The human potential of a recombinant pandemic influenza vaccine produced in tobacco plants

Åsne Jul-Larsen, 1,6 Abdullah S. Madhun, 1 Karl A. Brokstad, 2 Emanuele Montomoli, 3 Vidadi Yusibov 4 and Rebecca J. Cox 1,5

Influenza Centre, The Gade Institute; University of Bergen; Bergen, Norway; 2 Bredegård Research Laboratory; The Gade Institute; University of Bergen; Bergen, Norway; 3 University of Siena; Institute of Hygiene; Siena, Italy; 4 Fraunhofer USA Center for Molecular Biotechnology; Delaware, USA; 5 Department of Research and Development; Haukeland University Hospital; Bergen, Norway

Keywords: influenza, vaccine, recombinant antigen, plant-based, haemagglutinin, human, B cell, T cell, HI, SRH

Abbreviations: ASC, antibody secreting cell; CHMP; The Committee for Medicinal Products for Human Use; ELISA, enzyme linked immunosorbent assay; ELISPOT, enzyme linked immuno spot assay; GMA, geometric mean area; GM-CSF, granulocyte/macrophage colony stimulating factor; GMT, geometric mean titre; HA, haemagglutinin; HI, haemagglutination inhibition; IFN, interferon; IL, interleukin; M1, matrix 1; NA, neuraminidase; NP, nucleoprotein; PBMC, peripheral blood mononuclear cell; pdmH1N1, pandemic H1N1; SRH, single radial haemolysis; TLR, toll-like receptor; TNF, tumour necrosis factor

Introduction

The recent 2009 influenza pandemic clearly highlighted the limitations of the currently used egg-based influenza vaccine production system, in terms of producing enough vaccine antigen for the global market in a limited time window. Future strategies for pandemic vaccine manufacturing should therefore focus on increasing capacity and reducing cost and time required for antigen production. Several new technologies are under investigation and recombinant expression systems for vaccine antigen production are promising. Expression of antigen in tobacco plants is a rapid and up scalable system resulting in high concentration of antigen that can be recognized by the human immune system has however been poorly explored. In 2009 we conducted a clinical trial vaccinating frontline healthcare workers with oil-in-water, (AS03) adjuvanted, low dose pdmH1N1 vaccine. In the present study we have evaluated a recombinant haemagglutinin (HA) antigen (HAC1) derived from

Rapid production of influenza vaccine antigen is an important challenge when a new pandemic occurs. Production of recombinant antigens in plants is a quick, cost effective and up scalable new strategy for influenza vaccine production. In this study, we have characterized a recombinant influenza haemagglutinin antigen (HAC1) that was derived from the 2009 pandemic H1N1 (pdmH1N1) virus and expressed in tobacco plants. Volunteers vaccinated with the 2009 pdmH1N1 oil-in-water adjuvanted vaccine provided serum and lymphocyte samples that were used to study the immunogenic properties of the HAC1 antigen in vitro. By 7 d post vaccination, the vaccine fulfilled the licensing criteria for antibody responses to the HA detected by haemagglutination inhibition and single radial hemolysis. By ELISA and ELISPOT analysis we showed that HAC1 was recognized by specific serum antibodies and antibody secreting cells, respectively. We conducted a kinetic analysis and found a peak of serum HAC1 specific antibody response between day 14 and 21 post vaccination by ELISA. We also detected elevated production of IL-2 and IFNγ and low frequencies of CDA+ T cells producing single or multiple Th1 cytokines after stimulating PBMCs (peripheral blood mononuclear cells) with the HAC1 antigen in vitro. This indicates that the antigen can interact with T cells, although confirming that an effective adjuvant would be required to improve the T-cell stimulation of plant based vaccines. We conclude that the tobacco derived recombinant HAC1 antigen is a promising vaccine candidate recognized by both B and T cells.
the 2009 pdmH1N1 virus (A/California/4/2009), produced in tobacco plants. We used serum and lymphocytes from the 2009 study to investigate the recognition of the HAC1 antigen by human pdmH1N1 specific antibodies and to investigate the ability of HAC1 to be recognized by B and T lymphocytes in vitro. As such this study is a first step in taking these new generation vaccines into human clinical trials.

Results

We have utilized in vitro immunological assays for evaluating a recombinant influenza HA protein (HAC1) as a potential human vaccine candidate. The HAC1 antigen contained amino acids 18–530 of the influenza surface glycoprotein, HA, derived from the pdmH1N1 virus A/California/4/2009 (H1N1). The antigen was expressed recombinantly in tobacco plants using plant virus vectors. Serum and lymphocytes from 23 individuals was used for the immunological assays. The individuals were healthcare workers participating in a clinical study evaluating the safety and immunogenicity of the oil-in-water adjuvanted low-dose split vacce against the pdmH1N1 virus.14

Study group demographics.

The study group consisted of 20 women and three men, reflecting the female dominance among Norwegian healthcare workers (Table 1). The volunteers were between 25 and 64 y of age (mean age 50.2 y) when vaccinated between October 22nd and 27th 2009, two to three weeks prior to the peak in pdmH1N1 activity. Eight volunteers were concurrently immunized with the 2009 seasonal vaccine and 18 subjects had a history of previous seasonal influenza vaccination. Four volunteers had experienced influenza symptoms during the past 12 mo prior to vaccination, but none of the subjects had experienced clinical symptoms of pdmH1N1. Two volunteers reported taking an anti-influenza drug (Tamiflu) prophylactically during the 20 d post vaccination period. Two volunteers reported taking prophylactic Tamiflu.2 Both reported underlying respiratory disease.

