Towards improved and broadly protective influenza vaccines
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Chapter 7

Thesis discussion and perspectives
BACKGROUND

Influenza is one of the major respiratory diseases with a very high disease burden. Vaccination is the cornerstone for influenza control. Annually people are vaccinated with influenza vaccines made to protect against circulating strains of that particular season. There are many shortcomings of current seasonal influenza vaccines as discussed in detail in the introduction section of this thesis. The major problem is that the seasonal vaccines confer only strain-specific immunity by eliciting neutralizing antibodies against hemagglutinin (HA) of the vaccine strain. Accordingly, these seasonal vaccines fail to protect against antigenically drifted and shifted influenza virus strains which can be of pandemic potential. Since 1900, mankind has faced 4 major pandemics which led to havoc due to lack of broadly protective influenza vaccines. There is an urgent need for a universal or broadly protective influenza vaccine.

Preclinical vaccine evaluation of influenza vaccines is mainly done in mice or ferrets, both of which have their advantages and disadvantages. Hence influenza vaccine needs to be done in another small animal model. Much research effort is put into the field of influenza vaccine development to make better vaccines overcoming the shortcomings of the current vaccines. There are many new vaccine candidates in the pipeline which are in various stages of development. Despite all the efforts, we still don’t have an “ideal” influenza vaccine which would be 1) more immunogenic, universal/ broadly protective 2) stable, in different physiological conditions so that the immunogenicity and in turn the efficacy is not affected 3) safe to administer with low or no side effects. An ideal vaccine would also provide long term protection. Where are we going wrong? What needs to be improved in the ongoing research? Work put together in this thesis describes use of different vaccination strategies, delivery systems and immune mechanisms to achieve broad protection. It also describes suitability of cotton rats as a model for influenza vaccine evaluation and also suitability of this model for pulmonary vaccine delivery.

Vaccination strategies and delivery systems

A universal influenza vaccine is like the ‘holy grail’ for influenza vaccine research. Many different vaccination strategies are being tried and tested to improve cross-protection. In chapter 2, we combined WIV with four different adjuvants and clearly saw that the cross-reactive immune responses induced by adjuvanted WIV were significantly higher compared to non-adjuvanted WIV. Also, adjuvanted WIV conferred better cross-protection compared to non-adjuvanted WIV. It is known that sequential infection with influenza viruses can lead to cross-protection. However, less is known about whether sequential vaccination with different influenza vaccines can lead to cross-protection.

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One approach being employed is sequential vaccination with antigenically different strains to achieve cross-protection. In chapter 3, we describe that sequential vaccination with antigenically distinct influenza vaccines is a promising method of achieving cross-protection in mice. Another approach is use of virosomes as delivery vehicle for vaccine antigens as described in chapter 4. We combined virosomes with conserved NP of influenza and the adjuvant MPLA to improve activation of APCs and cross-presentation to achieve cross-reactive CD8 T cell immune responses for broad-protection upon live virus challenge. Targeting conserved proteins of influenza (HA stalk, NP, M1, M2e) is a promising approach to achieve broad-protection as these proteins can lead to cross-reactive antibodies and cytotoxic T cell responses which can in turn induce cross-protection.

Mechanisms of cross-protection and harnessing pre-existing immunity

It has been shown that both cross-reactive T cells and B cells are involved in cross-protection. In chapter 2 and chapter 3, we therefore investigated which immune mechanisms were critical for protection. We found that upon intranasal vaccination with (low dose) WIV combined with mucosal adjuvants, serum antibodies and CD4 T cells were crucial for optimal protection against heterosubtypic virus challenge. In contrast, upon sequential i.m. vaccination with (high dose) WIV, serum antibodies and memory CD8 T cells were involved in cross-protection. In both studies, we found that serum antibodies although not able to cross-neutralize the virus as measured by a classic neutralization assay, still protected naive animals upon adoptive transfer. This indicates the role of non-neutralizing antibodies which can work via mechanisms such as ADCC or ADCP. It has been shown that ADCC involves cells such as NK cells, monocytes/macrophages and neutrophils that bear the FcγRIIIa receptor and engage Fc receptors of the antibodies usually IgG1 and IgG3 in humans. This cross-linking of FcγRIIIa receptor leads to release of granzymes and perforins which in turn leads to DNA fragmentation and apoptosis of the target cells. HA stem specific antibodies employ ADCC function for the induction of cross-protection. In clinical studies, subjects with high ADCC titers before challenge, showed less viral load and reduced clinical symptoms. However more of such studies are needed as only three subjects had high ADCC titers. Additionally M2e based vaccines have also been shown to induce ADCC dependent cross-protection. Passive transfer of anti-M2e antibodies in humans was correlated with reduced clinical symptoms upon infection. We hypothesize that in chapter 2 and chapter 3, non-neutralizing antibodies mediated cross-protection via ADCC but further experiments are needed to confirm this hypothesis.

