

# Matrix M<sup>TM</sup> adjuvanted virosomal H5N1 vaccine induces balanced Th1/Th2 CD4<sup>+</sup> T cell responses in man

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T cellular responses play a significant role in mediating protective immune responses against influenza in humans. In the current study, we evaluated the ability of a candidate virosomal H5N1 vaccine adjuvanted with Matrix M<sup>TM</sup> to induce CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in a phase 1 clinical trial. We vaccinated 60 healthy adult volunteers (at days 0 and 21) with 30 µg haemagglutinin (HA) alone or 1.5, 7.5, or 30 µg HA formulated with Matrix M<sup>TM</sup>. To evaluate the T cellular responses, lymphocytes were stimulated in vitro with homologous (A/Vietnam/1194/2004 [H5N1]) and heterologous H5N1 (A/Anhui/1/05 or A/Bar-headed Goose/Qinghai/1A/05) antigens. The antigen-specific cytokine responses were measured by intracellular cytokine staining and by multiplex (Luminex) assays. An increase in CD4<sup>+</sup> Th1 and Th2 cytokines was detected 21 days after the first vaccine dose. No increase in Th cytokine responses was observed after the second dose, although it is possible that the cytokine levels peaked earlier than sampling point at day 42. Formulation with the Matrix M<sup>TM</sup> adjuvant augmented both the homologous and cross-reactive cytokine response. Antigen-specific CD8<sup>+</sup> T cell responses were detected only in a few vaccinated individuals. The concentrations of Th1 and to a lesser extent, Th2 cytokines at 21 days post-vaccination correlated moderately with subsequent days 35 and 180 serological responses as measured by the microneutralisation, haemagglutination inhibition, and single radial hemolysis assays. Results presented here show that the virosomal H5N1 vaccine induced balanced Th1/Th2 cytokine responses and that Matrix M<sup>TM</sup> is a promising adjuvant for future development of candidate pandemic influenza vaccines.

## Introduction

Highly pathogenic avian influenza (HPAI) viruses continue to pose a significant health threat. This threat is highlighted by recent studies showing that airborne transmission of HPAI viruses could be acquired with a limited number of mutations.<sup>1,2</sup> Vaccination is the best measure to prevent influenza, but meta-analyses have highlighted the need for better vaccines.<sup>3,4</sup> Due to the poor immunogenicity of candidate influenza H5N1 vaccines, there is a need for developing more effective vaccine and adjuvant formulations.