Table 1. Demographics of the enrolled volunteers

| Number of females | Age range | Mean age | Received seasonal influenza vaccine in 2009* | Received previous seasonal influenza vaccine* | Experienced influenza symptoms past year* | Received anti influenza drugs during trial* |
|-------------------|-----------|----------|---------------------------------------------|-------------------------------------------|------------------------------------------|-------------------------------------------|
| 20                | 25–64 y   | 50.2 y   | 8 (34.8%)                                   | 18 (78.3%)                                | 4 (17.4%)                                 | 2 (8.7%)                                  |

*Data presented as number of subjects reporting each variable. Both reported taking prophylactic Tamiflu. Both reported undergoing respiratory disease.

Vaccine-induced solicited adverse events. Vaccinees completed a diary card, registering any adverse events occurring during a 20 d post vaccination period and grading them in terms of severity. No serious adverse events were reported although the frequency of adverse events was high (Fig. 1). Twenty-two of the 23 vaccinees (95.6%) reported experiencing an adverse event of mild to moderate severity and no one reported any side reactions later than seven days after vaccination. Local side reactions were reported by 21 (91.3%) of the vaccinees, the most common being pain at the injection site experienced by 20 individuals (87.0%). Fourteen individuals (63.6%) experienced both systemic and local reactions after vaccination and only one individual (4.3%) reported only systemic reactions. The most common systemic reactions were headache and fatigue reported by 10 (43.5%) and 9 (39.1%) individuals, respectively. In summary the vaccine was well tolerated by the vaccinees, it had a good safety profile and no severe adverse reactions were observed.

Vaccine immunogenicity. The Committee for Medicinal Products for Human Use (CHMP) has defined a set of criteria for evaluating seasonal and pandemic influenza vaccine efficacy, defining single radial hemolysis (SRH) areas of ≥ 25 mm² and haemagglutination inhibition (HI) titres ≥ 40 as protective in humans. We investigated the level of surrogate protective antibodies to the vaccine antigen in the serum of our vaccinees according to these criteria. Three and two individuals in the study had pre-existing immunity to the vaccine antigen prior to vaccination, as measured by SRH and HI assays, respectively. This probably reflects that the volunteers were all frontline healthcare workers in the hospital. Already seven days post vaccination, protective antibody titers were observed in 14 and 12 of the 17 available serum samples by SRH and HI assays, respectively, and by 14 d post vaccination this had increased to 16 and 15, out of 16 subjects (Fig. 2A and B). At 14 d post vaccination the geometric mean area (GMA) for SRH was 35 mm² and the geometric mean HI titer (GMT) was 86. At 14 d post vaccination GMA had increased to 57 mm² and continued to increase to 66 mm² on day 21. For HI, the GMT increased to 272 at 14 d and remained stable at 21 d (GMT 277). A Spearman correlation test showed a good correlation between the HI and SRH data for each volunteer (r = 0.7315 and p < 0.0001) at day 21 post vaccination.

Figure 1. Solicited adverse events after vaccination in the enrolled volunteers. The frequency of local and systemic adverse events for volunteers included in the study. Data are based on diary cards completed by the 23 subjects in the study. *Any* refers to the report of an adverse event on one or more days after vaccination.
The longevity of the antibody response was measured by the HI assay. HI titers had fallen slightly at 3 mo post pdmH1N1 vaccination (GMT 164) and, for three individuals, titers were below the protective levels at three months. The HI titers continued to decrease by 6 mo (GMT = 109) and further by 12 mo (GMT = 25) and at these two last time points 68.4% and 38.0% of subjects still had protective HI titers (Fig. 2B and C).

Of the three subjects who were below protective HI antibody levels at three months, two were not sampled at 6 mo while one had a low but detectable HI titer (13) at 6 mo.

One subject did not reach a protective HI antibody titer after the first vaccination and was therefore given a booster dose of vaccine after the three-month blood sample. This person did, however, reach a protective SRH antibody titer both at 14 and 21 d post vaccination. This could reflect an increased sensitivity of the SRH assay in measuring low antibody responses.

Overall, the study group responded well to the vaccine as evaluated by the CHMP criteria.

The HAC1 protein was recognized by circulating IgG and IgA secreting B cells. The enzyme linked immunospot (ELISPOT) assay was used to investigate HAC1 recognition by specific antibody secreting B cells (ASCs) from 15 volunteers 7 d post vaccination and compared with the recognition of the vaccine antigen as a positive control. The number of ASCs producing each Ig class was enumerated per 100,000 peripheral blood mononuclear cells (PBMCs). We found that IgG (mean = 64.6) and IgA (mean = 32.7) but not IgM (mean = 1.2) secreting ASCs detected the HAC1 protein in vitro as shown in Figure 3. In agreement with previous findings, experiments on cells isolated from non-vaccinated individuals and on days 14 and 21 post vaccination showed very low numbers of HAC1 reactive ASCs per sample (mean numbers between 0 and 1, and no single measurement above 6 in 100,000 PBMCs) this was also similar to the results observed for the vaccine antigen (results not shown).

A non-parametric correlation test (Spearman’s test) also showed significant correlation between numbers of IgA secreting ASCs detecting HAC1 and the positive control (r = 0.6869, p = 0.0047) while the correlation for IgG secreting ASCs was not significant (r = 0.4844, p = 0.0673).

The HAC1 protein was detected by serum antibodies. The kinetics of the serum antibodies recognizing HAC1 after vaccination was investigated by Enzyme linked immunosorbent assay (ELISA) (Fig. 4). The level of serum IgG antibodies interacting with HAC1 was investigated prior to vaccination and 7, 14 and
Prior to vaccination the antibody level was low (mean = 12.6 μg/ml), it increased on day 7 post vaccination (mean = 70.8 μg/ml) and reached its peak between 14 d (mean = 157.1 μg/ml) and 21 d (mean = 158.5 μg/ml) post vaccination. The mean response then gradually declined throughout the rest of the sampling period (3 mo = 70.9 μg/ml, 6 mo = 62.0 μg/ml, 12 mo = 44.8 μg/ml).

