Cross-reactive antibody-dependent-cellular-cytotoxicity correlates with neutralizing, HA-stem reactive antibodies and cross-protection following influenza vaccination in humans
ABSTRACT

Broadly neutralizing hemagglutinin (HA) stem directed antibodies have been identified in humans vaccinated with seasonal influenza vaccine and have been suggested to mediate in vivo protection via antibody-dependent-cellular-cytotoxicity (ADCC). Here we evaluated the ability of a virosomal seasonal and a pandemic influenza vaccine, respectively, to induce cross-neutralizing and cross-reactive ADCC responses in healthy adults, and their ability to confer heterosubtypic protection. Healthy adults were vaccinated once with a high dose virosomal trivalent seasonal influenza vaccine, or twice with a virosomal pandemic H5N1 vaccine. We demonstrate that both virosomal seasonal and pandemic influenza vaccines induce cross-neutralizing antibodies that correlate with HA-stem binding antibodies and ADCC activity. Using a human-to-mouse serum transfer and challenge model we demonstrate for the first time that such antibody titers, as induced by seasonal influenza vaccine, correlate with survival after heterosubtypic challenge and can predict protection.
INTRODUCTION

Annually influenza viruses cause significant morbidity and mortality with 5 million cases of severe illness and between 250,000 and 500,000 deaths. To date, seasonal influenza vaccines are the most effective approach to lower the impact of influenza epidemics (1). Seasonal influenza vaccines are standardized based on their hemagglutinin (HA) content and characterized by their anti-HA mediated immunogenicity (2-4). Their protective efficacy is largely determined by the vaccine mediated serum hemagglutination inhibition (HI) antibody titer (5, 6). HI mediating antibodies bind near to the receptor binding site located on the globular head of the HA molecule and prevent interactions with host cells, thereby blocking viral entry (7-10). In part due to the pressure of pre-existing immunity, influenza type A viruses undergo a high rate of genetic drift, particularly in the HA head domain (11-14). When genetic drift results in at least a 4-fold drop in the HI titer between the predicted upcoming circulating strain and the vaccine strain, the composition of the vaccine is updated (15). Next to epidemics caused by seasonal influenza viruses, highly pathogenic zoonotic influenza viruses such as avian H5N1 viruses pose a global threat of causing influenza pandemics (16, 17). The protective efficacy of current influenza vaccines is narrow, and therefore, more effective, broadly protective influenza vaccines are needed. Numerous vaccine strategies are being evaluated for the development of a broadly protective influenza vaccine (18-20).

Several HA-specific broadly neutralizing antibodies (bnAb) have been discovered in the last decade (21-25). The majority of these bnAb’s are directed against the relatively conserved stem region of the HA protein (21-23, 26). While most HA-specific neutralizing antibodies are directed towards the immunodominant globular head region stem-antibodies are found at a lower frequency (27). To date a number of vaccine strategies to elicit high antibody titers against the HA-stem (28) including headless HA and chimeric HA are being explored (29-31). However, also current influenza vaccines can elicit broadly neutralizing antibodies directed to the HA-stem (32). While seasonal influenza vaccines in general only marginally boost such antibody responses (33, 34), it has been reported that humans exposed to the lately emerged pandemic H1N1A/California/07/09 virus (H1N1pdm) developed antibody responses that were mainly directed against the HA-stem (35-37).

Besides broadly neutralizing HA antibodies, Jegaskanda et al. recently demonstrated the presence of cross-reactive HA-specific antibody-dependent cellular cytotoxicity (ADCC)-mediating antibodies in human serum (38). ADCC has been suggested to play a role in controlling influenza infection in non-human primates (39) and can play a role in HA-mediated cross-protection (32). However, the ability of seasonal influenza vaccines to elicit cross-reactive HA-specific
ADCC-mediating antibody responses is less well understood. In influenza-naïve animals, seasonal influenza vaccines have not been able to induce detectable cross-reactive HA ADCC-mediating antibody responses (40, 41), but vaccination with seasonal influenza vaccine significantly boosted pre-existing cross-reactive HA ADCC-mediating antibody titers in healthy adults (42).

