Immune responses to conserved influenza epitopes : lessons for peptide vaccination strategies

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Chapter 8

Summarizing discussion

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Current seasonal influenza vaccines are effective inducers of antibody responses against matching circulating strains. However, the variable nature of HA and NA renders these vaccine-induced antibodies ineffective. In contrast, T cell responses are often directed towards internal proteins of influenza, such as NP, PB1, and M1, which contain a greater amount of conserved epitopes (1, 2). The relevance of T cell responses to conserved epitopes during influenza infection has been shown by studies describing the effect of the presence of cross-reactive T cell responses on influenza disease severity. Sridhar et al. described that a higher frequency of pre-existing $CD8^+$ T cells specific for conserved epitopes correlated with less severe illness after infection with pandemic H1N1 influenza virus (3). Wang et al. observed that the presence of early robust $CD8^+$ T cell responses correlated with faster recovery from H7N9 infection (4). Wilkinson et al. monitored T cell responses of healthy volunteers following influenza challenge, and observed lower virus shedding and less severe illness in the presence of influenza-specific cytotoxic $CD4^+$ T cells (5). In this thesis, we describe the current status of influenza vaccination and the role for vaccine-induced T cell immunity to influenza. We applied this knowledge to the development of new broadly protective influenza peptide-based vaccination strategies.

Current status influenza vaccination

In **Chapter 3**, a clinical trial is described in which we evaluated humoral and cellular immune responses during two consecutive influenza seasons from 2009 to 2011. The study was initiated in 2009 at the start of the influenza pandemic, following the introduction of the A(H1N1)pdm09 strain into the human population. This strain contains genes from swine, avian and human origin and was introduced into the human population through pigs. The virus had already infected individuals in different countries, and since individuals were expected to be naïve to this virus it was expected that the virus would spread easily and would be able to affect a high proportion of the human population worldwide. Therefore, the Dutch government decided to order pandemic vaccines in addition to the seasonal vaccine. The pandemic vaccine evaluated in this study was a monovalent MF59-adjuvanted subunit vaccine. The adjuvant was included to decrease the antigen-dose needed to obtain seroprotective antibody responses by half and induce a broader immune response in the form of enhanced functional antibody titers and greater breadth of antibody cross-reactivity (6). Based on studies on avian influenza vaccination the vaccine was registered for a two dose schedule to obtain seroprotective antibodies, which was also recommended by the Dutch government (7).

In the study described in **Chapter 3**, healthy adult participants received the MF59-adjuvanted monovalent pandemic vaccine in a two-dose setting. To account for background response and to be able to compare humoral and cellular immune responses, a statistical model was developed to analyze the data. Analysis of antibody titers showed that one dose would have

been sufficient to provide seroprotection, however, a second dose did further enhance antibody levels. In contrast, T cell responses were induced after the first dose, but were not boosted by a second dose. At the start of the second season, antibody levels and T cells were still detectable in vaccinated individuals. Administration of the unadjuvanted seasonal vaccine, also containing the pandemic strain of the previous year, boostered both these responses. Antibody levels of individuals that did not receive the seasonal vaccine in 2010 remained detectable until the end of the study indicating the presence of vaccine-specific antibodies for over 15 months. T cells remained detectable until one year after vaccination, but had reduced back to baseline level at 15 months post vaccination.

The finding that one dose of the A(H1N1)pdm09 pandemic vaccine is sufficient for providing seroprotection is supported by literature (8-10). We hypothesize that it may be possible to predict whether a two-dose setting is necessary during a pandemic threat, by predicting cross-reactive immunity. Cross-reactive immunity is in the first place dependent on the presence of cross-reactive responses in individuals. Ikonen et al. described the presence of cross-reactive antibodies to A(H1N1)pdm09 in elderly, which may have contributed to the low number of elderly affected by this pandemic (11). These data indicate that when cross-reactive immunity is present, one dose is sufficient (12), while in individuals that do not have pre-existing immunity even two doses might not be sufficient to provide seroprotective antibodies as shown by a study with H5 subtypes (13). However, characteristics of the virus itself should also be taken into account. While unadjuvanted vaccines for H5N1 subtypes already required a two dose schedule to induce seroprotective antibody titers (14-16), there are some viruses that have such a low immunogenicity that the design of an effective vaccine will be very difficult. The effect of immunogenicity of the virus itself on vaccine efficacy is demonstrated by data on H7N9, a subtype that emerged in China in 2013 and has a high lethality record (17). It was predicted by de Groot et al. that H7N9 had a low number of cross-reactive $CD4^+$ T cell epitopes, which are required for antibody induction. Indeed, unadjuvanted vaccines targeting H7N9 HA had a low efficacy (18), which underlines the importance of cross-reactive T cell responses and shows that these are not only dependent on pre-existing immunity present in the human population, but also on epitopes in the virus itself.