The increase in HAC1 detecting antibodies was statistically significant at all time points compared with pre vaccination concentrations. The peak response between day 14 and 21 reflected the rapid response as measured by HI and SRH (Fig. 2A and B) and clearly showed that the HAC1 protein was well recognized by human influenza specific serum antibodies. The ELISA results further appeared to follow the same trend as the HI results although there was no statistical significant correlation of the HI and ELISA results for each volunteer according to the Spearman’s test.

The HAC1 protein induced low levels of cytokine secretion from PBMCs isolated 21 days post vaccination. The ability of HAC1 to induce cytokine secretion in pdmH1N1 specific human PBMCs in vitro was measured by multiplex cytokine assay of 10 different cytokines. PBMCs from 19 volunteers isolated 21 d post vaccination were stimulated in vitro with the HAC1 protein or vaccine antigen (positive control) or medium alone (negative control). The time post vaccination was chosen based on previous time studies of in vitro stimulation.19 From Figure 5 it is evident that, although some of the human PBMC samples secreted high levels of cytokines after in vitro stimulation with the HAC1 protein, a number of samples did not respond to the HAC1 stimulation. By statistical analysis only IL-2 (p = 0.0243) and IFNγ (p = 0.0025) were significantly higher than the negative controls (Fig. 5). This indicates that although the protein seems to be well recognized by B-cells and their secreted antibodies, there are limitations as to the ability of the HAC1 protein alone to induce cytokine stimulation in human PBMCs in vitro. In comparison, the positive control elicited significantly higher cytokine secretion for the cytokines IL-2, IFNγ, IL-5, IL-10, IL-17, IL-18, GM-CSF and TNFα (p < 0.05) but not for IL-4 and IL-12. Even though HAC1 did not significantly stimulate the secretion of all these cytokines for the study group as a whole, for each cytokine there were nearly always a few individuals with elevated secretion indicating that HAC1 has the potential to also induce these cytokines but that further immunostimulatory agents might be needed when used as a vaccine. There was no significant correlation between the results obtained with HAC1 and the vaccine antigen for IL-2 and IFNγ, this could also be due to the poor stimulation by HAC1.

We examined if the same individuals were high expressers of each cytokine after stimulation. For HAC1 stimulation there did not...
Multifunctional CD4+ T cells were stimulated by the HAC1 protein in vitro. We also investigated the ability of the HAC1 protein to stimulate intracellular cytokine production in CD4+ and CD8+ T cells in vitro. We used PBMCs isolated from 19 vaccinees at 21 d post vaccination. The time point post vaccination was chosen to allow for comparison with reports in the literature in which CD4+ T cells responses have also been measured at day 21.20,21 The samples were stimulated with HAC1, the vaccine antigen or medium alone and subsequently analyzed for CD4+ and CD8+ T cells expressing the cytokines IFNγ, IL-2 and TNFα by multiparameter flow cytometry. The number of CD4+ lymphocytes expressing each of these cytokines was significantly elevated after HAC1 stimulation compared with medium alone (IFNγ, p = 0.0012; IL-2, p < 0.0001; TNFα, p < 0.0001) (Fig. 6A). We also found a significant correlation between the samples expressing elevated levels of each cytokine in response to HAC1 and the vaccine antigen, indicating that high responders recognized HAC1 (IFNγ, r = 0.7193, p = 0.0005); IL-2, r = 0.8811, p < 0.0001; TNFα, r = 0.6702, p = 0.0017). In contrast to CD4+ T cells, we did not see activation of CD8+ T cells in response to HAC1 or the vaccine antigen at this time point (data not shown). Previously published studies have shown that AS03 adjuvanted H5N120 and H1N121 vaccines induce an increase in the frequencies of influenza-specific CD4+ Th1 cells but not CD8+ T-cell responses.

By this method we were also able to investigate the quality of the T-cell response as indicated by the frequency of multifunctional CD4+ Th1 cells, producing more than one cytokine simultaneously. This parameter has been associated with vaccine protection22,23 and is shown to be an integral part of the response to influenza vaccination in murine models and in man.24,25 We measured the frequency of CD4+ cells producing one, any two or all three of the cytokines TNFα, IL-2 and IFNγ simultaneously. We found a significant increase both in single producing (p = 0.0136, mean increased from 0.061 to 0.079%, double producing (p < 0.0001, mean increased from 0.013 to 0.036%) and triple producing (p = 0.0004, mean increased from 0.002 to 0.018%) T cells after stimulation with the HAC1 protein (Fig. 6B). There was also a good correlation in multifunctional cells stimulated by HAC1 and the vaccine antigen (single producers, r = 0.7368, p = 0.0003; double producers, r = 0.9298, p < 0.0001; triple producers, r = 0.7474, p = 0.0002).

Discussion

Expressing recombinant HA antigens in tobacco plants is a highly promising next generation vaccine production system, shown to be rapid, cost effective and up scalable.26 The HAC1 protein as well as haemagglutinin plant antigens expressed in tobacco plants have been tested as vaccine candidates in animal models showing good immune responses in mice and ferrets.13,27-30 Medicago, Canada, have shown that plant produced influenza virus-like particles (VLPs) can induce protective immunity in mice and ferrets and show promising results in a phase one clinical trial.26,31 Together these studies demonstrate the potential for plants as biofactories for influenza antigens. The present study is the first to investigate the ability of human B and T cells to recognize recombinant influenza antigens produced in tobacco plants.