Using pre- and post-vaccination samples obtained from two distinct clinical trials, we evaluated the ability of a virosomal seasonal and a pandemic influenza vaccine, respectively, to elicit cross-neutralizing and cross-reactive HA ADCC-mediating antibody responses in humans. Furthermore, using a human-to-mouse serum transfer challenge model, we also evaluated how each of these parameters correlate to serum mediated heterosubtypic protection.

MATERIAL AND METHODS

Statement of Ethics

The clinical trials were approved by the regional ethical review boards; Universitair Ziekenhuis Antwerpen Comité voor Medische Ethiek, Committee for Medical Research Ethics, Northern Norway (REK Nord) and the Norwegian Medicines Agency. Informed consent was obtained from subjects prior to inclusion in the clinical trials.

Experiments in mice were performed in accordance with Dutch legislation on animal experiments. The experiment was performed at CVI Lelystad and approved by DEC Animal Sciences Group, Wageningen UR.

Human Sera/Plasma

High dose Seasonal Influenza Vaccine

In clinical trial Inf-V-A017 (EudraCT #2012-001693-28, ClinicalTrials.gov NCT01617239 ), 25 healthy male and female adults 18-50 years of age were vaccinated intramuscularly with an experimental high dose vaccine comprising 3 doses of the licensed trivalent virosomal seasonal influenza vaccine, Inflexal® V (Berna Biotech), from season 2011/2012 (composition: H1N1 A/California/07/09, H3N2 A/Victoria/210/09, and B/Brisbane/60/08, 15 µg HA per strain)(TVV). The study was performed in between influenza seasons to reduce the risk of the enrolled subject being exposed to influenza during the trial. Subjects who had received seasonal influenza vaccination for season 2011/2012 prior to enrollment were excluded from participation.

The vaccine-mediated humoral immune response was characterized by analyzing serum samples obtained from each of the subjects prior to vaccination (pre-vaccination serum) and 4 weeks post-vaccination (post-vaccination serum).
Pandemic H5N1 Influenza Vaccine

In clinical trial EudraCT no. 2008-006940-20, ClinicalTrials.gov NCT00868218 15 healthy adult male and female adults aged 20-49 years were vaccinated intramuscularly in a prime-boost regimen at day 0 and 21 with a non-adjuvanted virosomal H5N1 A/Vietnam/1194/2004 vaccine, (30 mg HA/ immunization). The subjects had never received an H5-based vaccine prior to enrollment. The vaccine-mediated humoral immune response was measured using plasma samples collected from each subject at day 0 (unvaccinated), 21 (post-prime) and 42 (post-boost)(43).

Influenza Challenge

The ability of the humoral immune response elicited by the high-dose seasonal influenza vaccine to confer heterosubtypic protection was assessed in a human-serum-to-mouse challenge model as previously described (42). In brief, from each of the 25 subjects in the Inf-V-A017 study, 400ul serum per time point was transferred via intraperitoneal injection into an individual, six-to-eight-week-old naïve female Balb/c (H2d) mouse (Charles River, Sulzfeld, Germany)., i.e. 1 mouse per serum sample. Twenty-four hours after receiving the serum, the mice were subsequently, under anesthesia, challenged intranasally with 25xLD_{50} of H5N1 A/Hong Kong/156/97 (Central Veterinary Institute, Wageningen University). Mice were kept under specific pathogen-free conditions.

To confirm successful transfer of human serum into the naïve mice a correlation analysis between the recombinant H1 HA (rH1) A/California/07/09 antibody titer measured in the human serum and in the pre-challenge mouse serum was performed. If the titer measured in the mouse pre-challenge serum was >100-fold lower than the titer in the corresponding human serum, the transfer was considered to have failed and the respective mouse was excluded from the correlate-of-protection analysis.

To assess the validity of the influenza challenge, groups of mice (n=8) were injected with mAb CR6261 (21) (15 mg/kg in PBS i.v. 24 hours prior to challenge) or PBS as positive and negative controls respectively. Only when a statistically significant difference between the survival proportions (Fisher’s exact-test, 2-sided) induced in the negative versus positive control group was demonstrated, was the influenza challenge considered valid (data not shown).