In **Chapter 3**, we also monitored T cell responses to the vaccine during the clinical trial. Vaccine-specific T cell responses were initially demonstrated by stimulation of PBMCs with the WT virus strains used in the vaccines to show the effect of vaccination on T cell responses following infection with influenza virus. IFN-γ production in response to whole virus stimulation was measured by ELISpot. Hereby, we showed that both pandemic and seasonal influenza vaccination induced T cell responses in vaccinated individuals. However, in contrast to antibody levels, T cell levels were not boostered by a second dose of the pandemic vaccine. The finding that booster vaccination is not favorable for T cell responses has been described earlier. McElhany et al. even found a negative correlation between antibody levels and cytokine ratios in elderly and proposed that a second dose might skew T cell responses

to an IL-10 dominated cytokine profile (19). IL-10 is associated with an anti-inflammatory response, which limits T cell and antibody responses. The presence of high levels of IL-10 is therefore not desired during a response to influenza (20). Others reported an inverse correlation between pre-vaccination IFN-γ production and the magnitude of responses post-vaccination (21, 22). In the clinical trial, a booster effect on T cell responses was, however, visible at the start of the second season; residual T cell levels of individuals vaccinated with the A(H1N1)pdm09 vaccine in the previous year, were further enhanced after vaccination with the seasonal vaccine. This apparent discrepancy in the ability of vaccination to boost T cell responses could be explained by timing of vaccination. The second dose of the pandemic vaccine was administered three weeks after the first dose, while the seasonal vaccine was administered approximately one year later. After three weeks, T cells will still have been activated or the memory T cell response may not have been fully developed (23, 24), thereby limiting further activation of T cells. However, the effect of vaccine dose administered cannot be excluded since the pandemic vaccine contained 7.5 μg HA, while the seasonal vaccine contained 15 μg HA.

To confirm that T cell responses detected in individuals that received pandemic and seasonal vaccination were vaccine-specific, T cell responses were also analyzed by stimulation with vaccine-specific peptide pools. During stimulation with live virus, T cells to the more conserved internal virus proteins induced by previous natural infections may have been activated. However, by stimulation of PBMCs with a peptide pool containing 15-mer peptides with 11 amino acid overlap spanning the entire HA or NA protein of the vaccine strains, only vaccine-specific responses were analyzed. A similar profile of responses was detected as with whole virus stimulation, showing that T cell responses were indeed vaccine-specific. Residual T cell levels were still detectable at the start of the second season, which implies that T cell memory is maintained for over a year. After 15 months T cell levels had reduced back to baseline, which is in line with findings that memory $CD4^+$ and $CD8^+$ T cells have a maximum life span of approximately one year (25, 26). Thereafter, a minimal compartment of memory cells may still be present, which is the background response or baseline of our assays. Following infection or vaccination, these T cells can be re-activated and this will be measurable as an induction of T cell levels (27). In **Chapter 3**, we described general T cell levels in response to influenza vaccination, however, it remains to be clarified by which cell type the measured IFN-γ levels were produced. Since peptide pools were used as a stimulating antigen, which are specifically designed to stimulate T cells, cytokines measured are most likely produced either by $CD4^+$ T cells, $CD8^+$ T cells, or both. Thus far, both types of T cells have been shown to be important in providing cross-reactive immunity during influenza infection (3, 5). However, the impact of each type of T cell following influenza vaccination remains to be elucidated. The impact of cellular responses also depends on specific characteristics of the T cell response, such as clonality of the T cells and their cytokine profile. As described in **Chapter 2**, for viral infections, a Th1-skewed T cell response, as characterized by the production of IFN-γ is more desirable than a Th2-skewed immune response as characterized by the production of IL-4,

IL-5, and IL-13. Furthermore, the response should be of magnitude and breadth to be able to clear virus and have an effect during influenza infection.