During the 2009 influenza pandemic we had access to a unique set of serum and lymphocyte samples, taken at sequential time points post vaccination, from vaccinees who elicited rapid and high protective antibody titers in response to the AS03 adjuvanted pdmH1N1 vaccine.27 These samples provided a unique opportunity to test the interaction between the plant-produced
pdmH1N1 specific HA antigen and specific human immune responses. 

The HI and SRH titers of the individuals in the study-group reflected the high antibody titers and rapid protection reported for the larger study and were comparable to reports from other pdmH1N1 vaccines and other clinical trials using the AS03 adjuvant. The frequency and severity of the solicited adverse effects were also similar to other influenza vaccine trials using the AS03 adjuvant, with transient, local pain at the injection site most commonly observed and no severe adverse events reported.

We found that the HAC1 antigen was well recognized by serum antibodies and antibody secreting cells from individuals in the study-group and that it stimulated cytokine secretion in lymphocytes from the same individuals, although at lower levels than the vaccine antigen. We also showed that HAC1 was able to induce secretion of IL-2 and IFNγ to statistically significant levels by in vitro stimulation of PBMCs from the vaccinated individuals. The in vitro stimulation of multifunctional Th1-cells from pdmH1N1 vaccinated individuals showed that the HAC1 protein was recognized and induced cytokine production. An important question for future studies is if plant produced antigens can induce the maturation of such polyfunctional T cells in man, as recent experiments conducted in mice point to this.

Importantly we saw a good correlation between the results obtained using the HAC1 antigen and those obtained using the vaccine antigen both in the ELISPOT assay and by intracellular cytokine staining, yet the results obtained for the vaccine antigen were always higher than those of HAC1. The X179a vaccine antigen was used as a homologous positive control commonly used for standard evaluation of the immune response after vaccination. It is important to note that the vaccine antigen is expected to induce a better response in our assays than the purified HA monomers of HAC1. In the ELISA and ELISPOT assays antibodies against the surface protein, neuraminidase (NA), with recognition of NA and additional internal proteins such as the nucleoprotein (NP) and matrix 1 (M1) proteins, will make a substantial contribution to the recognition of the whole virus vaccine antigen. In the T cell assays, trace viral RNA in the split virus vaccine antigen could induce innate mechanisms through activation of toll-like receptor 7 (TLR7) and together with recognition of NA and additional intracellular proteins such as the nucleoprotein (NP) and matrix 1 (M1), this could result in an increased T cell response. However, using the vaccine antigen as a positive control gives an indication of the immunological difference between the two antigens in a vaccination scenario, pointing to the need for an effective adjuvant for plant-based vaccines, especially for inducing a robust T-cell response. Oil-in-water formulated, squalene based compounds such as MF59, AF02 and AS03 are promising new proprietary adjuvants for intramuscular influenza vaccines. Triggering a local damage response from the muscle cells at the injection site, MF59 has been shown to activate macrophages and attract monocytes, resulting in the stimulation of dendritic cell maturation. Other oil-in-water formulated adjuvants are likely to function in a similar manner and both AS03 and MF59 have been shown to induce CD4 T cells after vaccination in humans. Both these adjuvants have shown good potential for enhancing systemic immune responses in clinical trials both for H5 and pdmH1N1 vaccines. Another attractive adjuvant is the 3rd generation immunostimulating complex, Matrix M, that recently showed promising results in a phase one clinical trial directed against an avian influenza H5N1 virus performed by our lab. The challenge in choosing a good adjuvant for a candidate vaccine today is, however, that all clinically approved adjuvants are proprietary, and hence not necessarily available for testing with a given antigen.

The field of developing targeted adjuvant technologies is still in its infancy, and a number of novel experimental adjuvants are under pre-clinical testing, some favoring alternative routes of administration. The bacterial second messenger, c-di-GMP, has shown a good immunostimulatory potential in particular as a mucosal adjuvant. We have previously found that intranasal administration of a plant produced H5 antigen together with c-di-GMP in a mouse model resulted in high antibody titers and good cytokine and T-cell responses, further demonstrating the ability of plant-produced antigens to induce a robust response in combination with an adjuvant. The c-di-GMP adjuvant is not yet suitable for clinical studies as it has yet to be approved for use in humans but preclinical studies of this and other new generation adjuvants indicate the potential strength of new adjuvant technologies. In addition to the use of adjuvants, the vaccine response could also possibly be enhanced by combining the HAC1 protein with additional plant produced viral antigens such as antigens against NA and perhaps internal proteins such as nucleocapsid (NP) and matrix (M1) proteins.

We conclude that the HAC1 protein has shown good vaccine potential as it was recognized by both human B- and T cells in vitro and should be combined with a safe and effective adjuvant for testing in clinical trials. Our results imply that the production of antigen in plants is a good strategy for future pandemic influenza vaccine production.