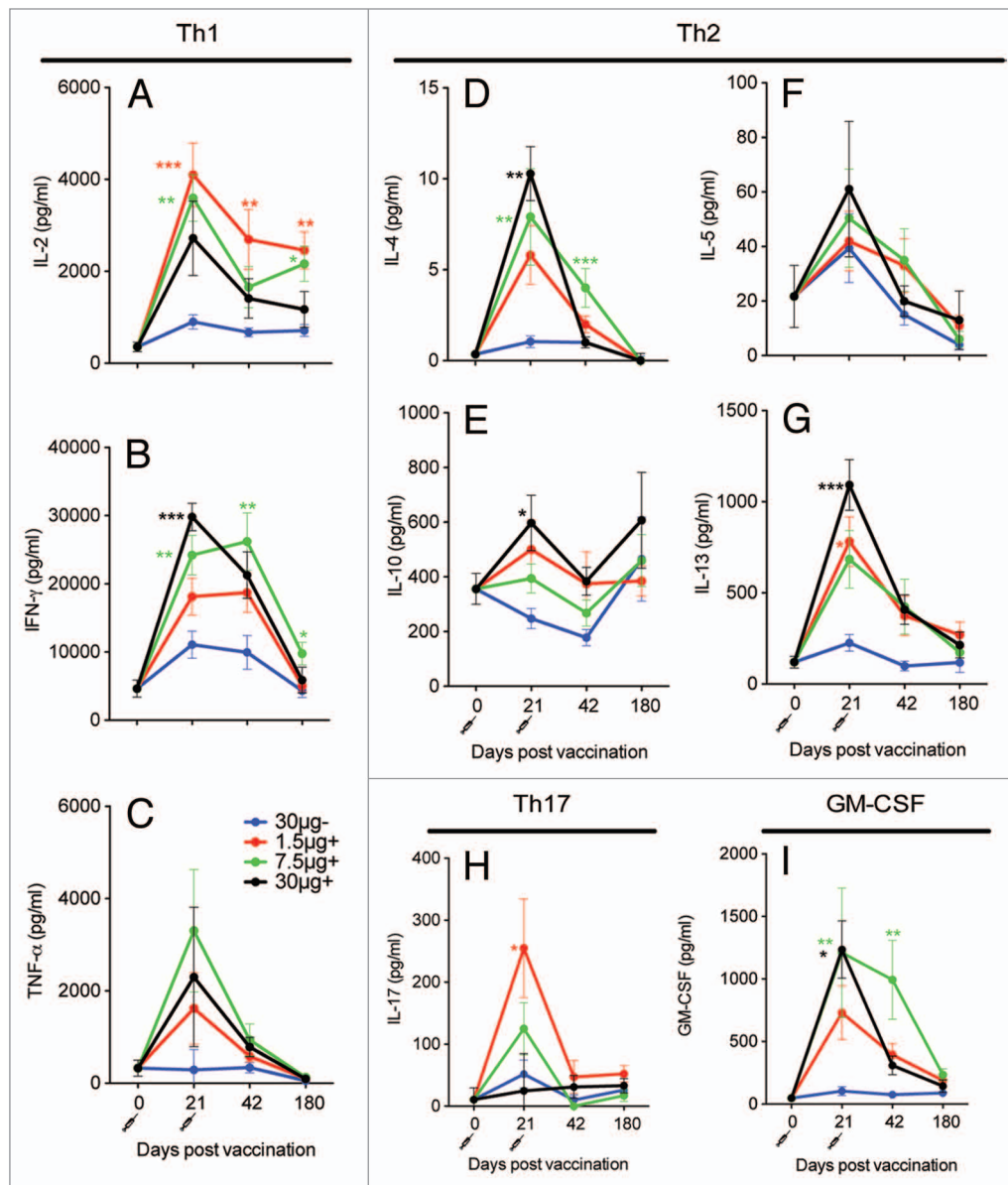
The immune response against influenza is multifaceted and both the humoral and cell-mediated arms are important. Antibody responses against the virus' HA protein are the major mediators of protection against influenza. Thus, traditionally vaccine immunogenicity is assessed by haemagglutination inhibition (HI) or microneutralisation (MN) assays that measure virus-specific antibodies. For seasonal influenza vaccines, an HI titer of 1:40 is considered a protective response according to the criteria defined by the EU Committee for Medicinal Products for

Human Use (CHMP). However, for pandemic vaccines, an HI or MN titer that correlates with protection has not been defined. There is growing evidence highlighting an important role for T cellular responses in anti-influenza immunity. In both mice and humans, viral clearance is associated with antigen-specific CD8<sup>+</sup> T cell responses<sup>5,6</sup> and CD4<sup>+</sup> T cells are required for maintaining the memory CD8<sup>+</sup> T and B cell responses.<sup>7</sup> Furthermore, recent studies have shown that pre-existing influenza-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells provide protection against heterologous strains by targeting the conserved internal proteins of the influenza virus.<sup>8</sup>

In response to antigenic exposure, naïve T cells differentiate into specific lineages, Th1, Th2, Th17, and regulatory T cells with distinct effector functions and cytokine profiles. Th1 cells have been shown to facilitate recovery from heterosubtypic influenza virus infection in mice.<sup>9,10</sup> Multifunctional Th1 cells (simultaneously producing IL-2, IFN-γ, and TNF-α) have been associated with protection against HIV,<sup>11</sup> *Leishmania Major*,<sup>12</sup> and tuberculosis.<sup>13</sup> While the importance of these cells has not yet been determined for influenza, they have been described as functionally superior to single cytokine producing CD4<sup>+</sup> T

