located in the S1 domain) of the Spike protein induced lower Spike-specific antibody and $CD4^+$ T cell responses compared to the DNA vaccine encoding the S1+S2 domains. This effect on immunogenicity was also found in a mRNA vaccine study (21). The difference in the number of B cell and $CD4^+$ T cell epitopes in the vaccines can possibly explain the corresponding difference in immunogenicity. The importance of $CD4^+$ T cell help in vaccines will be discussed later in this discussion. Furthermore, the importance of the number of epitopes on the vaccine-induced immune response is shown in **chapter 8**. Here, an SLP vaccine combining 11 different $CD8^+$ CTL and $CD4^+$ T helper epitopes, showed improved protection against SARS-CoV-2 challenge, compared to a single CTL epitope.

Designing a vaccine with broad epitope coverage is also the rationale behind the CMV SLP vaccines discussed in **chapter 4**. In this study, multiple SLPs were designed containing a CTL and helper CMV epitope in one SLP. In the human situation, this way of designing a multiple epitope SLP vaccine is important for broad HLA coverage. In the murine situation, SLPs containing multiple $CD4^+$ and $CD8^+$ T cell epitopes did not negatively affect the height of the CTL response, compared to single CTL epitope

vaccination. This result shows that this design can induce a broad T cell response without antigen competition. The importance of broad epitope coverage is also shown in **chapter 8**. Here, an SLP vaccine composed of multiple SLPs encoding different SARS-CoV-2 epitopes adjuvanted with the TLR1/2 agonist Amplivant improved protection to SARS-CoV-2 infection compared to a single epitope SLP vaccine. However, if the $CD8^+$ T cell response induced to a single epitope is strong enough (reached with multiple boosts and CpG as an adjuvant), such single epitope SLP vaccines can also protect against SARS-CoV-2 infection, as seen in **chapter 7**.

The choice of epitope is particularly critical for the immunogenicity of SLP vaccines. In **chapter 7**, SLP vaccines were designed with linear B cell epitopes, that based on literature should elicit neutralizing antibodies (22-24). Even though vaccination with these epitopes induced Spike-specific antibodies, no neutralizing antibodies were found. Non-neutralizing antibodies that opsonize the Spike protein have been described to be protective against SARS-CoV-2 (25). However, in our vaccination setting, no protection was found upon SARS-CoV-2 challenge. This discrepancy could be due to several reasons, ranging from incorrect studies, differences between the human and murine situation, the incapability of the SLP vaccine platform to induce neutralizing antibodies to the requirement of conformational proteins to induce neutralizing antibodies to linear epitopes. With respect to the latter, the Spike DNA vaccine studied in **chapter 6** encoded the whole protein, allowing the presentation of conformational epitopes (epitopes comprised of amino acids that form a three-dimensional structure) to the immune system and resulted in the induction of neutralizing antibody responses and protection against SARS-CoV-2 infection. This difference in type of epitope (linear vs. conformational) can potentially explain the presence or absence of virus neutralization and protection against SARS-CoV-2.

Additional $CD4^+$ T cell help and Adjuvants

In the introduction of this thesis, the importance of $CD4^+$ T cell help for the virus-specific humoral and cellular immune responses was described. Another method for improving the immunogenicity of a vaccine is by providing additional help to the immune system with helper epitopes. In **chapter 7**, SLP vaccines encoding linear B cell epitopes were combined with, or coupled to, the pan HLA DR-binding epitope (PADRE) (26). This additional help was beneficial for the Spike-specific IgG antibody response, as the absence of PADRE in the vaccine resulted in no induction of Spike-specific antibodies. With the presence of PADRE, Spike-specific antibodies could be found after vaccination. In **chapter 6**, a helper cassette was added to the Spike protein encoded in the DNA vaccine. This cassette contained a universal helper epitope derived from tetanus toxin P30 (27), PADRE and OVA17, a helper epitope from the widely experimentally used OVA

protein. Adding the helper cassette to the DNA vaccine improved the immunogenicity, shown by the improved Spike-specific IgG antibody and cytokine-producing $CD4^+$ T cell response. Though we did not formally test it, part of the improved immunogenicity of the Spike DNA vaccine encoding the S1+S2 domains compared to the RBD domain alone can possibly be explained by the presence of more helper epitopes.

