Cross-protection by conventional influenza vaccines
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Summarizing Discussion
Every year influenza viruses cause significant morbidity and mortality. Next to the seasonal influenza viruses, highly pathogenic zoonotic influenza viruses to which there is no herd immunity, pose a great threat. To date, the most effective way to prevent influenza infection is via vaccination. Considerable research is being undertaken to develop influenza vaccines that can elicit cross-reactive immune responses that broadly protect against both heterosubtypic influenza A and B viruses. While some of these vaccine candidates are being tested in clinical trials, the majority are still in early stage development, and it will be several years before they could conceivably reach the market. Contrary to long-held beliefs, currently available trivalent seasonal influenza vaccines (TIV) have been shown to elicit a degree of cross-reactive immunity, which appears to be at odds with the fact that they are expected to induce strain-specific protection.

In this thesis we explore the ability of trivalent virosomal seasonal influenza vaccines, TVV, to confer heterologous and heterosubtypic cross-protection. With the aim to enhance the effectiveness of available influenza vaccines until a universal influenza vaccine becomes available, we explored whether adjusting the vaccination regimen of a licensed seasonal vaccine improves its ability to confer cross-protection.

**FINDINGS PRESENTED IN THIS THESIS**

Throughout chapters 2-4 of this thesis we evaluated 3 different approaches to broaden and improve the cross-protective efficacy of TVV. In chapter 2 we demonstrated that the cross-protective efficacy of TVV increases with the number of vaccinations. After three consecutive immunizations TVV is close to fully protective against a lethal challenge with heterosubtypic H5N1 in naïve mice, as defined by survival. By passive transfer of immune serum into naïve mice, we demonstrated that cross-protective efficacy is at least in part serum mediated and correlated to the vaccine induced cross-reactive HA antibody titer. In chapter 3 we evaluated the cross-protective efficacy of TVV when given in a heterologous prime-boost regimen. We demonstrated that priming TVV with DNA encoding vaccine homologous HA improves the cross-protective efficacy of TVV against heterologous H1N1 strains. However, priming with DNA did not improve the cross-protective efficacy of TVV against heterosubtypic H5N1. In chapter 4 we co-administered TVV with an ISCOM-based adjuvant, Matrix M, and demonstrated an elevated cross-protective efficacy of TVV in mice and ferrets. After two immunizations TVV + Matrix M could induce protection or partial protection against heterosubtypic H5N1 and H7N7 in mice. Adjuvation enabled TVV to also protect ferrets against H5N1, but not H7N9.
In **chapter 5** we used the repeated vaccination approach to assess whether cross-protective immunity could also be elicited in humans. By using a human-to-mouse passive transfer challenge model we investigate the protective efficacy of the humoral immune response elicited after 1, 2 and 3 vaccinations with TVV in healthy adults. We showed that that a single vaccination with TVV significantly boosted homologous protection against H1N1 A/California/07/09. This protective efficacy was not further boosted by repeated vaccination but was maintained over the period of observation. Intriguingly, we also demonstrated that a single vaccination with TVV mediated a significant increase in heterosubtypic protection against H5N1. This protection is however, transient, irrespective of further vaccinations.

The humoral immune response against HA is thought to play an important role in serum-mediated protections. A mechanism by which broadly reactive antibodies against HA are thought to mediate cross-protection *in vivo* is ADCC. In **chapter 6** we evaluated the ability of both TVV and a pandemic influenza vaccine to induce cross-reactive ADCC-mediating antibody responses in humans. We demonstrated that a single vaccination with a high dose TVV or a pandemic influenza vaccine elicited a significant increase in cross-reactive HA-specific ADCC-mediating antibody titer. This antibody titer was strongly correlated with the broadly neutralizing antibody titer and serum-mediated cross-protection against H5N1.

In Table 1 we have summarized the results generated in chapter 2-6 on the protective efficacy of TVV when given according to various vaccination regimens in different species. Presented is the level of survival after either vaccine homologous, heterologous or heterosubtypic influenza challenge. In mice the cross-protective efficacy of TVV can be enhanced when administered according to either of the vaccination strategies evaluated, however with different efficacy and breadth. In ferrets protection against group 2 influenza viruses is less apparent. The cross-protective efficacy seen in mice after repeated vaccination is observed in humans after a single vaccination, potentially due to a boost of pre-existing immunity.