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is also shown in chapter 3 upon sequential vaccination with antigenically distinct WIV. Budimir et al showed that subcutaneous (systemic) vaccination with WIV led to induction of CTL's which were crucial for protection\textsuperscript{7}. Furthermore, it has been shown before that tissue resident memory CD8 T cells are essential for cross-protection\textsuperscript{24,25}. Using fluorescent microscopy, it was demonstrated that CD103\textsuperscript{+} CD8\textsuperscript{+} T cells remain attached to the walls of the large airways long after pulmonary immunization but are absent from systemically primed mice. Zens et al have also shown that upon intranasal immunization with LAIV, lung CD4\textsuperscript{+} TRM and virus-specific CD8\textsuperscript{+} TRM were generated and they conferred long term cross-protection\textsuperscript{25}. Vaccination in the upper respiratory tract of mice leads to the generation of TRMs in nasal epithelia and they can prevent the transmission of the virus to the lungs\textsuperscript{26}. Translating this to the humans and memory T cells, Purwar et al showed that a normal human lung contains abundant TRMs and they can respond to the recall antigen previously encountered via the lung mucosa\textsuperscript{29}. It would be important to target these cells through mucosal vaccination to achieve broader protection. In chapter 2, where we found that mucosally administered vaccine was superior to parenterally administered vaccine, the reason could be TRMs. We could not detect these cells in lungs due to technical problems and further studies are required to exploit this area more.

Pre-existing B and T cell immunity against influenza can influence vaccine efficacy. Most human beings have pre-existing immunity to influenza and it can really be decisive in shaping the immune system upon vaccination or subsequent infections. Pre-exposure to influenza can determine the specificity and magnitude of antibody responses to a subsequent exposure to a new strain\textsuperscript{29}. A lot of vaccine strategies are being designed to elicit broadly reactive memory B cells to induce cross-protection\textsuperscript{30}. It has been shown before that pre-existing T cell immunity can provide protection even in absence of antibodies\textsuperscript{30}. There are other studies which have shown that pre-existing T cells correlated with the reduced severity of the disease and also reduced spread of virus shed from the upper respiratory tract\textsuperscript{31–33}. It is quite crucial to understand how the performance of current vaccine candidates is influenced by pre-existing immunity to make better vaccines. Harnessing the pre-existing immunity in different populations might be a way to reach the goal of a universal or broadly protective influenza vaccine.

Use of suitable animal models for pre-clinical vaccine efficacy studies

Preclinical influenza research is mainly done in ferrets and mice\textsuperscript{34,35}. C57/BL6 and BALB/c mice are used widely for pre-clinical studies with influenza and also other infectious diseases. However, these two mouse models show differences in the susceptibility and immune responses against the invading pathogen\textsuperscript{36}. C57/BL6 mice show more Th1 skewed activity and Th1 cytokine responses, while BALB/c mice show Th2 skewed activity and Th1 cytokine responses against the invading pathogen\textsuperscript{36}. C57/BL6 mice show more Th1 skewed activity and Th1 cytokine responses, while BALB/c mice show Th2 skewed activity and Th1 cytokine responses against the invading pathogen\textsuperscript{36}.
immune profile\textsuperscript{39,40}. In chapter 2 and 3 we used CB6F1 mice which are F1 off-springs of a cross between C57/BL6 and BALB/c mice. Groves et al describe that they developed CB6F1 as infection model for a number of influenza viruses by characterizing disease progression, inflammation, cell influx and viral load. They could see clinical symptoms upon H1N1 and influenza B infection, however symptoms were less prominent with current seasonal H3N2 strain even upon challenge with high virus doses\textsuperscript{41}. Anderson et al also have shown that DNA vaccination in CB6F1 mice could enhance humoral as well as cellular immune responses in these animals\textsuperscript{42}. From the previous studies and studies described in chapter 2 and 3, CB6F1 is a robust mouse model for assessing both humoral as well as cellular immune responses in influenza vaccination-challenge studies.

In chapter 5 we describe the suitability of a cotton rat model for influenza vaccine evaluation. Cotton rats have been used for influenza infection and vaccination research\textsuperscript{43–46}. However, there was information missing in the literature about clinical disease progression of influenza in vaccinated cotton rats, which we tried to address. This animal model has both pros and cons as a model. Cotton rats can be infected with clinical isolates of influenza virus; however infection conditions need to be fine-tuned depending on strain of influenza virus, age of the animals, inbred/ outbred nature of the animals etc. All animals may not behave in similar way upon infection. We observed rather a high level of variation among animals especially with respect to clinical symptoms upon infection with clinical isolate of H1N1 pdm virus. Also, handling these animals can be time-consuming and laborious at times, due to their inherent aggressive behavior. Nevertheless, for specific research question the use of cotton rats can be advantageous. In chapter 6 we investigated pulmonary delivery of liquid and powder influenza vaccines in the cotton rat model. Since pulmonary immunization in animals requires intubation, due to their bigger size cotton rats are more suitable than mice and less prone to experience mechanical damage during the intubation process. We observed that liquid and powder WIV formulations could induce protective immunity in cotton rats upon pulmonary vaccination and we are the first to report about successful pulmonary vaccine administration in cotton rat model. Hence, cotton rats can be used as a model for influenza however only for certain aspects.