In addition, it will be interesting to identify to which epitopes the responses were directed. Much research is focused on T cell responses to internal influenza proteins, such as NP and PB1, since these proteins are more conserved and thus interesting for the development of universal vaccines. However, as elegantly shown in a model in which mice were infected with HK-X31, HA protein is, in addition to NP, one of the major contributors of MHC class II epitopes. Approximately 51% of the MHC class II epitopes submitted to the immune epitope database is HA-specific (28, 29). However, research on $CD4^+$ T cell epitopes may be focused on responses to antigens to which also antibodies were directed, explaining the large number of $CD4^+$ epitopes identified for HA. In concordance, the immune epitope database contains a large number of conserved $CD8^+$ T cell epitopes of internal viral proteins and only a minor percentage of epitopes derived from HA, which can be explained by the fact that many groups specifically investigate conserved $CD8^+$ T cell responses directed to these proteins (29). Therefore, a potential bias in literature cannot be excluded and it remains difficult to predict whether $CD4^+$ T cells or $CD8^+$ T cells are the main contributors to responses in **Chapter 3** and whether the T cell responses are directed to relatively conserved HA and NA epitopes or to subtype or strain specific antigens.

As of yet, no literature is available describing T cell responses to subunit vaccines in humans. One explanation is that current seasonal vaccines are designed to induce antibody responses to circulating strains, since antibodies are still the correlate of protection for influenza. Therefore, evaluation of these types of vaccines generally does not include an evaluation of T cell responses induced by vaccination. For example, Lambe et al. did evaluate the induction of T cell responses by a trivalent inactivated split vaccine, but focused only on conserved epitopes present in the remaining internal viral proteins M1, NP and NS1 (30). Other registered influenza vaccines that have been shown earlier to be capable of inducing T cell responses have components facilitating the induction of T cells. LAIV and WIV, for example, both contain ssRNA, which is a ligand for TLR7 (31). In addition, LAIV still has limited replicative potential, leading to presentation of viral antigens on MHC class I, while BPL-treated WIV has maintained fusion activity, facilitating the induction of T cell responses (32). However, we have shown that both the MF59-adjuvated pandemic vaccine and the unadjuvanted subunit vaccine induce T cell responses even though these vaccines did not contain internal viral proteins and were not designed to induce T cell responses. Apparently, administration of antigen alone is sufficient to induce a certain level of T cell responses, which could be due to activation of memory T cells already present due to earlier influenza infection or vaccination. For example, although the globular head of HA and NA is highly variable, these proteins do contain some conserved epitopes and individuals may have had T cells available directed to these epitopes explaining the induction of a response to the vaccine antigens. Although it could also be possible that the vaccine may have induced naïve T cells.

T cell-inducing influenza vaccines

Even though we have shown that current vaccines are capable of inducing a certain level of T cell responses, they are designed to induce antibody responses to circulating influenza viruses and do not induce a cross-reactive immune response. Therefore, current vaccines have several limitations; they need to be updated each year to match with circulating strains and if strains do not match, the vaccine is not as effective. In addition, antibody-induced vaccine-immunity might not be optimal for target groups of influenza vaccination, i.e., elderly and young children. McElhany et al. showed that T cell responses might provide a better correlate for protection than antibody responses in elderly (33). In addition, Bodewes et al. showed that although seasonal vaccination of children is effective in preventing disease, it does hamper the development of virus-specific $CD8^+$ T cells, normally acquired through natural infection (34). By specifically inducing T cell responses, an influenza vaccine would not only be more efficient in elderly, but by targeting conserved epitopes it would also provide additional immunity to a broader variety of influenza virus subtypes. Hereby, such a vaccine could limit disease and spread of infection when a new virus subtype emerges.

In scope of the development of such a new type of influenza vaccine, there are many strategies under development that are designed to induce influenza-specific T cell responses as reviewed by Soema et al. (35). Live attenuated influenza vaccines are already licensed and in use for vaccination of individuals of 2-49 years of age. LAIV has been shown to induce both $CD4^+$ and $CD8^+$ T cell responses in unprimed children (21, 36). T cell-inducing influenza vaccines that are under development include viral vectors, DNA vaccines, protein vaccines, and peptide-based vaccines. Viral vectors are replication-defective viruses capable of expressing sustained levels of antigens. As extensively reviewed by Tripp et al., there are many types of viruses that can be used as a vector to express high levels of influenza-specific antigens. (37). Recently, a vaccinia virus Ankara expressing NP and M1 was shown to induce T cell responses (38). DNA vaccines are also under development as a method to induce influenza-specific T cell responses. At the site of immunization, cells become transfected with plasmid DNA that encodes the protein of interest. This protein can then be processed and presented to the immune system in the context of MHC class I or II. Hereby, both $CD8^+$ and $CD4^+$ T cells can be activated (39). A third novel strategy are protein vaccines, recently a successful Phase I clinical trial has been described with a vaccine containing HA, M1, and NP that was capable of inducing cellular responses in adults and elderly (40). In this thesis, we focused on peptide vaccination as a means of inducing T cell responses to conserved influenza epitopes, which have the advantage of being fully synthetic and can therefore be readily produced for human use in a GMP setting. In addition, while the production of currently used vaccines first requires the generation of vaccine seed strains, which is time consuming and may result in low yield strains. To produce the vaccine, the virus seeds need to be grown on eggs, of which may be a shortage during a pandemic (41) and has the downside of possible contamination with egg antigens and extraneous agents. On the contrary, the production of peptides only requires the sequence of a new virus and is much faster and cleaner.