Mice were monitored daily for symptoms of disease by measuring body weight and assessing clinical score. The mice were monitored up to 21 days or until a humane endpoint (clinical score 4) was reached. Clinical scores are defined as: 0 = no clinical signs, 1 = rough coat, 2 = rough coat, less reactive, passive during handling, 3 = rough coat, rolled up, labored breathing, passive
during handling, 4 = rough coat, rolled up, labored breathing, unresponsive.
A schematic overview of the human-serum-to-mouse challenge model and the control groups is presented in figure 1 (Figure 1).

**Subject number**

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<td>Post Vac serum</td>
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PBS control (400 µl, i.p.)
Model control (FlumAb, 15 mg/kg i.n.
200 µl PBS, i.v.)

**Figure 1. Schematic figure of human-serum-to-mouse challenge model.**

**Hemagglutination Inhibition Assay**

Serum samples from high-dose seasonal vaccine recipients were analyzed for hemagglutination inhibition (HI) titers against both H1N1 A/California/07/09 (reassortant NYMC X-181) and H5N1 A/Hong Kong/156/97 (reassortant rgPR8-H5N1) using human red blood cells according to previously described protocol, Roozendaal et al. (42). Plasma samples obtained from subjects vaccinated with pandemic influenza vaccine were analyzed for HI titers against H1N1 A/California/07/09 using turkey red blood cells according to a previously described protocol, Roos et al. (44). Confirmed positive and negative human or sheep serum was used as controls (data not shown). Each sample was tested in duplicate. The HI titer was determined as the reciprocal of the highest dilution where no hemagglutination inhibition was observed.
**Virus Neutralization Assay**

Pre- and post-vaccination serum or plasma samples from vaccinated subjects were analyzed for neutralizing antibody titers using a virus neutralization assay (VNA) as earlier described (42). Heat-inactivated serum and plasma were mixed with 112 TCID$_{50}$ of H1N1 A/California/07/09 (reassortant NYMC X-181) or 200 TCID$_{50}$ of the H5N1 A/Hong Kong/156/97 (reassortant rgPR8-H5N1) virus followed by incubation with MDCK cells. Neutralizing antibody titers were expressed as IC$_{50}$ values, calculated after 4-parameter logistic curve fit. Indicated background levels are the lowest dilutions used in the assay.

**CR9114 Competition ELISA**

Serum and plasma samples were analyzed for HA-stem binding antibodies using a CR9114 competition ELISA assay as described (29). Serum/plasma was incubated with either His-Tagged HA of A/California/07/09 or A/Hong Kong/156/97 (produced and purified in-house). Biotinylated human IgG1 CR9114 (45) was produced and purified in-house. The IC$_{50}$ values of the CR9114 competing antibody titers induced by the pandemic influenza vaccine were calculated after 4-parameter logistic curve fit. Due to the lower level of CR9114 competing antibodies induced by the seasonal influenza vaccine, it was not possible to fit a 4-parameter logistic curve and a slope OD method was used. Positive and negative controls consisted of competition with monoclonal antibodies CR9114 (45) and CR8020 (22) respectively (data not shown).

**Surrogate Antibody-Dependent Cellular Cytotoxicity (ADCC) Reporter Assay**

ADCC-mediating antibody responses were measured using a surrogate ADCC reporter assay according to previously described protocol(29).