In **chapter 4**, human and murine SLPs containing both CMV-specific CTL and helper epitopes were designed. The effect of the additional helper epitope was studied experimentally in this chapter. However, there was no improvement of the $CD8^+$ T cell responses in the presence of an additional helper epitope. A possible explanation for this finding could be the use of the highly effective adjuvants CpG and OX40, that could overcome the help effect of the helper epitopes. In **chapter 7**, we also showed the possibility of inducing a protective $CD8^+$ T cell response against SARS-CoV-2 with an SLP vaccination adjuvanted with CpG and no helper epitopes.

The adjuvant CpG is a TLR9 ligand that induces an innate immune response characterized by T_H1 activity and proinflammatory cytokine production (28). Previously, our group has shown that CpG improves the height of an epitope-specific $CD8^+$ T cell response and protection against cancer compared to e.g. TLR4 ligands (29). This shows that CpG can partly overcome the requirement of $CD4^+$ T cell help for the $CD8^+$ T cell response. Indeed, it has been shown that CpG induces the expression of costimulatory ligands (CD70, CD80, CD86) known to be involved in $CD4^+$ T cell help to $CD8^+$ T cells (30).

In the MCMV setting, described in **chapter 4**, the booster SLP vaccination was adjuvanted with agonistic OX40, in addition to CpG. Agonistic OX40 activates the costimulatory OX40 receptor on T cells, resulting in T cell proliferation, cytokine production and survival (31). It was already established before by our group that for SLP vaccination adjuvanted with CpG the agonistic OX40 provided during booster vaccination improved the expansion and cytotoxic capacity of the T cell response (32). This finding was confirmed in **chapter 4**.

In **chapter 7**, SLP vaccines encoding linear B cell epitopes against SARS-CoV-2 were also adjuvanted with CpG, as CpG additionally results in the upregulation of CD40 on dendritic cells and is able to directly activate B cells (30, 33). This setting however, studied the effect of the combination with incomplete Freunds Adjuvant (IFA). IFA is an adjuvant that forms a depot that supports the slow release of antigen, induces inflammation and recruits immune cells (34). Combining IFA and CpG resulted in an improved Spike-specific IgG antibody responses to single adjuvant vaccinations. As

discussed before these antibodies were not neutralizing, nor did they protect against SARS-CoV-2 infection.

DNA vaccines are able to trigger pattern recognition receptors, and are, therefore, self-adjuvanting. In this thesis, providing extra adjuvants to DNA vaccines was not studied; however, in the DNA vaccine field, studies have been performed with (genetic) adjuvants to further optimize the immunogenicity of these vaccines (35).

Vaccination regimens

Since, the vaccination regimen used for administering a vaccine also affects the immunogenicity, different vaccination regimens were studied in this thesis. The dose of the vaccine is an important factor to induce an optimal immune response. In the case of the gB DNA vaccine studied in **chapter 5**, we found that using a higher dose improved the antibody response and reduced the viral load compared to a lower dose of the vaccine. This finding aligns with another study in which a higher dose of a DNA cancer vaccine improved the T cell response and protection upon prophylactic vaccination (36). The SLP dosages used in this thesis were based on previous research in our group and not further investigated (29, 37).

The site of vaccination is another factor that affects the immunogenicity of a vaccine. For T cell SLP vaccines, subcutaneous vaccination at the tail base results in an expanded T cell response, compared to subcutaneous vaccination in the flank (38). Therefore, the T cell SLP vaccines used in **chapter 4, chapter 5 and chapter 7**, were administered with a subcutaneous vaccination at the tail base. In **chapter 8**, different administration routes were studied to determine the optimal route for an SLP vaccine with a broad epitope coverage. In this chapter, intradermal vaccination at the tail base was resulted in the most optimal epitope-specific $CD8^+$ T cell response. In **chapter 5**, intramuscular vaccination was compared to intradermal vaccination, and the latter vaccination route resulted in an increased MCMV-specific IgG antibody response. These findings show that the optimal administration route can differ per vaccine modality. In the case of DNA vaccination, it is important that the vaccine targets cells such as keratinocytes for the antigen to be expressed and presented to the immune system (39). In the case of SLP vaccination, it is important that the route of administration provides direct access to secondary lymphoid organs, where the SLP can be presented to the immune system (40).

In addition to different administration routes and dosages, various vaccination timings were studied in this thesis. In **chapter 5**, a three time exponential priming regimen was investigated where 10, 20 and 30 µg of the sgB DNA vaccine was vaccinated on days 0,

3 and 6 respectively. Compared to the same total vaccine dose of 60 µg on day 0, the exponential dose priming regimen improved the MCMV-specific antibody response. Johansen *et al*. investigated the number of DCs and their activation upon exponential SLP prime vaccination and found that, compared to a single prime vaccination, the peak number of DCs and their maximum activation are delayed. The kinetics of the number of DCs and their activation also show that upon exponential prime vaccination there is a longer period where activated DCs are present, possibly explaining the improved immunogenicity upon exponential prime vaccination (41).