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**Figure 1.** The Matrix M<sup>TM</sup> adjuvanted boosted the Th1 and Th2 cytokine responses. Four groups of 15 vaccinees were given 2 immunizations with 30 μg HA of H5N1 NIBRG-14 virosomal vaccine alone (30 μg-) or increasing doses of virosomal vaccine (1.5, 7.5, or 30 μg HA) in combination with Matrix M<sup>TM</sup> adjuvant (+). PBMCs were isolated at indicated time-points and stimulated for 72 h with H5N1 virosomes. The supernatant was frozen at -80 °C and later thawed for analysis of Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-5, IL-10, IL-13), and Th17 (IL-17) cytokines and GM-CSF. The mean ± SEM is shown for each vaccine group. \*, \*\*, and \*\*\* indicate significant differences ( $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively) from the 30 μg- group, One-way ANOVA with the Dunnett post hoc test.

cells.<sup>14,15</sup> Th2 cytokines IL-4, IL-5, IL-10, and IL-13 enhance B cell proliferation,<sup>16</sup> and antibody secreting cell differentiation.<sup>17</sup> In mice, an aluminum-based adjuvanted influenza vaccine boosted Th2 responses, and HI titers, but did not confer superior protection to unadjuvanted vaccine<sup>18</sup> and IL-4 treatment of influenza-infected mice inhibited anti-influenza responses.<sup>19</sup> In another murine model, IgG2a and IgG1 antibodies, as stimulated by Th1 and Th2 cells, respectively had distinct roles for anti-influenza immunity.<sup>20</sup> Collectively, these experimental models suggest that anti-influenza immunity is associated with Th1 or balanced Th1/Th2 response. However, further studies, particularly in human

clinical trials are needed to better understand precise role of T cells in anti-influenza immunity and in vaccine immunogenicity. In contrast to alum-adjuvanted influenza vaccines that induced a Th2 skewed immune response in humans, clinical trials with emulsion-based influenza H5N1 vaccines showed that more balanced or Th1 type of responses can be elicited.<sup>21,22</sup>