Peptide-based vaccine strategies

In **Chapter 2**, we described the features of an effective cellular response against both acute and chronic infections, including an appropriate magnitude, breadth and quality. An effective immune response against viral infections largely depends on the activation of cytotoxic T cells capable of clearing infection by killing virus-infected cells. Proper activation of cytotoxic T cells depends on professional antigen presenting cells. In **Chapter 2** we discussed the potential of peptide-based vaccination as a strategy to prevent and treat viral infections. Many factors influence the efficacy of peptide vaccines, including choice of antigen, adjuvants and formulation. As already mentioned previously, it is desirable to specifically target conserved regions of influenza virus, making a vaccine efficacious against a broader range of influenza subtypes. For the selection of peptides there are databases available in which known CD4 and CD8 epitopes are listed, specific for a wide variety of pathogens including influenza virus (29). Furthermore, there are tools available to check whether selected regions are conserved, whether these regions contain T cell epitopes and whether selected sequences do not contain auto-antigens (42-44). Following successful selection, a choice needs to be made on the type of peptide used. Minimal epitopes only induce a $CD8^+$ or $CD4^+$ T cell response, while long peptides need to be processed by professional APCs and will induce both $CD8^+$ and $CD4^+$ T cells. In this thesis, we described both strategies. **Chapter 4** discusses the potential of a long overlapping peptide vaccine while **Chapters 5**, **Chapter 6** and **Chapter 7** describe the modification and formulation of minimal epitope-based vaccine concepts.

In **Chapter 4,** we set out to develop a vaccine that provides protection against a broad range of influenza virus subtypes. We selected 25 synthetic long overlapping peptides (SLP), derived from highly conserved regions of nucleoprotein (NP), polymerase basic protein 1 (PB1), and matrix protein 1 (M1). Length of the peptides ranged from 26-34 amino acids to ensure that known $CD4^+$ and $CD8^+$ T cell epitopes were included. Hereby, an immune response could be induced to a wide variety of antigens, thereby also providing a broad HLA coverage. In addition, we selected linear B cell epitopes directed to the highly conserved HA2 fusion peptide and to M2e peptide and conjugated these epitopes to a CD4 helper epitope. The CD4 epitopes were selected based on their known strong immunogenicity. By including a $CD4^+$ epitope, $CD4^+$ T cells can be activated in parallel to B cells. The activated $CD4^+$ T cells can then provide help to the B cell, resulting in enhanced immunogenicity of the B cell epitope. This will result in higher B cell epitope-specific antibody titers. C57BL/6 mice, BALB/c mice, and ferrets were vaccinated with the 25 SLP, the B epitopes, or a combination of both. We detected vaccine-specific antibodies in sera of both mice and ferrets. Vaccine-specific T cell responses were only analyzed in BALB/c mice and were shown to be directed to vaccine-specific peptides. Following challenge, a reduction of virus titers in the lungs was observed in mice vaccinated with the 25 SLP and the B epitope alone and in mice and ferrets vaccinated with the combined vaccine. Hereby, we have developed a promising universal influenza vaccine format capable of inducing both T and B cell responses. Although vaccination did not reduce disease severity, reduction of virus replication is valuable since it may reduce spread

of the virus in a pandemic situation.

In **Chapter 5**, we discuss a method in which immunogenicity of peptides itself can be enhanced. By substitution of amino acids in the wild type sequences with non-proteogenic amino acids, resulting in chemically enhanced altered peptide ligands (CPLs) the affinity for HLA can be increased. This method was applied to optimize minimal influenza-specific epitopes of varying natural affinity and dominance: the highly dominant GILGFVFTL ($M1_{58-66}$), the less dominant FMYSDFHFI (PA_{46-54}) and the low affine and subdominant NMLSTVLGV ($PB1_{413-421}$) epitopes. Based on these HLA-A*0201-specific epitopes, approximately 600 CPLs were designed and tested for binding affinity. For all three epitopes, we succeeded to design CPLs that exhibited greater binding affinity than their WT counterparts. FMYSDFHFI and NMLSTVLGV had the greatest window of improvement and therefore, the largest increase in binding score was obtained for modifications to these epitopes; however, even binding scores of the already high affinity GILGFVFTL epitope could be improved. To evaluate immunogenicity of these CPLs compared to their corresponding WT epitopes, IFN-γ responses in splenocytes of mice vaccinated with CPLs were compared to WT vaccinated mice. An increased response was measured for CPLs of GILGFVFTL and FMYSDFHFI and in addition to an increased response, modification of the low affinity epitope NMLSTVLGV also led to an increase in the number of mice that responded. Additionally, by optimizing three HLA-A*0301-specific epitopes: ILRGSVAHK ($NP_{265-273}$), SFSFGGFTK ($PB2_{322-330}$), and RMVLSAFDER (NP_{67-76}), we show that a general strategy is established that can be used to improve binding of essentially any class I epitope and to any allele.