Additionally, in **chapter 6**, the effect of one or two booster vaccinations on the immunogenicity and protective capacity of the Spike DNA vaccine was studied. In this setting, an additional booster resulted in an increased Spike-specific antibody and epitope-specific $CD8^+$ T cell response in the blood. Upon viral challenge, one booster vaccination was sufficient to entirely protect the vaccinated mice from SARS-CoV-2 infection and no significant difference in survival compared with a second booster vaccination was found. It would be of interest to also study the effect of extra booster vaccinations after an extended period after vaccination, when the antibody response has waned and the T cell response is contracted. In addition, it would be interesting to study the effect of single prime vaccination on the protective capacity of this Spike DNA vaccine.

The effect of different amounts of booster vaccinations was also studied in the SLP setting in **chapter 7**, where mice that were susceptible to SARS-CoV-2 infection were vaccinated either one, two or three times with an SLP vaccine that induces an exclusive single epitope-specific $CD8^+$ T cell response. In this setting, additional booster vaccinations improved the height of the (memory) T cell response and two booster vaccinations were required for complete protection against SARS-CoV-2 challenge. Fewer booster vaccinations (either one prime vaccination or one booster vaccination) resulted in reduced protection. Furthermore, differences in the phenotype of the vaccine-induced specific $CD8^+$ T cells were found. One or two booster vaccinations resulted in an increment of epitope-specific $CD8^+$ T cells with an effector-memory phenotype ($CD62L^-KLRG1^+CX3CR1^+Ly6C^+$). Prime vaccination resulted in the induction of epitope-specific $CD8^+$ T cells with a central-memory phenotype ($CD62L^+KLRG1^-$). These findings highlight that the determination of the most optimal vaccination regimen contributes to ideal immunogenic and protective capacities of vaccines.

Vaccine-induced immune responses

Understanding which types of immune responses are induced by vaccine modalities and are required for protection, is important to determine the choice of a vaccine for

a specific virus type. Both SLP and DNA vaccines can be used to induce humoral and cellular immune responses. The choice of epitopes included in the vaccine determines the type of immune response induced.

In **chapter 7**, an SLP vaccine was designed that induced a single epitope-specific $CD8^+$ T cell response, without vaccine-induced B cells or $CD4^+$ T cells. With sufficient booster vaccinations, this vaccine protected against SARS-CoV-2, highlighting the importance of the T cell response in viral protection. Moreover, in **chapter 8** it was demonstrated that the combination of SLPs encoding $CD4^+$ and $CD8^+$ T cell SLPs also protected against SARS-CoV-2 challenge, further emphasizing the importance of T cell responses in viral protection.

The SLP vaccines encoding linear B cell epitopes derived from the Spike protein combined with PADRE induced Spike-specific non-neutralizing antibodies and a PADRE-specific $CD4^+$ T cell response. In this setting, the non-neutralizing antibodies were not effective in viral protection. This is in line with numerous studies showing that neutralizing antibodies are crucial for protection (42-45). However, a different study shows that non-neutralizing antibodies are also able to protect against SARS-CoV-2, by opsonizing the virus by the non-neutralizing antibodies (25). Potentially, this difference in experimental outcome can be explained by the different epitope that is recognized by the non-neutralizing antibodies or the method used to induce these antibodies.

The DNA vaccines used in this thesis express whole proteins and therefore contain B cell, $CD4^+$ and $CD8^+$ T cell epitopes. In **chapter 6**, the DNA vaccine induced a broad immune response with Spike-specific (neutralizing) antibodies, $CD4^+$ and $CD8^+$ T cells. The gB DNA vaccine studied in **chapter 5**, induced an MCMV-specific antibody response. Since gB elicits basically no $CD4^+$ and $CD8^+$ T cell responses, the T cell responses induced by this DNA vaccine could not be studied. This chapter, also showed that additional SLP vaccination with dominant $CD8^+$ T cell epitopes (from other MCMV proteins) empowered the protective capacity of the antibody-inducing DNA vaccine.