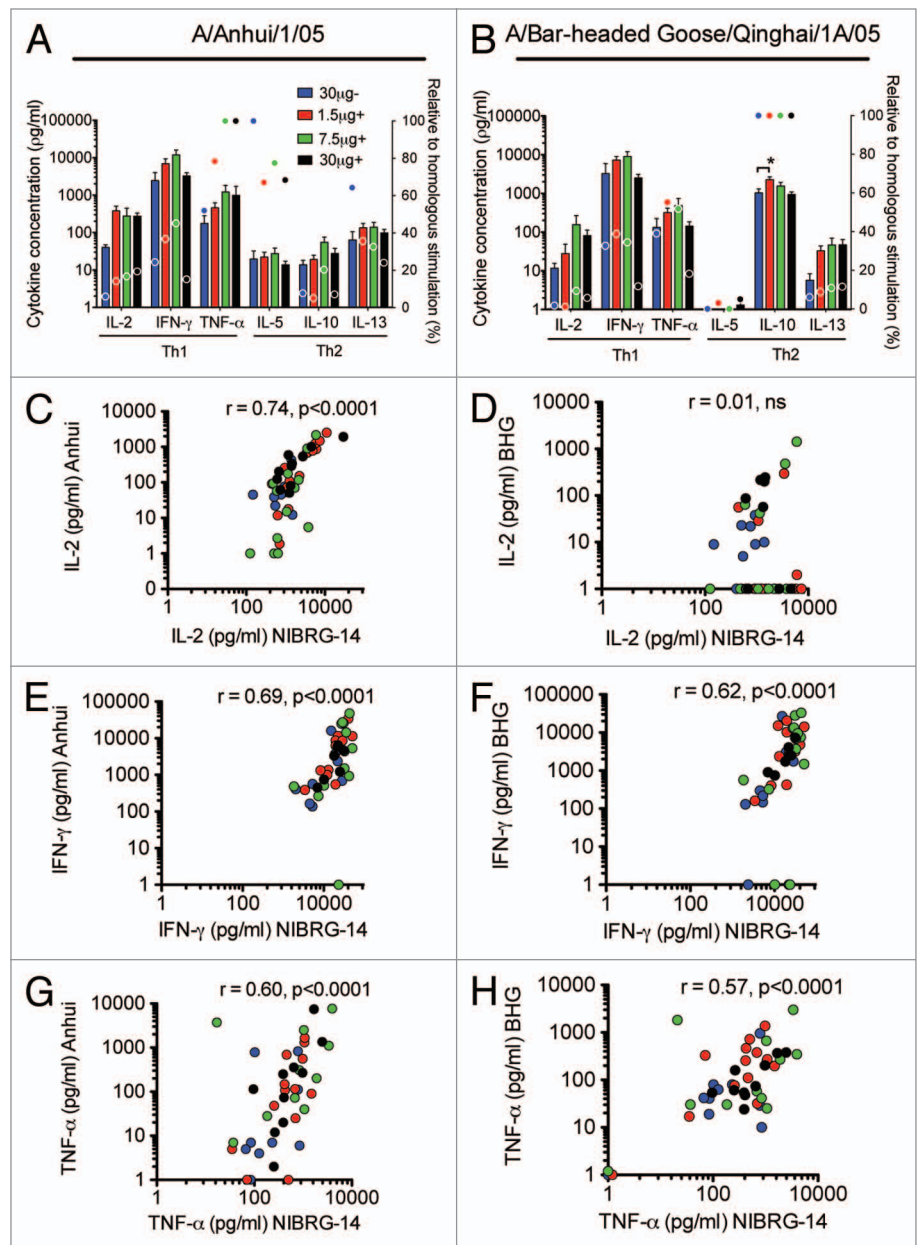
We have previously reported that a virosomal candidate H5N1 vaccine adjuvanted with Matrix M<sup>TM</sup> induced balanced Th1/Th2 responses in a mouse model<sup>23</sup> and protected the animals against HPAI H5N1 influenza challenge.<sup>14</sup> The vaccine was then tested in a phase 1 clinical trial where the HI and single

radial hemolysis (SRH) responses fulfilled all the EU CHMP immunogenicity criteria after 2 vaccine doses, even at the lowest antigen dose (1.5  $\mu\text{g}$  HA).<sup>24</sup> Notably, the early influenza-specific CD4<sup>+</sup> Th1 cell responses predicted subsequent seroprotection after the booster dose.<sup>25</sup> In the present study we investigated in detail the quality of the T cell response and found that the Matrix M<sup>TM</sup> adjuvanted virosomal vaccine induces balanced Th1/Th2 responses in humans and that CD4<sup>+</sup> T cell responses are cross-reactive toward antigenically distinct H5N1 virus clades.

