Formulation of influenza T cell peptides : in search of a universal influenza vaccine

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Chapter 8

Summary and perspectives

SUMMARY

The development of cross-reactive influenza vaccines remains a challenging ordeal. Several vaccine design approaches to tackle this problem are currently being followed, as described in **Chapter 2**. One of these approaches is the induction of influenza-specific T cell responses that lead to clearance of virus-infected cells. T cell peptides covering conserved epitopes of influenza are able to induce such T cell responses. However, these peptides are poorly immunogenic and require additional formulation with one or more adjuvants (i.e., a delivery system and/or immunopotentiator).

The aim of this thesis was to develop novel formulations that increase the immunogenicity of influenza T cell peptides. Three different types of peptide-based influenza vaccine formulations were investigated in this thesis; peptide-loaded virosomes, whole inactivated influenza virus (WIV)-adjuvanted peptides and peptide-loaded liposomes. Additionally, an alternative vaccine delivery system, the Bioneedle, was evaluated in this thesis for use in influenza vaccination.

Chapter 2 provides an extensive overview of formulation and production methods for current and future influenza vaccines. Key problems concerning current seasonal influenza vaccines, such as lack of cross-reactivity, lack of efficacy in high-risk populations and limited production capacity, were identified and possible solutions were suggested. Potentially cross-reactive immune responses and their formulation strategies for their respective antigens, which may lead to a new generation of influenza vaccines, were described. Finally, several novel influenza vaccine production methods were reviewed, and the production feasibility of novel influenza concepts was assessed.

Chapter 3 focused on the development of influenza virosomes as a delivery system for influenza peptide antigens. The influenza peptide GILGFVFTL was successfully associated with virosomes, without affecting virosome size, surface charge or fusogenicity. The immunogenicity of the peptide-loaded virosomes was tested in HLA-A2.1 transgenic mice. Peptide-loaded virosomes were able to induce peptide-specific T cells, and addition of Toll-like receptor (TLR) 9 agonist CpG-ODN 1826 to the virosomes significantly increased the T cell response. This formulation was also able to decrease morbidity and increase the recovery rate of mice infected with a heterologous influenza strain, which indicates that CpG-adjuvanted peptide-loaded virosomes are a promising vaccine formulation for the induction of effective T cells. Association between peptide and virosome as well as virosome fusogenicity were found to be necessary for effective uptake by dendritic cells and the subsequent induction of T cell responses.

The use of WIV as an adjuvant for peptides was investigated in **Chapter 4**. A physical mixture of WIV and the influenza peptide GILGFVFTL was able to induce high peptide-specific T cell responses in HLA-A2.1 transgenic mice. The dose for both WIV and peptides was optimized in a following study, and a prediction model could be generated from the dose-finding study. Next, it was found that WIV and peptide has to be co-localized at the site of injection to induce a T cell response; when WIV and peptide were injected at separate sites, the T cell response decreased significantly. Interestingly,

WIV did not have to be fusogenic to retain its adjuvanticity. Finally, WIV was used as an adjuvant for peptide pools of three wild-type influenza peptides (GILGFVFTL, FMYSDFHFI and NMLSTVLGV), or three chemically enhanced peptide ligands (CPL) derived from the three WT peptides. WIV was able to increase the immunogenicity of both WT and CPL GILFGFVFTL and FMYSDFHFI, while incomplete Freund's adjuvant was only effective for the GILGFVFTL peptide and its CPL derivative. The results of this study indicate that WIV is an effective adjuvant for influenza peptides and might also be useful as an adjuvant with other antigens.

In **Chapter 5** the influence of the lipid composition on liposome size, surface charge and liposome-induced dendritic cell maturation was studied by using a design of experiments (DoE) approach. Liposome size and surface charge could be modeled and accurately predicted. The surface charge of the liposome was mostly influenced by the inclusion of DOTAP, a cationic lipid, in the lipid composition. The other cationic lipid included in the study, DC-Chol, had much less influence on the liposomal surface charge. Aside from physicochemical characteristics of the liposome, the ability to mature dendritic cells (DCs) by the liposomes was investigated. CD40, CD80, CD83 and CD86 maturation responses could be modeled as a function of lipid composition. The DOTAP lipid positively affected CD40 and CD80 expression, while the other lipids did not influence the expression of these maturation factors. CD83 expression was controlled by the presence of DC-Chol and DOTAP, while DOPE negatively affected CD83 expression. None of the lipids had a significant impact on the CD86 expression. The prediction models for all four maturation markers were able to accurately predict the DC maturation responses induced by a liposome composed of a previously untested lipid composition. Liposome size and surface charge as function of lipid composition also could be accurately predicted. This method could therefore be a valuable tool to rapidly screen the immunogenicity of various liposomal formulations *in vitro*, using a minimal number of experiments with the DoE approach.