In addition to the experiments described in **Chapter 5**, a challenge experiment was performed in HLA-A2 transgenic mice to assess the efficacy of a CPL-based vaccine and to determine whether modified epitopes have an additional value compared to WT epitopes. To this end, mice were vaccinated with a combination of the three HLA-A*0201-specific WT epitopes described above or with a combination of the three optimal CPLs derived from these epitopes (G1 ([am-phg]ILGFVFTL), F5 ([4-FPHE]MYSDFHF[2-AOC]), and N53 (N[NLE] LSTVLGV)). An identical formulation was used as in the immunogenicity experiments, i.e., adjuvation with IFA and the TLR9-adjuvant CpG. As a negative control, mice were vaccinated with adjuvants alone, while positive control mice received a low dose of the mouse adapted HK-X31 (H3N2) influenza A strain. Two weeks following booster vaccination, at the peak of the T cell response, mice were challenged with the HK-X31 strain. No significant differences were observed between the lung viral load of mock-vaccinated mice and both groups of peptide-vaccinated mice. In addition, all mice lost bodyweight after challenge, however, CPL-vaccinated mice demonstrated significantly less bodyweight loss than mock vaccinated mice and WT epitope-vaccinated mice. Pathological examination of lungs of mice showed a reduction in relative lung weight (a measure for edema formation), percentage affected tissue, and the end score of histopathological markers such as inflammation of tissues and damaged epithelium, in CPL-vaccinated mice compared to mock-vaccinated mice. WT epitope-

vaccinated mice also had a lower percentage of affected tissue, but other histopathological markers were not decreased as much. These results indicate a small advantage of CPLs over the WT epitopes, although additional experiments should be performed to strengthen these findings. Altogether, modification of epitopes provides a valuable tool to improve the range and immunogenicity of preventive T cell-targeted peptide vaccines, although optimization of these vaccines is needed to obtain a vaccine also capable of reducing viral load in the lungs.

However, the limited immunogenicity of peptides requires strong adjuvation. In the vaccines described in **Chapters 4** and **5**, peptides were formulated in Incomplete Freund's Adjuvant in combination with a TLR1/2 and TLR9 adjuvant, respectively. IFA is a depot-forming adjuvant, which is highly effective for peptide-based vaccines, however, it is not desirable for human use (45, 46). Therefore, other methods need to be developed to enhance immunogenicity of peptide-based vaccines. In **Chapter 6**, we formulated the GILGFVFTL-peptide with virosomes and assessed its immunogenicity in HLA-A2 transgenic mice. We showed that vaccination of mice with peptide-loaded virosomes (P-V) induced peptide-specific $CD8^+$ T cells. Addition of TLR9 ligand CpG to the formulation increased IFN-γ production of $CD8^+$ T cells in response to stimulation of splenocytes with the GILGFVFTL-peptide. Furthermore, IgG2c titers measured to HA of the virosome were increased in mice vaccinated with P-V in combination with CpG. The presence of IgG2c-antibodies indicates a Th1-skewed response, which is advantageous for the induction of $CD8^+$ T cell responses. Mice vaccinated with P-V in combination with CpG demonstrated less weight loss and increased recovery compared to PBS and mice vaccinated with peptide and CpG formulated with IFA after challenge with influenza. **Chapter 6** shows that virosomes are an effective peptide delivery system, however, depending on characteristics of the peptide, encapsulation of peptides can be challenging. Therefore, virosomes are not yet broadly employable and additional development is needed before it can be considered for clinical testing in humans.