## Results

### The Matrix M<sup>TM</sup> adjuvanted vaccine induces a balanced Th1/Th2 response

In this study we evaluated T cell responses after H5N1 virosomal vaccine administered alone or in combination with Matrix M<sup>TM</sup>. PBMCs were isolated pre-vaccination (day 0) and at days 21, 42, and 180 and stimulated with H5N1 NIBRG-14 virosomes. Th1 (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ), Th2 (IL-4, IL-5, IL-10, and IL-13), Th17 (IL-17) cytokines, and IL-12 and GM-CSF were measured in the supernatant. Low concentrations of all Th1 cytokines were detected pre-vaccination. At day 21 the concentrations of IFN- $\gamma$  and IL-2 were boosted in all groups, while increased TNF- $\alpha$  concentrations were only observed in the Matrix M<sup>TM</sup> adjuvanted groups (Fig. 1A–C). The highest concentrations of Th1 cytokines were detected in the Matrix M<sup>TM</sup> adjuvanted groups and significantly higher concentrations of IFN- $\gamma$  were observed in the Matrix M<sup>TM</sup> adjuvanted 30  $\mu\text{g}$  ( $P < 0.001$ ) and 7.5  $\mu\text{g}$  ( $P < 0.01$ ) groups than the virosomal alone group at day 21. Significantly higher concentrations of IL-2 were found in the 1.5  $\mu\text{g}$  ( $P < 0.001$ ) and 7.5  $\mu\text{g}$  ( $P < 0.01$ ) Matrix M<sup>TM</sup> adjuvanted groups as compared with the virosomal alone group. For the Th2 cytokines, the highest response was also generally found in the Matrix M<sup>TM</sup> adjuvanted groups (Fig. 1D–G). Low concentrations of IL-4 (mean 2–10 pg/mL) were measured, but vaccinees receiving 7.5  $\mu\text{g}$  or 30  $\mu\text{g}$  adjuvanted with



**Figure 2.** The cross-reactivity of the Th1 cytokine responses measured by Luminex and correlation with homologous responses. Four groups of 15 vaccinees were given 2 immunizations with 30  $\mu\text{g}$  HA of H5N1 NIBRG-14 virosomal vaccine alone (30  $\mu\text{g}$ -) or increasing doses of virosomal vaccine (1.5, 7.5 or 30  $\mu\text{g}$  HA) in combination with Matrix M<sup>TM</sup> adjuvant (+). PBMCs were isolated at day 42 into the study and stimulated for 72 h with influenza H5N1 antigen. The homologous response was evaluated toward the NIBRG-14 antigen, using the H5N1 virosomes. The cross-H5 clade response was measured against A/Anhui/1/05 (Anhui) and A/Bar-headed Goose/Qinghai/1A/05 (BHG). The supernatants were frozen at  $-80^{\circ}\text{C}$  and later thawed for analysis of Th1 (IL-2, IFN- $\gamma$ , TNF- $\alpha$ ) and Th2 (IL-4, IL-5, IL-10, IL-13) cytokines. (A and B) Mean cytokine concentrations  $\pm$ SEM after Anhui or BHG stimulation (bars, y-axis) and response in percentage relative to that observed for homologous stimulation as shown in Figure 1 (circles, second y-axis) are shown. \*Indicates significant difference ( $P < 0.05$ ) from the 30  $\mu\text{g}$ - group in a One-way ANOVA with Dunnet's post hoc test. (C–H) Individual IL-2, IFN- $\gamma$ , and TNF- $\alpha$  cytokine concentrations after NIBRG-14 stimulation plotted against those after Anhui or BHG stimulation as indicated on the y-axis. The correlation was tested by Spearman rank test and the correlation coefficient ( $r$ ) and  $P$  value are shown on the graphs.