**Mucosal vaccination and mucosal adjuvants**

Mucosal immunization is gaining a lot of attention as it can induce local as well as systemic immunity\textsuperscript{47–49}. Furthermore, mucosal vaccination can induce long lasting B and T cell memory\textsuperscript{50}. IgA has been shown to be more cross reactive than IgG antibodies against influenza\textsuperscript{51}. Other advantages of mucosal vaccination are that the vaccines are easy to administer, especially in people having needle phobia, and there is a low risk of current seasonal H3N2 strain even upon challenge with high virus doses\textsuperscript{52}. Anderson et al also have shown that DNA vaccination in CB6F1 mice could enhance humoral as well as cellular immune responses in these animals\textsuperscript{53}. From the previous studies and studies described in chapter 2 and 3, CB6F1 is a robust mouse model for assessing both humoral as well as cellular immune responses in influenza vaccination-challenge studies.

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cross-contamination\textsuperscript{47,52}. However to date mucosal vaccination has only been successful with live attenuated vaccines while inactivated vaccines have been suboptimal\textsuperscript{53,54}. Live attenuated vaccines pose a problem of safety, while inactivated vaccines are associated with low immunogenicity and hence a lot of research is now going on in mucosal adjuvants\textsuperscript{47}. Safe and effective adjuvants are needed for the development of mucosal vaccines. In chapter 2, we describe that WIV combined with the mucosal adjuvants CAF09, CTAA-3M2e-DD given via the i.n. route conferred better cross-protection compared to non-adjuvanted or CAF01 adjuvanted WIV given via the i.m. route. Although, mucosally adjuvanted vaccines induced high levels of local IgA, interestingly this antibody class was not crucial in inducing cross-protection as observed in a study conducted in IgA KO mice.

Certain parenteral vaccine adjuvants also work as mucosal adjuvants and many are being tested in vivo in animal models\textsuperscript{65}. Bacterial toxins such as \textit{Escherichia coli} heat labile enterotoxin (LT), cholera toxin (CT), and their derivatives were also used as mucosal adjuvants. But, LT was associated with diarrhea in adults and CT adjuvanted influenza vaccine was linked to Bell’s palsy in humans\textsuperscript{56,57}. However with more research, CTAA1DD, a safer nontoxic cholera toxin based protein adjuvant was formulated and has shown a great promise in pre-clinical studies\textsuperscript{38–42}. A number of toll like receptor agonists such as Muramyldipeptide, PolyIC, MPL, flagellin, and CpG have been shown to work as mucosal adjuvants\textsuperscript{43–46}. Apart from these other mucosal adjuvants could be nonmicrobial-derived products, including liposomes, oil emulsions, and several kinds of nanoparticles\textsuperscript{66}. Taken together adjuvants studied in chapter 2 and other mucosal adjuvants mentioned show promising results and need to be investigated further.

Pulmonary immunization is as an attractive alternative to intranasal immunization due the large surface area of the lungs which contains a large number of antigen presenting cells\textsuperscript{47–65}. In chapter 6, we assessed whether the site of deposition for pulmonary administered liquid or powder influenza vaccines can affect their immunogenicity and protective efficacy using the cotton rat model. We found that liquid formulations deposited mainly in the lungs, while powder formulations deposited almost exclusively in the trachea. After two pulmonary vaccinations, liquid vaccines induced significantly higher immune responses, especially mucosal immune responses, than powder formulations. However, upon live virus challenge, protection conferred by both liquid and powder vaccines in terms of reduction in breathing frequency and lung viral load were similar. Hence, the site of deposition was of minor importance for the overall protection conferred by these two vaccine formulations. Powder vaccine formulations would be preferred over liquids as they are much more stable, less prone to degradation by varying temperatures and are easy to stockpile in situations like pandemics. Also, in humans, delivery of powder to the lungs would not...
be a problem as suitable devices (Twincer, Torus) are available and are already used successfully [68,69].

**CONCLUDING REMARKS**

There is a need of an influenza vaccine which is immunogenic, stable and can induce broadly protective immunity. There are many factors as discussed above that add to the complexity of formulating the ideal influenza vaccines. Apart from them, host factors can largely affect vaccine efficacy. Studies need to be carried out in which physiological factors like age, sex, obesity, and immune-compromised status should be taken into consideration when formulating and testing new influenza vaccine candidates. With work described in this thesis we tried to address some of the problems using several approaches. However, there is still a lot of research needed in order to reach the goal of making better influenza vaccines. There are several universal vaccine candidates and vaccination strategies being tested or used by the vaccine manufacturers or research institutions independent of one another. Thus, relative effectiveness of these candidate vaccines or approaches compared to the other approaches remains elusive. Comparative approaches especially head-to-head comparison studies are much more favorable. Collaboration among researchers within and across countries would facilitate such a platform for the evaluation of their candidates under similar condition. Such collaborative studies will be much more effective and speed up the process of vaccine evaluation.
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