Improving existing influenza-vaccine formats may also be a possibility. In **Chapter 7**, we investigated whether T cell responses can be improved by using whole inactivated influenza virus (WIV) as an adjuvant for influenza peptides. Immunogenicity of WIV mixed with minimal peptides was investigated in HLA-A*0201 transgenic mice. WIV was formulated with GILGFVFTL alone or with a peptide pool containing the three HLA-A*0201-specific WT epitopes described in **Chapter 5** and the three optimal CPLs corresponding with these epitopes. WIV was found to be a potent adjuvant for GILGFVFTL alone, even at low concentrations and for the WT and CPL peptide pools. In mice vaccinated with CPLs adjuvanted with WIV the IFN-γ response was enhanced compared to mice vaccinated with CPLs formulated in IFA. When WIV was used as an adjuvant, responses to GILGFVFTL and FMYSDFHFI were higher than with IFA. Especially the response to the more subdominant FMYSDFHFI peptide was increased. In addition, injecting peptide and WIV separately at different sites or mixed at a single site, showed that co-localization of the peptide antigen and WIV adjuvant was important for an efficient peptide-specific immune response. The relatively simple formulation and the use of a

known influenza vaccine component make WIV an attractive adjuvant. However, in contrast to the vaccination strategies described in **Chapters 4** and **5**, which describe the development of possible universal influenza vaccines, the strategy described in **Chapter 7** is meant to improve current vaccines. By adding T cell inducing components to current vaccine formats, the response to vaccines can be broadened thereby improving vaccine-specific responses.

Future perspectives

One of the major issues for T cell inducing vaccines is designing a vaccine that provides a broad HLA-coverage. The minimal epitopes described in this thesis are directed to HLA-A*0201 and HLA-A*0301. Both are alleles frequently occurring in the Caucasian population, however allele frequencies differ between different ethnicities as described in an online database (47). Minimal epitopes are described that are recognized by a broader set of alleles,

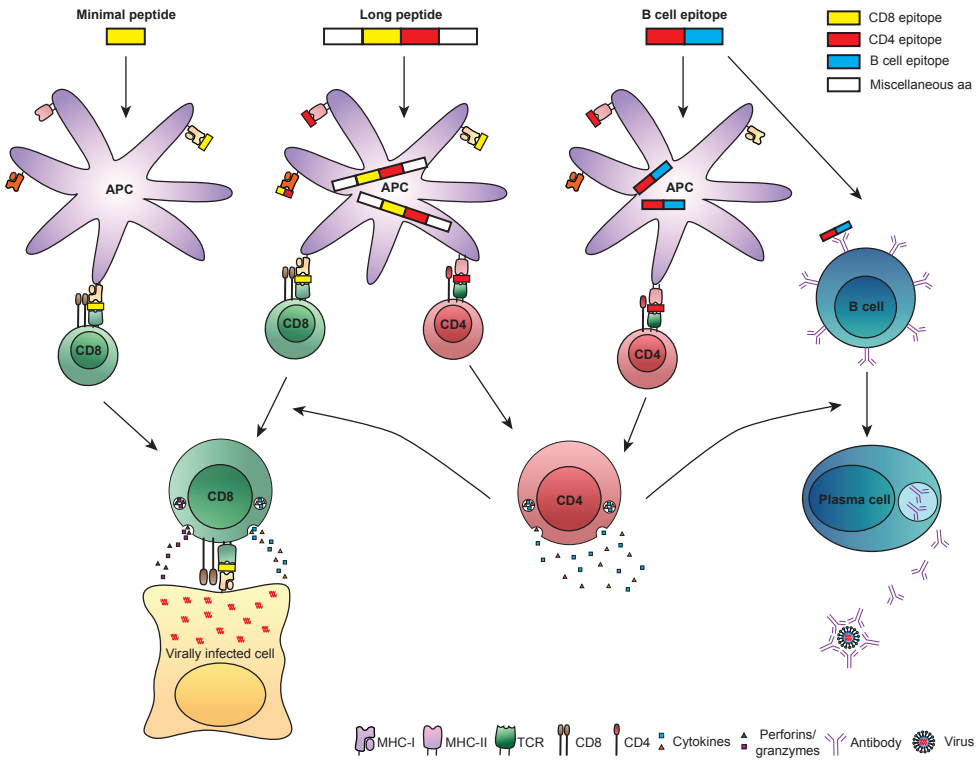

Figure 1: Overview of peptide vaccination strategies.
This Figure provides an overview of the peptide vaccination strategies described in this thesis. Minimal peptides (left) fit directly into a specific MHC class I molecule, require no processing by APCs and will therefore only induce $CD8^+$ T cells. Compared to WT peptides, CPLs will induce an enhanced $CD8^+$ T cell response compared to WT epitopes. Long peptides (middle) do need to be processed by a professional APC and are capable of inducing both $CD8^+$ and $CD4^+$ T cells. The B epitopes (right) described in this thesis contain both a $CD4^+$ T cell epitope and a B cell epitope and are therefore capable of activating $CD4^+$ T cells and inducing the production of B cell epitope-specific antibodies.