**DISTINCT REGIMENS OF TVV RESULT IN CROSS-PROTECTION WITH DIFFERENT MECHANISM OF ACTION**

With the recent finding that seasonal influenza vaccines can boost the HA stem directed antibody titer in humans much focus has been directed towards cross-reactive, HA specific immunity when studying the cross-protective efficacy of TIV. In chapter 2-4 we have assessed three different strategies which all could boost the HA specific immunity induced by TVV.
In our experiments we saw an increase in cross-protective efficacy according to either of the vaccination strategies evaluated, however with different efficacy and breadth. In ferrets protection against group 2 influenza viruses is less apparent. The cross-protective immune response was observed in mice and humans after repeated vaccinations, potentially due to a boost of pre-existing immunity.

In a repeated vaccination regimen of naive animals the immune response is likely to be boosted in direct correlation with the number of vaccinations. With no pre-existing memory B-cells, the primary immunization activates a germinal center (GC) response. Depending on the interval between consecutive vaccinations, the GC’s may still be present, and a second or third immunization will enhance the ongoing GCs, thereby increasing the antibody titer.

In our experiments we saw an increase in cross-protective efficacy according to the number of vaccinations. Though we have not been able to identify the correlate of protection the humoral response is likely to play a key role in vaccination-mediated cross-protection, as confirmed by passive transfer experiments. TIVs predominantly elicit HA-head reactive antibodies, and frequently only low titers...
of broadly neutralizing antibodies directed against the HA stem are detected(1). In fact, in naïve animals a single vaccination with a TIV alone has not been able to elicit detectable broadly neutralizing HA stem directed antibodies. Even after triple vaccination we did not detect cross-reactive antibodies in the VNA or CR9114 competition ELISA. Cross-reactive HA antibody titers were however detected in the ELISA, increasing with the number of immunizations, indicating the presence of low HA stem directed antibody titers. These cross-reactive HA antibody titers correlated with the serum mediated protection induced by TVV suggesting that a seasonal influenza vaccine can induce HA antibody mediated cross-protection.

Co-administration with adjuvants is a strategy known to boost the vaccine mediated immune response, as demonstrated by earlier studies co-administrating TIV with MF59(2). Adjuvating TVV with Matrix M demonstrated not only a significant increase in the anti-HA cross-reactive immune response but also significantly enhanced and broadened the cross-protective efficacy of TVV. While the cross-protective efficacy of TVV + MM extended to protection against heterosubtypic group 2 viruses in mice this was less apparent when assessing the cross-protective efficacy using ferrets as animal model. With the ferret model being considered a better model than mice for mimicking a human influenza infection, these results could indicate a less robust protection against group 2 influenza viruses mediated by the vaccine. However, it should be noted that the cross-protective efficacy was assessed using two different H7 viruses and a direct comparison between the two challenge and animal models should not be made. Follow up studies should more extensively map the breadth of group 2 protection mediated by TVV+ MM. However, for a universal influenza vaccine, robust cross-protection against influenza B and group 2 viruses as well as group 1 viruses is needed. CR9114 is a monoclonal Ab which targets the stem region of HA and can broadly protect against both influenza A and B viruses(3). In recent studies by Cox et al.(8) it was demonstrated that although Matrix M enhances the cross-reactive immune response elicited by TVV, no CR9114-like antibodies were induced. It has been suggested that certain adjuvants not only boost, but broaden cross-reactive antibody responses (9, 10). It would, in follow up studies, be interesting to assess whether a different adjuvant than Matrix M could further broaden the response mediated by TVV to also induce CR9114-like antibodies.

An approach specifically aiming at boosting the HA specific antibody response is the heterologous prime-boost regimen earlier described by Nabel et al.(8) in which TIV is primed with DNA encoding vaccine homologous H1 HA. While the regimen enhanced the cross-reactive T-cell response induced by TVV, no cross-reactive antibodies against H5 HA were detected in post-vaccination serum. This is in contrast with earlier published results which showed detectable
cross-neutralizing antibodies in a pseudo-particle assay. It is possible that this difference is due to the vaccine composition or type of vaccine. However, the lack of improved protection against heterosubtypic H5N1 compared to TVV alone also suggests that this strategy is not a robust approach to broadly improve the cross-protective efficacy of TIVs. To better steer the antibody response toward the conserved HA stem, one could likely improve the heterologous prime-boost regimen by having the DNA encoding a divergent HA from that in the seasonal influenza vaccine. To broaden the cross-protective efficacy not only against group 1 viruses, follow up studies could assess the effect of DNA priming with a mix of divergent influenza A, group 1 and 2, and influenza B antigens.