A549 epithelial cells (ATCC CCL-185) were transfected with plasmid DNA encoding H1 A/California/07/09 HA or H5 A/Hong Kong/156/97 using Lipofectamine 2000 (Invitrogen Waltham, Massachusetts, USA) in Opti-MEM (Invitrogen). Transfected cells were mixed with heat-inactivated serum or plasma sample dilutions and ADCC Bioassay effector cells gene(46)) (Promega, Madison, Wisconsin, USA) at a target-effector ratio of 1:4.5. Monoclonal antibody CR9114 (human IgG1, produced in-house) and plasma from influenza naïve macaques were used as a positive and negative controls, respectively (data not shown). Indicated background levels are the lowest dilutions used in the assay. The EC$_{50}$ values were calculated after 4-parameter logistic curve fit.
Human NK-cell Activation Assay

ADCC-mediating antibody responses were also evaluated with a human NK-cell activation assay, using a protocol similar to that described by Jegaskanda et al (38). In brief, 96-well MaxiSorp U-bottom ELISA plates (Nunc™, Thermo Scientific) were coated with 600 ng recombinant HA of H5N1 A/Hong Kong/156/97 (rH5) (in-house produced). Heat inactivated serum was added to the plate at a starting dilution of 1:10 followed by 2-fold serial dilutions. Healthy human donor PBMCs, at a density of 1x10^6 cells/well were added to the plate. Frozen PBMCs were used due to unavailability of fresh PBMCs at the volume and time needed. The PBMCs were processed from fresh buffy coats (Australian Red Cross Blood Bank) using Ficoll (GE Healthcare, Paramatta, NSW, Australia) and subsequently frozen at a concentration of 5x10^6 cells/ml. On the day of the experiment, the PBMCs were thawed, counted and re-suspended in medium. Following 5 hour incubation with anti-human CD107a-APC (BD), Golgi Stop (Becton and Dickinson) and BFA (Sigma Aldrich) Anti-human CD3 PerCP (Becton and Dickinson), anti-human CD56 PE-Cy7 (Becton and Dickinson) and 0.5 M EDTA (Invitrogen) was added. After fixation and permeabilization the cells were incubated with anti-human INFγ AF700 (Becton and Dickinson). The cells were acquired on a LSRII flow cytometer (Becton and Dickinson). The NK-cells were gated as CD3-CD56+ cells. NK-cells expressing the degranulation marker CD107a and producing INFγ were regarded as activated NK-cells.

As the NK-cell activation assay is susceptible to inter- and intra-assay variation and NK-cell activation can be donor-dependent, PBMCs from 3 buffy coats were used and the samples were re-tested in total 6 times. Due to the constraints of 1) cell number availability per donor and 2) the sample size limitation of the assay for the reason of inter-assay variation, we were not able to screen all individual serum samples for pre- and post-vaccination in one assay. We therefore pooled the serum from all 25 subjects per time point (pre- and post-vaccination). The NK-cell activation titer induced by the serum pools was determined by performing an analysis combining the results from all 6 experiments. A four-parameter logistic curve was fitted for each dilution series of antigen/antibody stimulated PBMCs. Background NK-cell activation in the absence of antigenic stimulation was set as the average percentage of CD107a and INFγ double positive cells when the PBMCs were added to plates coated with PBS alone. The NK-cell activation titer was defined as a 0.75% increase in the serum dilution of CD107a and INFγ double positive cells on the fitted curve compared with the CD107a, INFγ double positive cell background level. The 0.75% increase above background was chosen so that a titer could be calculated for all samples.
Statistical Analyses

Influenza Challenge Study
Serum-mediated protection against H5N1 A/Hong Kong/156/97 was analyzed using the same method as previously reported by Roozendaal et al. (42). In brief, mouse survival was analyzed up to day 21 using the Sign Test of the difference in survival state between each pair of mice receiving the post- and pre-vaccination serum of the same human donor, respectively. Differences in survival duration were calculated similarly but tested with the Wilcoxon signed-rank test. On the AUC bodyweights, analysis-of-variance was done with vaccination group and human donor as explanatory factors. Clinical scores were summarized per mouse as percentage of days at each score level and analyzed by logistic regression with score level, vaccination group and human donor as explanatory factors and mouse as subject factor.

Correlate of Protection Analysis
To determine a potential correlation between protection and the serum antibody titers, we made use of the Receiver-Operator Characteristic (ROC) curve methodology. The ROC curve was calculated by using the survival status of each individual mouse and the antibody titer measured in the corresponding human serum sample. A correlation between the antibody titer and survival was determined by the area under the ROC curve. Using an asymptotic Mann-Whitney test, the null hypothesis was tested for equality to 0.50 (no information or no correlation with protection).