however these epitopes are not presented equally efficient by the different alleles (28). Long peptides provide part of the solution, since they need to be processed by professional APCs and are thus not specific for one allele (48). However, a large enough selection needs to be made to provide for sufficient epitopes for each allele and even if long peptides can be selected that provide a broad HLA-coverage, the question still remains how many and which type of epitopes are sufficient for providing at least a reduction of disease burden. There are publications describing potent trials with synthetic peptide vaccines containing immunodominant epitopes (49, 50). However, these trials have only shown that synthetic peptide vaccines are immunogenic in a small population. It remains to be evaluated whether this vaccine is applicable worldwide and whether it is effective against natural infection with influenza. A recent paper describes in an in vitro model of human epithelial cells that although the immunodominant GILGFVFTL epitope is present in a high number of copies per cell following infection, GILGFVFTL-specific T cells are unable to effectively recognize infected human epithelium (51). These results warrant caution when selecting epitopes for use as a vaccine, since some epitopes might induce an unwanted T cell response.

As already recognized as early as 1969, efficacy of influenza vaccines is also influenced by the route of immunization (52). With the exception of LAIV, which is administered intranasally, registered influenza vaccines are administered either intramuscularly or intradermally. As also discussed in **Chapter 2** and evaluated in **Chapter 4**, route of immunization will play an important role in efficacy of peptide-based influenza vaccines. Lung-resident T cells will naturally play an important role in clearance of influenza virus and as Rose et al. described, lung-resident immune cells will be primed more easily via intranasal vaccination (53). In **Chapter 4**, no major differences in efficacy were observed between the different routes of immunization with the SLP vaccine in ferrets. A study in mice, however, which compared pulmonary immunization to systemic immunization found that while circulating memory $CD8^+$ T cells are efficiently induced by systemic immunization these cells do not prevent viral replication and damage to the lung epithelium. In pulmonary vaccinated mice, a T cell response is observed followed by efficient clearance of virus from the lungs (54). These findings demonstrate the potential advantage of inducing T cell immunity at the site of entry of the pathogen. This can be obtained by selecting a certain route of immunization, as described above. However, other factors also contribute to the induction of an efficient T cell response in the lungs. Lung-primed DCs, for example, play an important role in recruiting T cells by inducing upregulation of the CCR4 receptor on these cells. CCR4 was shown to be an important homing marker on T cells for lung tissue, specifically (55), illustrating that additional actions are needed to obtain a localized immune response.

However, a risk of inducing T cell responses too efficiently is that overstimulation of the immune response may lead to immune pathology. To examine the influence of vaccination on pathological effects induced by a virus challenge, lungs of mice and ferrets collected five days after infection in studies described in **Chapter 4**, were fixed in formalin after which

haematoxilin and eosin (HE) stained sections were examined for general histopathology. In C57BL/6 mice, the overall pathology in the vaccine groups and the mock vaccinated group was similar. In BALB/c mice, however, lung pathology in the group vaccinated with B epitopes, but mostly in the group vaccinated with the 25 SLP alone was higher than that of mock vaccinated group. Surprisingly, the pathology in mice vaccinated with 25 SLP + B epitopes was not enhanced. In addition, in the group vaccinated with 25 SLP alone, inflammation around the bronchi, bronchiole, and blood vessels was increased. In ferrets, gross pathology, as examined by percentage affected lung tissue and relative lung weight, was increased in the vaccinated groups. An increase in the latter represents edema formation and plays a role in acute respiratory distress. On the microscopic level, an increase in pathology was also observed although there was a large lobular variation and variation between animals. Although the increased parameters were mainly of inflammatory nature, in some animals increased damage to the epithelium was also observed.