The current state of the development of T cell-based influenza vaccines is reviewed in **Chapter 6**. In contrast to antibody responses, influenza-specific T cell responses have the potential to be cross-reactive against many epidemic and pandemic influenza strains. Many different concepts are currently under development, such as whole virion-based vaccines (live-attenuated and whole inactivated influenza vaccines), and small subunit antigens such as short or long influenza peptides. However, certain aspects of T cell responses have yet to be elucidated. The priming status of the vaccinated individual seems to be of great importance for the induction of T cells; naïve individuals generate better cellular responses than individuals who are already primed with antibody-inducing influenza vaccines. Moreover, the location of the cellular response is important; local T cell responses at the site of infection (i.e., the lungs) are more effective than systemic T cell responses. Furthermore, there are some concerns about the induction of cellular responses, because they can lead to severe lung inflammation and pneumonia. Therefore, the induction of cellular responses by vaccines should be adequate but not excessive. Furthermore, it should be noted that T cell responses do not

provide protection against influenza infection, but merely accelerate viral clearance and decrease disease morbidity after infection. Thus, T cell-based influenza vaccines have great potential, but special attention should be given during their development to the immunological aspects of such vaccines.

Chapter 7 describes the development of an alternative delivery method for influenza vaccines, the Bioneedle. Bioneedles were successfully filled and freeze-dried with four types of inactivated influenza antigens (WIV, virosome, split and subunit), while maintaining vaccine antigenicity. The immunogenicity of the influenza antigen-filled Bioneedles was assessed in C57BL/6 mice and compared to that of intramuscular and subcutaneous administered influenza vaccines. Bioneedle-delivered vaccines induced high HI and IgG titers, comparable to i.m. administered vaccines. Bioneedle-delivered virosome vaccine performed even better than s.c. administered virosome vaccine, which indicates a beneficial effect of Bioneedle delivery. It was also found that, in line with previous literature, WIV was able to induce influenza-specific T cell responses, contrarily to the other vaccine formulations. An accelerated stability study showed that vaccine-filled Bioneedles remained antigenicity after exposure to 60°C for one month, indicating that Bioneedles are superior to liquid vaccines under harsh environmental conditions, which can be beneficial in developing countries.

PERSPECTIVES

Peptide-based influenza vaccines

Peptide antigens are an interesting group of antigens for influenza vaccines. Compared to traditional protein antigens, they are relatively small, which comes with an inherent poor immunogenicity. However, peptide antigens come with several benefits. They encompass one or several epitopes, which can be arranged at will for the most optimal immune response. This gives researchers the opportunity to design the ideal peptide antigen, which can induce both humoral or cellular immune responses. This is especially useful for T cell-inducing influenza vaccines, since priming with antibody-inducing influenza vaccines can even affect the efficacy of subsequent cellular-based vaccines, as demonstrated with LAIV in children and adults [1]. Furthermore, peptide antigens can be produced synthetically, while most other antigens are produced on biological platforms. Synthetic-based vaccines can be changed more easily from a production point of view than vaccine produced on biological platforms such as cell lines. This offers more flexibility in terms of altering the epitopes to be included in the peptide vaccine.

Selection of the epitope(s) to be included in the peptide is arguably the most crucial for the effectiveness of the vaccine, but remains a tricky business. While many epitope discovery strategies exist, e.g., *in silico* prediction, mass spectrometry-based discovery and other methods [2-4], not a single immunological parameter, such as epitope immunodominance, is yet correlated to protective T cell epitopes [5]. Indeed, this was recently demonstrated by Keskin et al., who concluded that the $M1_{58-66}$ influenza peptide, which was the peptide of choice in this current thesis because of its immunodominance, induced T cell responses with such a poor avidity that the T cells did not recognize influenza infected epithelium [6]. With this knowledge, careful consideration should be given to pursue further optimization of epitopes, such as increasing epitope binding properties [7].

Nonetheless, peptide-based influenza vaccines remain promising. Concepts that use long peptides comprised of multiple influenza epitopes have recently seen considerable success, with Multimeric-001, Flu-v and FP-01.1 passing phase I studies [8-10]. The efficacy of these vaccines however has not been evaluated yet in humans, and phase II and III studies with these vaccines are thus highly anticipated.

Short peptide antigens still remain in the preclinical phase of development as of yet. Due to their poor intrinsic immunogenicity, short peptides need additional formulation with drug delivery systems and/or other adjuvants to be effective. In this thesis, three types/categories of formulations for short peptide antigens have been studied. In the following sections, the prospects for each of these formulation types will be briefly discussed.

Influenza virosomes as peptide carriers

Virosomes were originally developed as an inactivated influenza vaccine [11]. While subunit and split influenza vaccines dominate the market, they are unable to induce a cellular response, which

greatly reduces their cross-reactivity. Virosomes are able to induce both humoral and cellular responses, similar to WIV formulations. Virosomes were initially marketed by Berna Biotech as a seasonal influenza vaccine in Europe since 1997 [12], but have recently been discontinued for reasons yet to be disclosed.