Cross-protection against divergent influenza viruses can be mediated via several mechanisms targeting not only the HA protein but also other influenza proteins. With TVV’s also incorporating influenza proteins such as NA, M2 and NP it is plausible that cross-reactive immune responses directed against these proteins could contribute to cross-protection mediated by TVV. Earlier studies have demonstrated that by co-administrating a TIV with NA(4) protein the cross-protective efficacy of TIV could be improved. Though the triple vaccination regimen of TVV in chapter 2 demonstrated to correlate with the vaccine mediated cross-reactive HA antibody titer, a TVV from a different season was able to confer a comparable level of cross-protection however without inducing detectable cross-reactive HA antibodies. Instead, antibodies against NP appeared to play a key role in mediating the observed cross-protection for this vaccine. NP is a highly conserved internal influenza protein known to induce strong cross-reactive T-cell responses(5). Intriguingly, recent literature has suggested that also antibodies against NP can play a role in cross-protection via Fc-mediated effector mechanisms (6, 7). This confirms the findings that not only HA specific immunity can mediate cross-protection. Interestingly, these findings suggest that the level of cross-protective efficacy as well as the mechanism of action is dependent on the vaccine composition. To fully understand the differences between the cross-protective efficacies mediated by the different TVV compositions, the mechanism of action associated with protection needs to be further elucidated. Such studies could involve serum as well as cellular depletion studies.
CAN SEASONAL INFLUENZA VACCINES MEDIATE CROSS-PROTECTION IN HUMANS AND DOES SUCH PROTECTION CORRELATE WITH THE LEVEL OF BROADLY NEUTRALIZING ANTIBodies AGAINST THE HA STEM?

The ability to induce cross-reactive antibodies against HA has been shown to be strongly dependent on the immunological memory of an individual. The pre-existing B-cell memory will shape the antibody response against a vaccine and its ability to induce cross-reactive anti-HA antibodies. In the literature it has been reported that when vaccinating with a novel antigen such as a pandemic virus, relatively higher levels of broadly neutralizing anti-HA antibodies are elicited(8). When vaccinating with antigens that have been circulating among the human population for a longer period of time, cross-reactive antibody responses are not elicited.

By vaccinating with a standard dose TVV we demonstrated a significant boost in both the vaccine homologous antibody response and the cross-reactive antibody response against heterosubtypic H5N1. Unlike observations in naïve mice, repeated vaccination did not further boost the cross-reactive antibody responses. A similar pattern was seen when assessing the cross-reactive immune response elicited by a pandemic influenza vaccine, confirming the result and supporting the concept recently presented by Andrews et. al.(9); that repeated vaccination steers the response against the immune-dominant strain specific HA head. The majority of subjects who participated in the clinical trial had low, or no pre-existing HI antibody titer against the H1N1 vaccine strain; pH1N1 A/California/07/09. In the presence of low pre-existing serum titers, vaccinating with a novel antigen preferentially re-activates cross-reactive memory B cells. By the second immunization with the same vaccine, B cells directed against strain-specific epitopes in the HA head will be preferentially activated.

Though the presence of cross-reactive HA antibodies and their ability to be induced by TIV has been established, little is known about their cross-protective efficacy. We demonstrated that the humoral immune response elicited after a single vaccination in humans conferred protection against H5N1 in mice. The protective efficacy of the humoral response was not boosted by repeated vaccination, but was instead transient. While we have not been able to identify the mechanism of protection, we did identify a positive correlation with the cross-reactive antibody titer induced. For broadly reactive antibodies bearing the ability to protect against heterosubtypic viruses, focus has been on broadly neutralizing antibodies directed against the HA stem. In vitro, these antibodies have shown to neutralize the influenza virus by blocking the conformational change
of HA, thereby preventing endosomal escape and viral propagation. When given at a high enough concentration, these antibodies mediated protection in vivo via direct neutralization. At lower or suboptimal concentrations, Fc-mediated mechanisms such as ADCC have shown to play a key role in antibody-mediated protection (10).

By vaccinating once with a high dose TVV we confirm the cross-protective efficacy observed after a single immunization with a standard dose. The serum mediated protection against H5N1 significantly correlated with both the HA stem-directed, neutralizing and ADCC-mediating antibody response. The read-out of several of the humoral assays tested for correlation were strongly correlated making it difficult to determine whether any of these parameters is actually a mechanistic correlate rather than a co-correlate of protection, and whether the assays capture distinct subsets correlated to the overall magnitude of the immune response.

We have here focused on HA stem-directed antibodies for mediating cross-protection. Cross-reactive, HI negative antibodies directed against the HA head have also been described and have recently been shown to mediate ADCC (11). As with vaccine mediated HA stem antibodies, little is known about the cross-protective efficacy of these HI-negative antibodies and the ability of vaccines to induce them. To confirm the identity of the serum component that actually mediates protection, future studies will depend on serum transfer studies, using serum depleted of certain antibody specificities.