While there was a large variation in lung pathology observed in the mice and ferret studies, the observations described above indicate enhanced lung pathology after challenge. Based on the BALB/c mice study, this appears to be linked to the T cell component of the vaccine. Increased pathology, and especially the increased inflammation, may be explained by the induction of lymphocytes by this type of vaccine. In addition, the timing of influenza challenge in these studies is approximately at the peak of the T cell response and it could be that a completely developed effector T cell response in combination with an unnaturally high challenge dose induces exaggerated pathological effects. Recently, when we used the intratracheal challenge model in ferrets to investigate an H7N9 LAIV, also known to induce cellular responses, increased inflammation compared to the placebo group was observed (manuscript in preparation). In addition, when mice vaccinated with a low dose of BPL-inactivated WIV (5 µg), also known to induce T cell responses, with or without the addition of T cell epitopes as described in **Chapter 7** were challenged with influenza virus, enhanced pathology in the lungs was observed compared to the mock vaccinated group. This indicates that increased pathology is not an effect of the peptide vaccine specifically, but it might be the result of the design of the challenge model in combination with vaccines that induce T cell responses. Future research, in which we, a.o., want to identify the cells involved in pathology by histochemical examination and look into a model which addresses a different timing of the challenge and a lower challenge dose, will hopefully reveal the mechanism behind these observations. However, when developing T cell inducing vaccines for respiratory infections, the risk of the induction of immune pathology should be taken into account and proper models to examine these side effects should be developed.

Vaccine-related immunopathology has also been reported by others. Studies in mice indicate a role for $CD4^+$ T cells in influenza-related immunopathology. Mice that had memory $CD4^+$ T cells available specific for the challenge virus, exhibited more clinical signs, greater lung damage, and died sooner than mice that did not have $CD4^+$ memory T cells available. It was

hypothesized that memory $CD4^+$ T cells tend to cluster together during a secondary challenge, resulting in a concentrated secretion of antiviral cytokines. These localized clusters resulted in more extreme damage of the epithelium than during a normal immune response in the lungs (56, 57). Another group demonstrated that influenza infection in aged mice resulted in increased pathology, which was contributed to $CD8^+$ T cells. Enhanced lung pathology has also been observed for other respiratory pathogens. A mouse model to elucidate the mechanisms behind the failure of the 1960 trial with formalin-inactivated RSV, which induced enormous vaccine-related morbidity and mortality, a Th2-skewed $CD4^+$ T cell response as the cause of enhanced disease (58). In other RSV virus models, there are indications that $CD8^+$ T cells provide protection at the cost of pulmonary immune pathology (Knudson, Keystone 2014, unpublished data) (59).These results illustrate that the presence of memory T cells in the lungs may aid in efficient clearance of the virus, but may also induce immunopathology. This leaves us with an apparent paradox, in which important antiviral cytokines, such as IFN-α and IL-6 are also inducers of pathology in the lungs (60). Therefore, an efficient antiviral response requires the presence of T cells that, upon activation, will produce a balanced set of cytokines. Antiviral cytokines such as interferons are needed to aid in clearance of the virus, but should be followed by cytokines regulating this cytotoxic response, such as IL-10, to restrict pathological effects induced by the production of pro-inflammatory cytokines (61).

Summarizing conclusion

When a successful T cell inducing vaccine would be available, a constraint is that even if memory T cells induced by this vaccine are present, time is needed for a T cell response to develop. Therefore, a vaccine only focused on the induction of T cells, will not clear an influenza virus infection before the onset of symptoms. This is in contrast to the current antibody-inducing vaccines that are capable of providing neutralizing immunity. While we showed that current subunit vaccines are capable of inducing both humoral and cellular responses, they do not offer cross-protection. As long as no neutralizing cross-reactive antibodies can be induced by vaccination, it will already be an advantage to induce T cell responses towards conserved parts of influenza virus. A few studies describe the role of cross-reactive T cells in reducing disease burden during infection with a new virus subtype. However, a vaccine inducing cellular responses is not only desired in case of a possible pandemic, in which T cells can aid in limiting spread of the virus, but it may also be the key to improve vaccines for children and elderly. In this thesis, we have described several peptide vaccination strategies targeting conserved influenza epitopes (summarized in **Figure 1**) and we investigated formulation strategies to enhance immunogenicity of peptide-based vaccines.

Therefore, we propose that while a new T cell inducing format will not replace current seasonal vaccines, it is desirable to add T cell inducing components to current seasonal vaccines. This will not only enhance immunogenicity of these vaccines in elderly and young children, but may also aid in providing cross-protection when the humoral components of the seasonal vaccine do not match circulating vaccine strains. In parallel, completely new vaccine formats

should be developed that ideally induce humoral and cellular immunity to highly conserved influenza antigens to be able to protect against a wide variety of influenza virus subtypes. Optimally, such vaccines will induce memory T and B cells at the location of influenza virus entry, i.e., the respiratory tract, to ensure a quick response upon infection. One can apply a different route of immunization or make use of adjuvants to obtain a memory response at the desired location. Concluding, there is still a long road ahead, but the findings in this thesis may contribute to the development of the next generation influenza vaccines, which offer broad and cross-reactive protection in the form of both humoral and cellular responses to conserved influenza epitopes.

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