**Materials and Methods**

**Study design.** Inclusion of health care workers in the main study, which was a clinical study of pandemic H1N1 (pdmH1N1) split virus, AS03 adjuvanted, vaccine at Haukeland University Hospital, was performed as described in. The study was approved by the Regional Committee for Medical Research Ethics, Western Norway (REKvest) and the Norwegian Medicines Agency and is registered in the European Clinical Trials Database (2009-016456-43), and National Institute for Health database Clinical trials.gov (NCT01003288). The exclusion criteria (a history of anaphylaxis or hypersensitivity to vaccines, an oral temperature > 38°C in the preceding 72 h, an acute respiratory infection up to 7 d prior to immunization or virologically confirmed pdmH1N1) were reviewed for each subject before administration of the vaccine and written informed consents were provided from all subjects prior to inclusion in the study. All volunteers were asked to fill in a diary card reporting any adverse effects, their seasonal influenza vaccination status and suspected or confirmed pdmH1N1 infection.
Single radial hemolysis (SRH). SRH was based on a modified reference method standardised by Schild and colleagues.18 Two different sets of SRH plates were prepared using turkey erythrocytes at final concentration 10%, to which were added 2,000 HAU/ml (Haemagglutinin Units/ml) inactivated whole virus X179a, provided by NIBSC/HPA UK. All samples were heat inactivated at 56°C for 30 min before the test, and 6 μl volumes were added to each well. Antibody titration was made in duplicate in two different assay sets. Diameters of hemolysis area for each sera tested were measured using a Transidyne Calibrating Viewer (Transidyne General Corporation). Both pre and post-vaccination sera were titrated simultaneously. A hemolysis area less than 4 mm² was considered as seronegative, between 4 and 25 mm² was considered positive but not protective, sera with areas of hemolysis equal to or higher than 25 mm² were considered seroprotected, as defined in the CHMP guidelines. Undetectable SRH areas were assigned an area of 4 mm². In each test a negative control sample and a positive control serum (sheep hyperimmune sera provided from NIBSC) were included.

Expression and preparation of recombinant antigen. HAC1 was expressed recombinantly in tobacco plants, using plant virus vectors as described previously.16–18 In brief amino acids 18-530 of the HA protein of the A/California/4/09 (H1N1) virus were cloned into a plant virus expression vector and transformed into Agrobacterium tumefaciens together with a helper plasmid. The transformed bacteria were introduced into 6-week-old Nicotiana benthamiana plants by vacuum infiltration. After seven days leaves were homogenized and extracts clarified by centrifugation, the protein was purified by immobilized metal affinity chromatography and anion exchange chromatography. The purity of the HAC1 protein was measured to > 90% by reverse-phase ultra-performance liquid chromatography (RP-UPLC).5

Enzyme linked immunospot (ELISPOT) assay. The ELISPOT assay was conducted as previously described18 with the following modifications. Ninety-six well ELISPOT plates were coated with inactivated whole virus X179a (2 μg HA/ml) or recombinant HAC1 antigen (2 μg/ml) diluted in PBS overnight at 4°C. After blocking 100,000 PBMCs were added to duplicate wells and incubated at 37°C and 5% CO₂ overnight. Secreted antibodies were detected with biotinylated goat anti-human class specific antibody [IgG (Sigma), IgA and IgM (both Southern Biotech)] for two hours at room temperature, followed by detection with extravidin peroxidase. Following development, the number of spots was counted using the ELISPOT reader (Immunoscan19) and the ImmunoSpot software (both from Cellular Technology, Ltd.).

Enzyme linked immunosorbent assay (ELISA). The ELISA assay was performed as previously described.18 In brief wells were coated with 100 μl 0.2 μg/well recombinant HAC1 protein (0.1 μg goat anti-human IgG for the standard) at 4°C overnight. Wells were blocked with 150 μl PBS with 20% NCS at room temperature for one hour. Serum samples and human IgG standard were prepared in two-fold dilutions series and 100 μl was added to each well and incubated for two hours at room temperature. IgG was detected with a biotinylated goat

Serum and lymphocytes from 23 healthcare workers were included in the present study. Volunteers were included randomly based on time of vaccination and arrival of lymphocytes in the lab.

Vaccination was performed by mixing one vial of split virus vaccine antigen suspension (strain X179a) with one vial of AS03 emulsion according to the manufacturers instructions. The vaccine was administered intramuscularly (IM) by injecting 0.5 ml of vaccine into the deltoid muscle. Blood samples were collected prior to vaccination, 7, 14, 21 d and 3, 6 and 12 mo post vaccination.

All volunteers were sampled on days 0 and 21, but due to unavailability of some volunteers on certain sampling days and a variation in sampling based on differences in vaccination days, not all volunteers were included in all assays.

Samples were collected on the indicated days except for the day seven samples (one sample collected on day six) and the day 21 samples (one sample collected on day 20 and one on day 25). Three month samples were collected three to four months post vaccination, six month samples were collected six to seven months post vaccination (one sample at six months minus eight days) and 12 mo samples were collected at 12 mo +/- six weeks.

Sample preparation. Lymphocytes were isolated by centrifugation of blood collected in cell preparation tubes (CPT) according to manufacturer’s instructions. Plasma was removed and lymphocytes were washed twice in 15 ml PBS containing 5% fetal bovine serum (FBS). Cells were reconstituted in 1 ml/CPT and lymphocytes were washed twice in 15 ml PBS containing 5% FBS.

Samples were collected on the indicated days except for the day seven samples (one sample collected on day six) and the day 21 samples (one sample collected on day 20 and one on day 25). Three month samples were collected three to four months post vaccination, six month samples were collected six to seven months post vaccination (one sample at six months minus eight days) and 12 mo samples were collected at 12 mo +/- six weeks.

Sample preparation. Lymphocytes were isolated by centrifugation of blood collected in cell preparation tubes (CPT) according to manufacturer’s instructions. Plasma was removed and lymphocytes were washed twice in 15 ml PBS containing 5% fetal bovine serum (FBS). Cells were reconstituted in 1 ml/CPT and lymphocytes were washed twice in 15 ml PBS containing 5% FBS.
anti-human IgG antibody (Sigma) for 1.5 h at room temperature and detected by extravidin-peroxydase.