While a large part of our study cohort gain serum-transferable cross-protective efficacy after a single vaccination which is thereafter gradually lost, other subjects were not able to transfer cross-protection at any time point throughout the vaccination regimen. Subjects who were able to mediate cross-protection prior to vaccination either retained their cross-protective efficacy, or it was gradually lost. Intriguingly, there were also subjects who did not transfer any cross-protection prior to vaccination, but gained serum transfer protective efficacy after 3 vaccinations. To understand the differences in vaccine-mediated cross-protective efficacy between these subjects, the mechanism of protection first needs to be identified. It is possible that differences in cross-protective efficacy between individuals could be explained by genetic differences in the VH1-69 germline. Many of the broadly neutralizing HA stem-directed antibodies such as CR9114 use the VH1-69 gene to encode the heavy chain variable region (12). However, these antibodies are not produced by all individuals: Pappas et al. demonstrated the requirement for 1) an allele polymorphism of VH1-69 encoding phenyl alanine at position 54 and 2) a tyrosine at position 98 in the HCDR3, for antibody development (12). Recently Avnir et al. demonstrated a
VH1-69 polymorphism dependent effect on the broadly neutralizing HA antibody repertoire induced after vaccination with an H5N1 vaccine (13). It would therefore be important to in follow up studies to map the allelic polymorphism in the VH1-69 locus of the subjects in our cohort. To further understand whether such polymorphism have had an impact on the broadly reactive HA stem directed antibody repertoire induced by TVV. Should there be a correlation between allele polymorphism and the cross-protective efficacy of the vaccine mediated antibody response, this should be considered in the context of seasonal vaccination campaign. To ensure the ability of the vaccine to mediate protection against divergent influenza viruses, patients would have to be screened for VH1-69 genotype prior to vaccination, which would be very challenging and costly to implement. At a research level, analyzing the B-cell repertoire before and after vaccination one could possibly generate a better understanding of underlying serological memory and its role in the dynamic antibody response induced after vaccination.

**FUTURE PERSPECTIVE**

While we demonstrated cross-protective efficacy against H5N1 of the humoral immune response induced by TVV in humans, such cross-protective efficacy is only likely to be possible in the setting of low pre-existing immunity against the HA antigen of the vaccine. For a future HA-based universal influenza vaccine to be fully effective in inducing broadly protective antibodies (covering both influenza A and B strains) against the HA stem, it will need to 1) overcome pre-existing memory and immune dominance of the variable HA head and 2) induce VH1-69 based broadly influenza reactive, or similar antibodies.

Immunizing with a novel HA antigen could technically overcome pre-existing memory. However, if a full length HA protein is incorporated into the design, a second immunization with the vaccine is likely to boost the HA head-directed antibody response. Novel strategies which are currently being evaluated for their ability to steer the immune response towards the HA stem epitopes, thereby circumventing pre-existing immunity involve chimeric (14) and truncated (headless) HA proteins (15). In chimeric HA proteins the conserved stem is kept constant while coupled to an “exotic” HA head domain. Consecutive vaccinations with such a construct will, however, require variants of the chimeric protein, coupling the stem domain to HA head regions of divergent origin. For headless HA proteins the vaccine construct could remain constant. For both strategies it will be important to assess whether exposure
to influenza viruses after vaccination will affect the pool of cross-reactive memory B cells.

Many of the broadly reactive HA stem-directed antibodies such as CR9114 are encoded by the VH1-69 germline. These antibodies are highly mutated, suggesting that they have undergone repeated somatic mutations. Current strategies aim to induce CR9114-like antibodies by presenting the immune system with the epitope to which the VH1-69 antibodies bind. However, this may not be the optimal strategy to select for these antibodies, since the epitope to stimulate the germline of these antibodies might look different than the epitope to which they bind after continuous somatic hypermutation. It would be worthwhile for future studies to investigate the selection of broadly influenza reactive antibodies based on the VH1-69 germline.

As mentioned earlier, cross-protection can be mediated by several mechanisms and universal influenza vaccines targeting cross-protective immune responses against more conserved influenza proteins are under investigation. It is possible that the most promising strategy for a universal influenza vaccine will be to combine these approaches, inducing cross-protective immunity which can both prevent infection and mediate clearance of infected cells. Thus, increased presence and control of NA and internal influenza proteins such as NP and M1 in seasonal influenza vaccines could therefore be an attractive approach to further enhance and standardize their protective efficacy against influenza viruses highly divergent from those in the vaccine. Finally, the challenges that lie ahead for broadly protective influenza vaccines include overcoming and understanding pre-existing immunity and inducing long-lived immunity.
REFERENCES