Serological Assays
For assays using human sera or plasma, the log titers at the pre- and post-vaccination visits were compared using an analysis-of-variance model, with visit and human donor as factors. Titers at the detection limit were analyzed as censored observations.

For the human NK-cell activation assay, a mixed model was used with serum pools as fixed effects and with experiment, dilution series and residual error as random effects. The post- vaccination serum pool was compared with the pre-vaccination serum pool and the p-value was adjusted with a 6-fold Bonferroni correction (additional post-vaccination serum pools were analyzed in parallel with the post-vaccination serum samples presented here).

Linear Correlation
Correlations between vaccine-mediated antibody titers measured in different serological assays were calculated using the Spearman rank correlation model.
RESULTS

Cross-neutralizing H5N1 antibody titer induced by a high-dose seasonal influenza vaccine in humans correlates with serum mediated H5N1 protection in mice

A high-dose seasonal influenza vaccine induced a significant increase in vaccine homologous H1N1pdm HI antibody titers compared to pre-vaccination titer in human volunteers (p<0.001, Fig S1).

Antibody-mediated cross-protective capacity of the vaccine was assessed by transferring pre- and post-vaccination serum into naïve mice which were subsequently challenged with a lethal dose of H5N1 A/Hong Kong/156/97 virus. A significantly larger proportion of the mice that received post-vaccination serum survived the challenge compared to mice receiving pre-vaccination serum (p = 0.002 Figure 2A). Although mice that received post-vaccination serum developed severe symptoms of disease, they had a significantly smaller reduction in body weight loss and lower clinical scores compared to mice that received pre-vaccination serum (p=0.002 and p<0.001 respectively, Figure S2).

While no cross-reactive H5N1 HI-mediating antibodies were detected in either pre- or post-vaccination serum (Figure 2B), a significant increase in H5N1 VNA titers were detected in post-vaccination serum compared to pre-vaccination serum (p<0.001, Figure 2C). To evaluate whether the vaccine-mediated cross-neutralizing antibodies to H5N1 (A/Hong Kong/156/97) virus correlated with serum mediated heterosubtypic protection, we used the Receiver-Operator Characteristics (ROC) curve analysis method. The H5N1 VNA titers correlated significantly with survival against the H5N1 challenge (area = 0.73 and p <0.05, Figure 2D).

Vaccine mediated cross-reactive ADCC responses correlate to HA-stem directed, cross- neutralizing antibodies and heterosubtypic H5N1 protection in mice

The presence of cross-neutralizing antibodies that are not capable of inhibiting hemagglutination suggests that these antibodies, at least in part, are directed to the HA-stem region. To assess whether the high-dose seasonal influenza vaccine induced HA-stem antibodies, we measured the ability of the sera to compete with the broadly neutralizing stem-specific CR9114 mAb (45). CR9114 competing antibodies in the post-vaccination serum were significantly increased, compared to pre-vaccination titers (p<0.001, Figure 3A). A ROC analysis revealed that the CR9114-competing antibody response correlated significantly with serum-mediated survival against H5N1 (area = 0.87 and p < 0.05, Figure 3B). Correlation analysis furthermore demonstrated that the post-vaccination CR9114-competing antibody response also correlated with the post-vaccination VNA response
Cross-reactive antibody-dependent-cellular cytotoxicity following influenza vaccination in humans

Broadly neutralizing HA-stem antibodies have also been shown to mediate protection against influenza viruses in vivo via Fc-mediated mechanisms (47). In a surrogate reporter assay we measured the titer of cross-reactive ADCC against the HA of H5N1 (A/Hong Kong/156/97). The high-dose seasonal influenza vaccine