Next to the use as an antigen, influenza virosomes have been also identified as a possible vaccine delivery system [13]. Virosomes have been extensively studied as a carrier for malaria peptide vaccines [14]. Furthermore, hepatitis C peptides were tested in combination with influenza virosomes [15]. Next to a publication of Arkema et al. [16], this thesis contains the only other study utilizing influenza virosomes as a delivery system for influenza T cell peptides [17]. The formulation was able to decrease disease morbidity and accelerate recovery in mice. However, the low encapsulation efficiencies and extensive formulation procedures may make the virosome concept less attractive for the delivery of influenza peptide antigens. Especially the inclusion of several peptide antigens, which is necessary to ensure proper HLA coverage [18], could prove to be too challenging. Thus, while the preclinical performance of peptide-loaded virosomes is promising, it is unlikely that this concept will be a vaccine formulation viable for human use.

WIV as a vaccine adjuvant

Whole inactivated influenza vaccine was the first influenza vaccine formulation that entered the market in the 1940s [19]. WIV was eventually replaced by split, subunit and virosome vaccines, because the use of WIV was associated with a higher incidence of adverse effects. However, improvements in the production and purification processes have made WIV equally safe as split vaccines [20]. The immunogenicity of WIV is however still superior to that of other inactivated influenza vaccines, most likely because it contains the viral ssRNA, which is a natural TLR7 ligand [21, 22]. This inherent immunogenicity of WIV was the rationale behind the study using WIV as an adjuvant for peptide antigens, as described in this thesis. WIV proved to be an effective adjuvant for most of the tested influenza epitopes, both for immunodominant and less dominant epitopes. It is likely that WIV can increase the immunogenicity of non-influenza antigens as well, for both humoral and cellular responses. WIV should therefore be combined with other vaccine antigens in future studies to investigate its effect on non-influenza antigens.

The ease of formulation (simple mixing) of WIV makes it an attractive adjuvant. Formulations such as virosomes and liposomes are more sophisticated, but require formulation steps that are sometimes difficult to scale up for industrial scale production. Simple mixing of adjuvant and antigen means that both components can be produced separately. Furthermore, the components can be mixed just moments prior to administration, which is useful when adjuvant and antigen combined are only temporarily stable. It thus might be feasible to use WIV as both an antigen and an adjuvant in a combination vaccine.

Liposomes as peptide carriers

Liposomes have been used extensively as delivery systems for various pharmaceuticals and biologics, including vaccine antigens. How liposomes affect the immune system themselves is however unclear. Liposome formulations that include PAMPs in the lipid layer, such as CAF01, clearly serve as an immunopotentiator. However, studies have shown that cationic liposomes induce DC maturation without the inclusion of PAMPs or other immunostimulatory molecules [23, 24]. In this thesis, we attempted to evaluate and model the effect of the liposomal lipid composition on the expression of DC surface markers. While the mechanism(s) behind this immunostimulatory effect of cationic lipids on DCs were not investigated, this study confirmed that cationic liposomes can activate DCs on their own, and that the DC maturation responses can be predicted when the lipid composition is known. The method described in this thesis could be an excellent tool to extensively screen liposomal formulations *in vitro* with minimal formulation efforts.

Influenza Bioneedles

Many alternative administration methods for influenza vaccines have been developed and studied over the years [25]. One of these alternatives are Bioneedles, which are hollow injectable implants which can be filled with a vaccine formulation. Bioneedles have been tested with numerous antigens, including alum-adjuvanted tetanus toxoid [26], LpxL1-adjuvanted hepatitis B surface antigen [27], CAF01-adjuvanted BCG and inactivated polio vaccine [28, 29]. In this thesis, Bioneedles were made with four influenza vaccine types [30]. Influenza vaccine-filled Bioneedles proved to be remarkably thermostable (minimal loss of antigenicity after expose to 60°C for 1 month). This could be especially useful in developing countries, where the logistics for vaccine refrigeration are often unreliable, hampering the distribution of vaccines. Furthermore, if an appropriate implantation device is developed, Bioneedles can be quickly administered by untrained personnel, which is useful in mass vaccination campaigns. Bioneedles are therefore a promising alternative to conventional needle-based vaccination, and further development of this system should be pursued.

Universal influenza vaccines

T cell-inducing influenza vaccines are just one of the concepts that are currently in development to reach the ultimate goal: a universal influenza vaccine, which protects against all influenza strains with a limited number of immunizations. Other concepts involve induction of mucosal IgA at the lungs or cross-reactive antibodies directed against HA stalk regions or M2e protein. While all concepts are promising, it is unlikely they will result in a truly universal influenza vaccine in the next 5 to 10 years. However, existing seasonal vaccines might be supplemented with additional antigens that induce cross-reactive T cells or antibodies to expand the breadth of the induced immune response. Indeed, already two different T cell-inducing vaccines have been combined with seasonal influenza vaccines in preclinical and clinical studies [31-33]. Such combinations of seasonal and T cell vaccines have the potential to be the next generation of influenza vaccines.

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