Multiplex cytokine detection. We quantified the concentration of cytokines secreted in the supernatants from stimulated peripheral blood mononuclear cells (PBMCs) using Bio-plex software (Bio-Rad) according to the manufacturers instructions. In brief cells isolated on day 21 post vaccination were stimulated with 100 μl/well, 10 μg HA/ml of split virus X179a antigen or plant protein (HAC1) for 72 h before supernatants were harvested. A 10-plex kit (Biozol) was used to detect the cytokines IL-2, INF-γ, IL-4, IL-5, IL-10, IL-12, IL-13, GM-CSF, TNFα and IL-17 and plates were read with the Bio-Plex 200 reader using Bio-Plex manager 5.0 software (Biozol). Intraspecific cytokine staining and multiparameter flow cytometry. Fresh lymphocytes were incubated at 37°C and 5% CO2 overnight with 100 μg/ml Brefeldin A (BD Biosciences) to allow intracellular cytokine staining. Negative controls were incubated in lymphocyte medium alone (no antigen) and lymphocytes from pre vaccination samples incubated in medium with or without influenza antigens. Subsequently, cells were stained for CD3, CD4, CD8, IFN-γ, IL-2 and TNF-α (BD Biosciences) using the BD Cytofix/Cytoperm kit according to the manufacturer’s instructions and as described in reference. Finally, cells were re-stimulated in PBs containing 5% FCS and IL-2 (500 U/ml) to maintain the cell quality and the cell numbers. Single producers were classified based on cytokine secretion as single producers (1 cytokine), double producers (2 cytokines) and polyfunctional triple producers (simultaneously secreting IFN-γ, IL-2 and TNF-α).

Intracellular cytokine staining and multiparameter flow cytometry. Fresh lymphocytes were incubated at 37°C and 5% CO2 overnight with 100 μg/ml of HA or split virus X179a antigen (10 μg/ml HA), 2 μg/ml anti-CD28 and CD49d antibodies (PharMingen) and Golgistop (monomeric) and Golgiplug (10 μg/ml Brefeldin A) (BD Biosciences, USA) in lymphocyte medium before intracellular cytokine staining. Negative controls were incubated in lymphocyte medium alone (no antigen) and lymphocytes from pre vaccination samples incubated in medium with or without influenza antigens. Subsequently, cells were stained for CD4, CD8, IFN-γ, IL-2 and TNF-α (BD Biosciences) using the BD Cytofix/Cytoperm kit according to the manufacturer’s instructions and as described in reference. Finally, cells were re-stimulated in PBs containing 10% FCS and IL-2 (500 U/ml) to maintain the cell quality and the cell numbers. Single producers were classified based on cytokine secretion as single producers (1 cytokine), double producers (2 cytokines) and polyfunctional triple producers (simultaneously secreting IFN-γ, IL-2 and TNF-α).

Statistics. Statistical analysis were performed using the GraphPad Prism 5.0c for Mac OS X. Significant changes were determined with a paired student t test (two-sided with 95% CI) except for the ELISA results where a non-paired t test was used (two-sided with 95% CI). All correlations were determined using the Spearman test (two-sided with 95% CI).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
The project was funded by The Norwegian Research Council program, GLOBDVAC (854412) and intramurally by the Influenza Center, University of Bergen, the Norwegian Directorate of Health and the Ministry of Health and Care Services. We thank Diane Major at the National Institute for Biological Standards and Control (NIBSC), UK, for supplying the whole virus X179a antigen and GlaxoSmithKline for supplying the split virus X179a antigen. We also thank the Bergen Clinical Vaccine Consortium for help with the clinical study, and the staff at the Influenza Centre for help with laboratory analysis.