Figure 2. Cross-neutralizing H5N1 Ab titer induced by a high-dose seasonal influenza vaccine in humans correlate to serum-mediated H5N1 protection in mice. (Human) serum drawn pre- and post-vaccination (presented in grey and blue respectively) was transferred into naïve mice, followed by a lethal challenge with H5N1 A/Hong Kong/156/97. Shown are Kaplan-Meier survival curves (A). The neutralizing antibody response against H5N1 A/Hong Kong/156/97 (rgPR8-H5N1) was determined by HI assay (B) and VNA assay (C). Pre- and post-vaccination titers are presented separately, and with closed circles for mice that succumbed to challenge and open triangles for mice that survived the challenge. Group means are shown. A receiver-operator characteristic (ROC) curve was calculated to assess the correlation between survival and VNA titers (D). * = p < 0.05

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Figure 3. Vaccine-mediated cross-reactive ADCC responses correlate to HA-stem directed, cross-neutralizing antibodies and heterosubtypic H5N1 protection in mice.

CR9114-competing Ab titer were determined in pre- and post-vaccination serum (A). A receiver-operator characteristic (ROC) curve was calculated to assess the correlation between survival and CR9114 competition titers (B). Linear regression between the post-vaccination CR9114 competition titer and the VNA titer is plotted. Correlation determined with Spearman rank correlation method. (C). In a surrogate ADCC assay, the H5 HA ADCC-mediating Ab titer were determined in pre- and post-vaccination sera (D). A receiver-operator characteristic (ROC) curve was calculated to assess the correlation between survival and H5 HA ADCC-mediating Ab titer (E). Linear regression between the post-vaccination CR9114 competition titer and the H5 HA ADCC-mediating Ab titer is plotted. Correlation determined with Spearman rank correlation method. (F). H5 HA-specific activation of human NK-cells measured by CD107a and INFγ expression, mean titer with 95% confidence interval (G). In (A) and (D), pre- and post-vaccination titers are presented separately and with closed circles for mice that succumbed to challenge and open triangles for mice that survived challenge. Group means are shown. * = p < 0.05
significantly increased the cross-reactive ADCC-mediating antibody response compared to pre-vaccination responses ($p<0.001$, Figure 3D). A ROC analysis demonstrated that the ADCC-mediating antibody response also correlated with the serum-mediated survival against H5N1 virus challenge (area = 0.84 and $p<0.05$, Figure 3E). Correlation analysis furthermore demonstrated that the H5-specific ADCC-mediating antibody response correlated strongly with the CR9114-competing antibody response induced by vaccination ($p=0.001$, Figure 3F), suggesting that ADCC, at least in part, is mediated by HA-stem directed antibodies.

Next, we employed an influenza-specific NK-cell activation assay using primary human PBMC to verify the ADCC-mediating antibody responses measured in the surrogate reporter assay and measured a significant increase in NK-cell activation (CD107a and IFN-$\gamma$ positive) to rH5 HA protein in the post-vaccination compared to in the pre-vaccination serum ($p<0.05$, Figure 3G). This data further confirms the results of surrogate ADCC assay, by using primary NK cell from healthy donors.

**HA-stem directed antibodies induced by pandemic H5N1 influenza vaccine in humans correlate to vaccine-mediated cross-reactive H1 ADCC responses**

Cox et al. have previously shown that the H5N1 A/Vietnam/1194/04 virosomal vaccine administered to volunteers in our study is able to induce homologous H5N1 A/Vietnam/1194/2004 neutralizing antibodies (43, 48). Here, we measured cross-neutralizing HI and VNA antibody responses against heterosubtypic H1N1pdm induced by the pandemic H5N1 vaccine. Since, the clinical trial was conducted prior to the pandemic Influenza A(H1N1)pdm09 outbreak, subjects can be considered naïve to the virus. Indeed, while no cross-reactive HI antibody responses were detected in pre- and post-vaccination serum to the H1N1pdm virus (Figure 4A), a significant increase in neutralizing antibodies against H1N1pdm were detected after a single vaccination ($p=0.008$) compared to the pre-vaccination titer. Neutralizing antibody titers did not increase further after the second vaccination, but rather decreased in some subjects. Subsequently, also day 42 neutralizing titers were not significantly higher than the pre-vaccination neutralizing titer (Figure 4B).