A different way to study the importance of the adaptive immune subsets, is by cellular depletion during vaccination. Hints on the role of adaptive immune cells in the human situation are obtained by the vaccination of patient groups that receive immunosuppressive drugs. In the case of SARS-CoV-2, patients receiving anti-CD20 treatment (a drug that depletes B cells) had a decreased humoral response and augmented $CD8^+$ T cell response compared to healthy individuals (46). In experimental mouse models, controlled depletion of immune subsets is possible. In **chapter 5**, $CD4^+$ T cells were depleted during the combinatorial vaccination of the sgB DNA vaccine

and CD8⁺ T cell SLP vaccination. Depletion of CD4⁺ T cells resulted in the absence of the MCMV-specific antibody response, but the CD8⁺ T cell response was not affected, possibly because of the CpG adjuvant. In **chapter 6**, more cell types were depleted in the context of vaccination. In this setting, both B cell and CD4⁺ T cell depletion during vaccination lowered the Spike-specific IgG antibody response, and CD4⁺ T cell depletion also reduced the CD8⁺ T cell response (no additional adjuvant was present in this vaccination). Also, the survival was reduced by the depletion of CD4⁺ T cells and B cells during vaccination. CD4⁺ and CD8⁺ T cell depletion during viral infection upon vaccination, did not affect survival. Possibly, the presence of the neutralizing antibodies compensates for the effect of the removal of the CD4⁺ and CD8⁺ T cells, if there is any effect in this setting at all.

Overall these studies show that different arms of the immune system have important roles in protection, and that when one part is depleted, another can compensate and still provide protection.

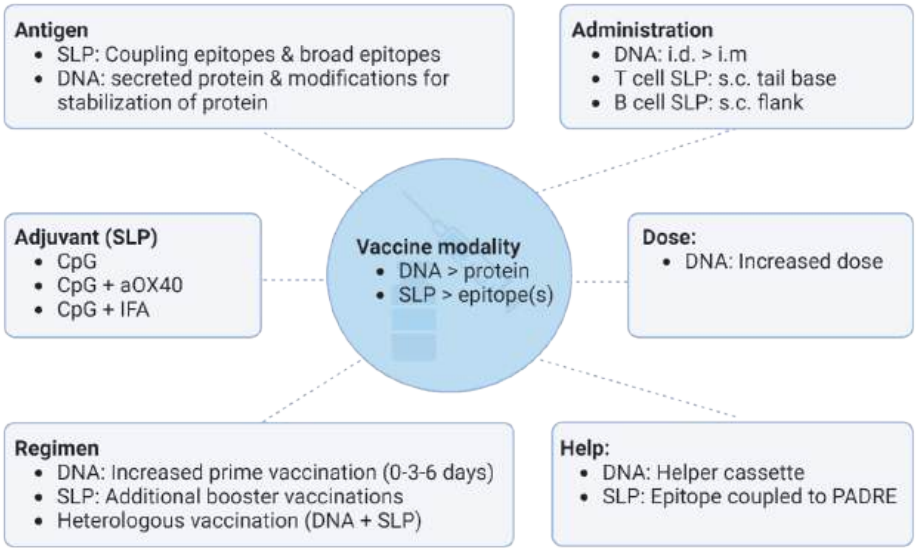


Figure 1. Methods studied in this thesis for improving vaccine-induced immunity against viral infection

Concluding remarks

Although each virus has unique characteristics and induces a different type of immune response (47), this thesis shows that vaccinology and immunology knowledge experimentally obtained by studying one type of virus (CMV) can be translated into protective vaccines for a separate virus (SARS-CoV-2). This indicates that the principles

of protective immunity are more universal. For this translation, it is fundamental to understand the viral immune response and mechanisms of vaccine-induced immunity. Preclinical studies as performed in this thesis are decisive for acquiring this knowledge, since the experimental mouse models used here provide the opportunity to study the separate factors important for viral protection provided by vaccine-induced immunity. An overview of the factors improving vaccine-induced immunity studied in this thesis is provided in Figure 1. Moreover, this thesis shows that there should be more emphasis on T cell responses in the development of prophylactic vaccines, since T cell responses, if strong in quantity and quality, can protect against viral infection as well. Historically, vaccines focus on the induction of (neutralizing) antibodies, but in the case of SARS-CoV-2, these antibodies tend to lose their protective capacity against newer mutated variants of the virus. T cell responses can be induced against a broad range of epitopes that are less susceptible to mutation, because they are essential for the survival of the virus. Designing a vaccine that (also) induces a strong and broad T cell response is more armed against evolution of the virus, and thus more future proof. Overall, this thesis provides an overview of mechanisms of DNA and SLP vaccine-induced immunity against viral infection.

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