References
1. Chichester JA, Yeohe Y, Plante as detoxified vaccines for production of H5 vaccines. Vaccine 2007; 5:1648-8; PMID:17643895, http://dx.doi.org/10.1016/j.vaccine.2007.04.456.
2. Chichester JA, Hadfield LR, Yeohe Y. Using plant cells as influenza vaccine substrates. Expert Rev Vaccines 2009; 8:1-18; PMID:19345496, http://dx.doi.org/10.1586/erv.09.83.
3. Shoji Y, Chichester JA, Jones M, Manceva SD, Damon E, Pett Y, et al. Plant-based rapid production of recombinant native homotetrameric vaccine targets H1N1 and H1N1 influenza. Virus Res 2011; 7 (Suppl 1): S8-S16; PMID:21626846, http://dx.doi.org/10.1016/j.vaccine.2011.07.1458.
4. Lu, N, Trumper TM, Moseley T, Zakeri C, Gao N, Kim J. M: a mouse model for the evaluation of pathogen and immunity to influenza A (H5N1) virus isolated from humans. J Virol 1999; 73(5):3993-4001; PMID:10176933.
5. Lippay AS, Hemllman E, Salomon H, Yee H-L, Wolinetz KG. Cross-reactivities and immunogenicity of influenza A/Singapore/57(1957) vaccines against infection with A/Singapore/1(1973) in mice inoculated from humans. J Virol 1975; 73(5):3993-4001; PMID:10176933.
6. Lanier LL, Maio, M, Salzman, LA, Cheng, S-Y, et al. Phenotypic characterization and immunogenicity of influenza A/Beijing/252/2005 (H5N1) vaccine antigen. J Virol Methods 2006; 136(1-2): 71-78; PMID:16510764, http://dx.doi.org/10.1016/j.jviromet.2006.07.009.
7. Nicholson KG, Goldsack AE, Paddon A, Stephenson I, Wood J, Ypma G, et al. Safety and antigenicity of cross-adjuvanted and M929-adjuvanted influenza A(H1N1) (H5N1) vaccine in various age groups. N Engl J Med 2008; 361:226-35; PMID:18440103, http://dx.doi.org/10.1056/NEJMoa0805090.
8. Zhu FC, Wang H, Fang H-H, Yang JG, Liu XJ, Long SF, et al. A novel influenza A(H5N1) vaccine in various age groups. N Engl J Med 2008; 361:226-35; PMID:18440103, http://dx.doi.org/10.1056/NEJMoa0805090.
9. Zhu FC, Wang H, Wang J, Fang H-H, Wu J, Li X, Zhu FC, et al. Safety and immunogenicity of 2009 pandemic influenza A/H1N1 vaccines in China: a randomized, double-blind, randomized, placebo-controlled trial. Lancet 2010; 375:575-84; PMID:20348555, http://dx.doi.org/10.1016/S0140-6736(10)60152-1.
10. Greenberg ME, Lai MH, Hartel GF, Wichems CH, Gittleson C, Bennet J, et al. Response to a monovalent influenza A (H1N1) monovalent MF59-adjuvanted vaccine. N Engl J Med 2009; 360:2242-50; PMID:19745216, http://dx.doi.org/10.1056/NEJMoa0907650.
11. Malathi A, Ashkenazi PR, Simon P, Podda A, Stephenson I, Staubesand S, Nembhard JK, et al. Adjuvanted pandemic influenza A(H1N1) vaccine produced using in-laboratory grown ferrets. Vaccine 2010; 28:237-44; PMID:20494288, http://dx.doi.org/10.1016/j.vaccine.2010.01.050.
12. Step J, Chichester JA, Bi H, Moritzsch K, de la Rosa P, Goldschmidt L, et al. Plant-expressed HA as a novel influenza vaccine candidate. Vaccine 2008; 26:3930-8; PMID:18440350, http://dx.doi.org/10.1016/j.vaccine.2008.03.040.
13. Step J, Bonnafous CE, Bi H, Hamblot M, Green B, Manceva S, et al. Immunogenicity of homoglutinin from Arabidopsis. Green 2008; 2(4):229-37; PMID:18208414, http://dx.doi.org/10.1016/j.vaccine.2008.01.051.
14. Step J, Bi H, Horwich K, Ruse A, Honey A, Roy G, et al. Plant-derived hemagglutinin protects ferrets against challenge infection with the A/H5N1 virus produced in Nicotiana benthamiana plants. Vaccine 2009; 27:3447-56; PMID:19352085, http://dx.doi.org/10.1016/j.vaccine.2009.11.108.
17. Mori T, Marchant C, Kihara H, Tanaka R, Tsuda M, et al. Parenteral influenza vaccine induces long-lasting memory T cells and strong antibody responses in aged mice. Vaccine 2006; 24:5180-9. PMID: 16782683.

18. Cox RJ, Del Rio-Tsonis K, Zuckerman MA, Wood JH, Lindblad I, et al. A single immunization with highly pathogenic avian influenza vaccine protects mice against lethal challenge. Vaccine 2005; 23:2810-5. PMID: 15724357.

19. Cox RJ, Madhusuthan S, Haneke S, Sjursen H, Major D, et al. Parenteral influenza vaccine induces long-lasting memory T cells and strong antibody responses in aged mice. Vaccine 2006; 24:5180-9. PMID: 16782683.

20. Morel P, van der Meire B, Lavoie P-O, Couture MM-J, Trépanier A. Evaluation of a single dose of a humanized, inactivated, matrix-matched influenza vaccine in healthy adults during two randomized controlled trials. Clin Vaccine Immunol 2011; 18:1031-5. PMID: 21450978.

21. Roman F, Clément F, Dewé W, Walravens K, Maes C, et al. Effect of live attenuated influenza vaccine formulated with adjuvants on local blood monocyte and granulocyte chemoattractants and monocyte and granulocyte chemoattractant receptor expression in adults after vaccination: a randomized controlled trial. Clin Vaccine Immunol 2011; 18:1031-5. PMID: 21450978.

22. Seder RA, Darrah PA, Roederer M. T-cell quality in Th1 CD4+ cells and strong antibody responses in mice. Vaccine 1999; 17:555-65. PMID: 10215135.

23. Forrestal DL, Del Rio-Tsonis K, Lavoie P-O, Couture MM, et al. The potential of 3',5'-cyclic adenosine monophosphate as an immunostimulatory molecule. J Immunol 2007; 179:6039-47. PMID: 17985178.

24. Forbes EK, Sander C, Ronan EO, McShane H, Hill ME, et al. Adjuvanted H5N1 vaccine induces high frequencies of multifunctional and polyfunctional CD4 T-cell responses. J Clin Invest 2004; 113:1235-44. doi: 10.1172/JCI200417111. PMID: 15175421.

25. Mett V, Musiychuk K, Bi H, Farrance CE, Horsey A, et al. Molecular and cellular signatures of vaccine-mediated protection against Leishmania major. Nat Med 2007; 13:843-50. PMID: 17558415.

26. Madhun AS, Haaheim LR, Nøstbakken JK, Ebensen LR. Influenza virus-like particle vaccine formulated with AS03 induces strong, cross-neutralizing and polyfunctional CD4 T-cell responses. J Clin Invest 2011; 121:3157-67. doi: 10.1172/JCI45334. PMID: 21688874.

27. Coxe PJ, Del Rio-Tsonis K, Zuckerman MA, Wood JH, Lindblad I, et al. A single immunization with highly pathogenic avian influenza vaccine protects mice against lethal challenge. Vaccine 2005; 23:2810-5. PMID: 15724357.