In agreement with the results obtained with the seasonal influenza vaccine, the absence of cross-reactive HI titers could suggest that the cross-neutralizing antibody response is mediated by HA-stem antibodies. To evaluate the presence of HA-stem antibodies, we analyzed pre- and post-vaccination serum in the CR9114 competition ELISA. A single H5N1 vaccine administration resulted in a significant increase in CR9114-competing antibodies compared to the pre-vaccination (day
0) titer (p<0.001, Figure 4C). The CR9114-competing antibody response was not boosted by a second vaccination, but remained comparable to the levels obtained after the first vaccination and was overall significantly higher than the pre-vaccination titer (p < 0.0001). Comparable to the seasonal influenza vaccine, there was a significant correlation between the CR9114-competing antibody response and the H1N1pdm-neutralizing antibody response at day 21 (p = 0.0307, Figure 4D). Due to the larger number of samples with undetectable VNA titer at day 42, the correlation between the VNA and CR9114-competing antibody titer was not significant after the second immunization.

Figure 4. HA-stem directed antibodies induced by pandemic H5N1 influenza vaccine in humans correlate to vaccine-mediated cross-reactive H1 ADCC responses. Serum drawn at day 0, 21 and 42 (presented in grey, light green and dark green respectively) was characterized for anti-HA immunity. Neutralizing antibody response against H1N1 A/California/07/09 (NYMC X-181) was determined in a HI assay (A) and VNA assay (B). CR9114-competing Ab titers were measured in an ELISA (C). Linear regression between the CR9114 competition titer and the VNA titer at day 21. Correlation determined with Spearman rank correlation method (D). In a surrogate ADCC assay, the H1 HA ADCC-mediating Ab titers were determined (E). Linear regression between the CR9114 competition titer and the H1 HA ADCC-mediating Ab titer at day 21. Correlation determined with Spearman rank correlation method (F). Group means are shown. * = p< 0.05
A single H5N1 vaccination induced cross-reactive H1N1pdm ADCC responses that were higher than the responses in the pre-vaccination samples ($p=0.001$, Figure 4E). Similar to the CR9114-competing antibody responses, no booster effect was observed after the second vaccination, but the titer remained significantly higher than the pre-vaccination titer ($p=0.0010$). A significant correlation was demonstrated between the vaccine induced CR9114-competing antibody titers and the ADCC responses induced at day 21 ($p=0.0003$, Figure 4F). A significant correlation between the CR9114-competing antibody titers and the ADCC responses was also seen at day 42.

**DISCUSSION**

We have demonstrated the ability of seasonal and pandemic virosomal influenza vaccines to induce HA-specific, cross-reactive ADCC-mediating antibody responses in humans. These ADCC-mediating antibodies correlate with CR9114-competing HA-stem binding antibody responses and cross-neutralizing antibody response. The functional antibody titers measured in serum furthermore correlate with survival of mice following lethal H5N1 challenge after serum transfer, suggesting that besides virus neutralization, ADCC responses may play a role in the observed protection.

Neutralizing antibodies that target the conserved stem region of HA have received a lot of attention as a pathway for the generation of a universal influenza vaccine. These antibodies have been demonstrated to cross-neutralize influenza A and/or B strains in vitro by blocking the conformational change of HA, thereby preventing endosomal escape and viral propagation (49). Recent data from Di Lillo et al. demonstrated that these HA-stem antibody require, in a dose-dependent manner, FcγR interactions to mediate protection in vivo (47). As classical influenza neutralization assays predominantly assess neutralization involving the Fab region of the antibody and do not take into account Fc-mediated mechanisms, these assays cannot fully mimic the mechanisms by which HA-stem antibodies mediate protection.