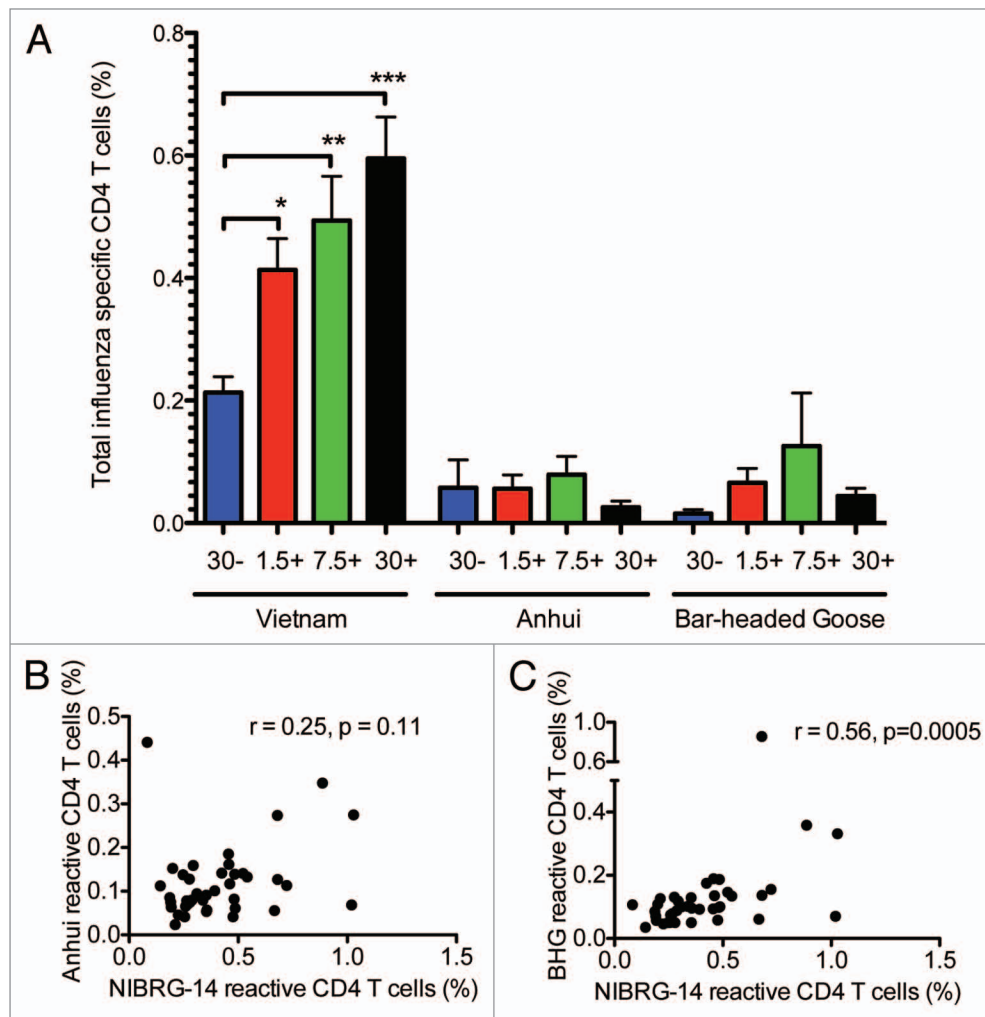
Matrix M<sup>TM</sup> had significantly higher IL-4 concentrations than those who were immunized with the virosomal vaccine alone at day 21 ( $P < 0.001$ ). Similarly, Matrix M<sup>TM</sup> significantly boosted IL-13 and IL-10 concentrations in the 30  $\mu\text{g}$  group ( $P < 0.05$ ) at day 21.

The day 42 cytokine responses were lower than at day 21. The IFN- $\gamma$  and IL-4 concentrations were significantly higher in the 7.5  $\mu\text{g}$  Matrix M<sup>TM</sup> adjuvanted group ( $P < 0.01$ ) and the IL-2 response was significantly higher in the 1.5  $\mu\text{g}$  Matrix M<sup>TM</sup> adjuvanted groups than in the virosomal vaccine alone group ( $P < 0.01$ ). The responses generally continued to decline at day 180, but the concentrations of IL-2 and IFN- $\gamma$  were still significantly higher in the Matrix M<sup>TM</sup> adjuvanted 7.5  $\mu\text{g}$  groups than in the 30  $\mu\text{g}$  virosomal vaccine alone group ( $P < 0.05$ ) at day 180. IL-17 was also found at increased levels at 21 days post-vaccination. In contrast, few vaccinees produced IL-17 at day 42. Subjects with high cytokine concentrations at day 21 generally also had the highest concentrations at days 42 and 180 (Table S1). In summary, an increase in Th1, Th2, and Th17 cytokines was observed post-vaccination and higher concentrations of Th1 and Th2 cytokines were observed in the Matrix M<sup>TM</sup> adjuvanted groups as compared with the virosomal vaccine alone group.