28. Forrestal DL, Del Rio-Tsonis K, Lavoie P-O, Couture MM, et al. The potential of 3',5'-cyclic adenosine monophosphate as an immunostimulatory molecule. J Immunol 2007; 179:6039-47. PMID: 17985178.

29. Dupuis M, Dusin-Mercier L, Lafaille A, Pinet W, Chau H, McDonald DM, et al. Immunoconjugates with the adjuvant MP01 induce macrophage phagocytosis and antigen presentation. Eur J Immunol 2001; 31:4959-70. PMID: 11619501.

30. Sheehan AL, Armentano L, Hanafee W, Zambon MC, et al. Contamination of a monospecific, sheep red blood cell-based hemagglutination assay for influenza hemagglutinin. Applications for diagnosis and seroepidemiologic surveillance of influenza. Bull World Health Organ 1975; 52:43-50. PMID: 1082381.

31. Roman F, Vaman T, Gerlach B, Markendorf A, Gillard C, et al. Recognition of unglycosylated HNHA1a by TLR7-deficient mice. Proc Natl Acad Sci U S A 2005; 102:5388-93. doi: 10.1073/pnas.0503664102. PMID: 15792219.

32. Seubert A, Monaci E, Pizza M, O Brien R, et al. Molecular and cellular signatures of vaccine-mediated protection against highly pathogenic avian influenza H5N1 viruses after a single dose of influenza A H1N1v 2009 vaccine formulated with and without AS03-A: preliminary report of an observational, blinded, randomized trial. Vaccine 2010; 28:1740-5. doi: 10.1016/j.vaccine.2010.04.029. PMID: 20239797.

33. Doherty GS, Kanda T, Hosomi H, Akira S, Ogra P. Cross-priming and antigen presentation by dendritic and macrophages differentiate transplant donor dendritic cells. J Immunol 2006; 176:6084-92. PMID: 16597722.

34. Cox RJ, Madhusuthan S, Beverley PCL, et al. Multifunctional, high-level immunization with a humanized, inactivated, H5N1 vaccine protects ferrets against virus challenge. Vaccine 1996; 14:1359-66. PMID: 8798520.

35. Czajka M, Emmerich M, Bertsch G, Kuhlmann R, et al. Parenteral influenza vaccination induces a rapid and cross-reactive immunity with an adjuvanted H5N1 prototype pandemic influenza vaccine: a randomized controlled trial. Lancet 2007; 369:763-70. PMID: 17707773.

36. Bancroft DE, Edwards KM, Dekker CL, Belshe R, Perera NS, et al. Effect of adjuvants on the safety and immunogenicity of an avian influenza vaccine in adults. J Infect Dis 2008; 197:1067-70. PMID: 18526876.

37. Leirisu B, Niedobitek G, Vannier-Baselguni T, Dumitru C, Clément F, Howes E, et al. Antigens-priming and cross-reactive immunity with an adjuvanted H5N1 prototype pandemic influenza vaccine: a randomized controlled trial. Lancet 2007; 369:763-70. PMID: 17707773.

38. Stephenson IA, Regina B, Nakahrizi K, Police A, Wood JH, Zambon MC, et al. Cross-reactivity to highly pathogenic avian influenza H5N1 vaccine after vaccination with inactivated and 2009-adjuvanted influenza A(H1N1): vaccine: a randomized proportion study. J Infect Dis 2008; 199:1240-1. PMID: 18277694.

39. Leirisu B, Niedobitek G, Vannier-Baselguni T, Dumitru C, Clément F, Howes E, et al. Antigens-priming and cross-reactive immunity with an adjuvanted H5N1 prototype pandemic influenza vaccine: a randomized controlled trial. Lancet 2007; 369:763-70. PMID: 17707773.

40. Bancroft DE, Edwards KM, Dekker CL, Belshe R, Perera NS, et al. Effect of adjuvants on the safety and immunogenicity of an avian influenza vaccine in adults. J Infect Dis 2008; 197:1067-70. PMID: 18526876.

41. Bernstein DI, Edwards KM, Dekker CL, Belshe R, Perera NS, et al. Effect of adjuvants on the safety and immunogenicity of an avian influenza vaccine in adults. J Infect Dis 2008; 197:1067-70. PMID: 18526876.

42. Seder RA, Darrah PA, Roederer M. T-cell quality in Th1 CD4+ cells and strong antibody responses in mice. Vaccine 1999; 17:555-65. PMID: 10215135.

43. Forrestal DL, Del Rio-Tsonis K, Lavoie P-O, Couture MM, et al. The potential of 3',5'-cyclic adenosine monophosphate as an immunostimulatory molecule. J Immunol 2007; 179:6039-47. PMID: 17985178.

44. De Pinna S, Schad G, Finn P, Mazzarino A, Solomon T, Voller D, et al. Effect of TLR7 and TLR8 agonists as influenza vaccine adjuvants in a phase I clinical trial. Vaccine 2011; 29:6880-93. PMID: 21792722.

45. Chen W, Kuolee R, Yan H. The potential of 5',3'-cyclic monophosphate as an effective vaccine adjuvant. Vaccine 2010; 28:6129-35. PMID: 20197316. doi: 10.1016/j.vaccine.2010.04.021.

46. Schad GC, Finn P, Mazzarino A, Solomon T, Voller D, et al. Effect of TLR7 and TLR8 agonists as influenza vaccine adjuvants in a phase I clinical trial. Vaccine 2011; 29:6880-93. PMID: 21792722.

47. De Pinna S, Schad G, Finn P, Mazzarino A, Solomon T, Voller D, et al. Effect of TLR7 and TLR8 agonists as influenza vaccine adjuvants in a phase I clinical trial. Vaccine 2011; 29:6880-93. PMID: 21792722.