Since in vitro ADCC assays have not been formally established to correlate with vaccine-mediated cross-protection, we elected not to estimate vaccine-mediated protection from cross-reactive immunogenicity but rather determine the actual level of protection. By using a novel human serum transfer and mouse challenge model we have previously demonstrated that a single dose of seasonal influenza vaccine in healthy adults was able to confer serum-mediated protection against H5N1 in mice (42). Although there was a positive correlation between survival and the cross-neutralizing antibody titers, the relationship
was not significant or predictive for survival. By vaccinating with a higher dose of seasonal influenza vaccine, the current study confirms that cross-protective efficacy occurs and we demonstrate a strong correlation between antibody titer and survival. We furthermore demonstrate a significant boost in cross-reactive antibodies as measured in VNA, CR9114 competition ELISA and ADCC assays. In the absence of detectable cross-reactive HI antibodies, it is fair to assume that the majority of neutralizing HA-specific antibodies are directed against the HA stem. This is supported by the demonstrated correlation between the cross-neutralizing antibody titer measured by VNA and HA-stem binding antibody titer measured by CR9114 competition ELISA. A stronger correlation was however demonstrated between the HA-stem binding antibodies and ADCC-mediating antibody responses. A conceivable explanation might be that not all HA-stem antibodies are neutralizing, but are able to mediate ADCC (50). Both, the VNA and ADCC-mediating antibody titers significantly correlated with the vaccine-mediated protection, and thus, could both be used as predictors of serum-mediated survival against H5N1 virus infection.

In contrast to seasonal influenza vaccines, pandemic vaccines based on HA of non-circulating strains, have been suggested to more effectively boost cross-reactive antibody responses (35). Here, we confirm these earlier findings by demonstrating that a virosomal, pandemic H5N1 vaccine boosts pre-existing cross-neutralizing H1N1pdm antibody responses. The pandemic H5N1 vaccine induced higher levels of CR9114-competing antibodies than the seasonal influenza vaccine. Interestingly, the primary response of H1N1pdm neutralizing antibodies and CR9114-competing antibodies could not be boosted with a second H5N1 immunization, which is comparable to previous findings (33, 51). These results provide further support for the concept that repeated vaccinations with the same vaccine directs the response towards strain-specific epitopes, predominantly located in the immune dominant globular head of HA (33). This is illustrated by an increase in homologous H5N1 HI antibody responses after two doses of a H5N1 vaccine as has previous been shown (43). Using pooled serum from the same pandemic vaccine trial (EudraCT no. 2008-006940-20) containing high HA-stem neutralizing antibody titers, Nachbagauer et al. demonstrated serum-mediated protection against a lethal challenge with H9N3 chimeric virus in mice (51). Besides confirming the high levels of HA-stem reactive antibodies, our results show that cross-reactive ADCC-mediating antibodies may have contributed to the observed protection against the H9N3 virus.

Cross-protection against influenza viruses has been shown to be highly complex and may involve a number of mechanisms, including immune responses
against other influenza proteins than HA. While we do not exclude the possible contribution of cross-reactive immunity against other influenza proteins, such as NA, we have in this manuscript focused on the vaccine-mediated antibody response against HA, their functionality and possible role in mediating protection.

CONCLUSION

We have demonstrated that a seasonal and pandemic influenza vaccine can induce HA cross-reactive ADCC mediating antibodies. For the first time we show that such antibody titers correlate with survival after heterosubtypic challenge and can predict protection in a passive mouse transfer model. Although the current study does not allow us to formally conclude which mechanism is involved in serum transferable protection, our results suggest that HA-stem binding antibodies play a role, likely through neutralization and/or ADCC. Further work is needed to better understand these mechanisms to facilitate development of the next generation of broadly protective influenza vaccines.
REFERENCES


Figure S1. High-dose seasonal influenza vaccine induces vaccine-homologous H1N1 HI titers in humans.
Pre- and post-vaccination serum was analyzed for neutralizing antibody response against H1N1 A/California/07/09 (NYMC X-181) in an HI assay. Group means are shown. * = p < 0.05

Figure S2. Immune human serum induced by a high-dose seasonal influenza vaccine protects mice against H5N1 challenge.
Serum drawn pre- and post-vaccination (presented as grey and blue respectively) was transferred into naïve mice followed by a lethal challenge with H5N1 A/Hong Kong/156/97. Shown are mean bodyweight change (A) and median clinical score (B). Error bars indicate 95% confidence interval (bodyweight) or interquartile range (clinical scores). * = p < 0.05