IL-12 mediates a Th1 response,<sup>26</sup> but in the present study, no IL-12 was observed (data not shown). The Granulocyte-macrophage colony-stimulating factor (GM-CSF) is another regulator of T cell responses,<sup>27</sup> which plays a role in protection from influenza.<sup>28</sup> Significantly higher concentrations of GM-CSF were found in the 7.5  $\mu\text{g}$  and 30  $\mu\text{g}$  HA adjuvanted groups than in the non-adjuvanted group at day 21 ( $P < 0.05$ ) (Fig. 11).

#### Cross-reactive T cell responses

Influenza viruses are undergoing constant antigenic change. Vaccines stimulating cross-reactive T-cells are therefore needed. The cross-reactivity of the cytokine response after vaccination with the H5N1 virosomal vaccine was evaluated by stimulating PBMCs with HA from A/Anhui/1/05 (clade 2.3.4) (Anhui) or A/



**Figure 3.** The Matrix M<sup>TM</sup> adjuvant boosted the frequencies of Th1 cells specific for the homologous strain, while lower frequencies of cells were cross-reactive toward HA of heterologous clades as measured by intracellular staining. Four groups of 15 vaccinees were given 2 immunizations with 30  $\mu\text{g}$  HA of H5N1 NIBRG-14 virosomal vaccine alone (30  $\mu\text{g}$ -) or increasing doses of virosomal vaccine (1.5, 7.5 or 30  $\mu\text{g}$  HA) in combination with Matrix M<sup>TM</sup> adjuvant (+). PBMCs were isolated at day 42 into the study and stimulated for 16 h with influenza H5N1 antigen, co-stimulators, brefeldin A, and monensin. The homologous response was evaluated toward the NIBRG-14 antigen (clade1), using the H5N1 virosomes. The cross-H5 clade response was measured against A/Anhui/1/05 (Anhui) (clade 2.3.4) and A/Bar-headed Goose/Qinghai/1A/05 (BHG) (clade 2.2). (A) The mean percentage of influenza-specific CD4<sup>+</sup> T cells  $\pm$  SEM is shown for each vaccine group. \*, \*\*, and \*\*\* indicate significant differences ( $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively) from the 30  $\mu\text{g}$ - group, One-way ANOVA with Dunnet's post hoc test. (B and C) Correlation between NIBRG-14 and Anhui/BHG reactive CD4<sup>+</sup> T cells. The correlation was tested by Spearman rank test and the correlation coefficient ( $r$ ) and  $P$  value are shown on the graphs.

Bar-headed Goose/Qinghai/1A/05 (Clade 2.2) (BHG) and measuring the production of Th1 and Th2 cytokines by Luminex. Except for IL-4 (data not shown), all the measured cytokines were produced in response to Anhui stimulation, while BHG induced production of all cytokines except IL-4 and IL-5 (Fig. 2A and B). Lower levels of cytokines were observed after heterologous than homologous virus stimulation. There was a correlation between Th1 cytokines produced after stimulation with NIBRG-14 and the heterologous Anhui virus,  $r = 0.60$  for TNF- $\alpha$  to  $r = 0.74$  for IL-2 (both  $P < 0.0001$ ) (Fig. 2C, E, and G). Significant ( $P < 0.0001$ ) correlations were also found between the NIBRG-14 and

BHG antigens for IFN- $\gamma$  ( $r = 0.62$ ) and TNF- $\alpha$  ( $r = 0.57$ ) but not IL-2 ( $r = 0.01$ ) (Fig. 2D, F, and G). For the Th2 cytokines, there was no significant correlation between cytokine concentrations after NIBRG-14 and BHG stimulation, but IL-5 and IL-13 responses correlated between NIBRG-14 and Anhui stimulated samples (data not shown).

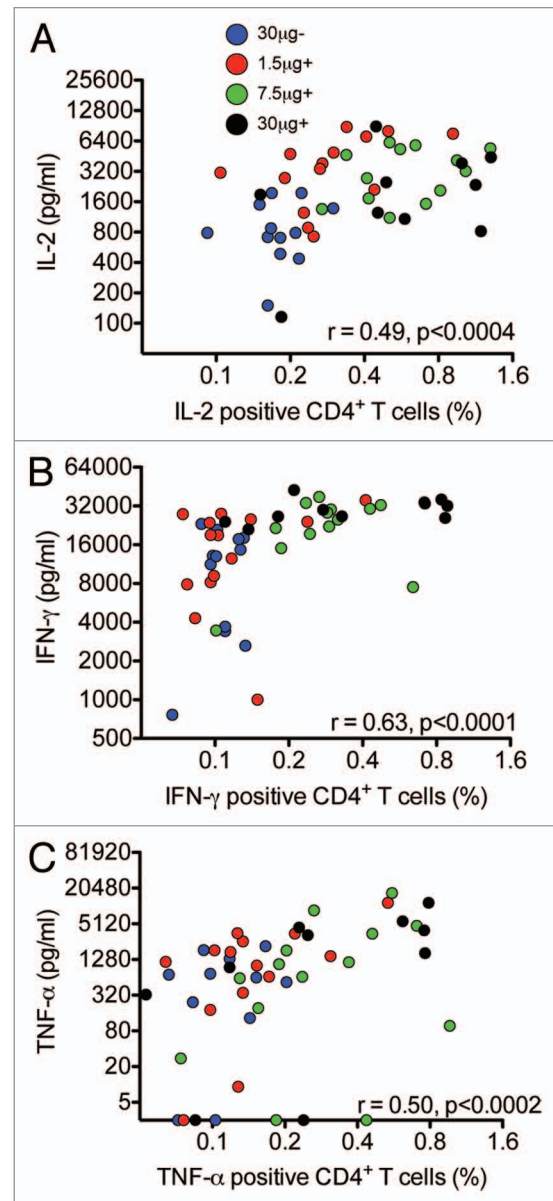
#### Th1 and CD8<sup>+</sup> T cell cytokine responses measured by intracellular cytokine staining

Measuring cytokine responses on a single-cell level allow for phenotyping cytokine producing cells. CD4<sup>+</sup> T cells producing one or more of the cytokines IL-2, IFN- $\gamma$  and TNF- $\alpha$  upon influenza stimulation were measured by flow cytometry at 21 days after the second vaccine dose (day 42). In the 30  $\mu$ g HA virosomal vaccine alone group, >0.2% of the CD4<sup>+</sup> T cells produced one or more of the measured cytokines after NIBRG-14 stimulation (Fig. 3A). Significantly higher frequencies of influenza-specific CD4<sup>+</sup> T cells were observed in all the adjuvanted groups ( $P < 0.05$ ) with frequencies of NIBRG-14 specific cells ranging from 0.4% in the 1.5  $\mu$ g HA adjuvanted group to 0.6% in the 30  $\mu$ g HA adjuvanted group. In contrast to CD4<sup>+</sup> T cell responses, low frequencies of CD8<sup>+</sup> T cells producing IL-2, IFN- $\gamma$  and TNF- $\alpha$  were observed (Fig. S1). The cross-reactive response was measured toward the HA from the Anhui and BHG strains (Fig. 3A). For both strains, low frequencies of influenza-specific CD4<sup>+</sup> Th1 cells were observed and there were no significant differences in responses between the adjuvanted and non-adjuvanted groups. Figure 3B and C shows the correlations between NIBRG-14 and Anhui or BHG reactive CD4<sup>+</sup> Th1 cells, respectively. There was a significant correlation ( $r = 0.56$ ,  $P = 0.0005$ ) between CD4<sup>+</sup> T cells reactive toward NIBRG-14 and BHG, but not between NIBRG-14 and Anhui ( $r = 0.25$ ,  $P = 0.11$ ).

To compare the cytokine responses measured by the multiplex cytokine assay to ICS, we plotted the cytokine concentrations found at the peak of the cytokine response (day 21) by multiplex cytokine staining against the percentages of influenza-specific CD4<sup>+</sup> T cells producing the respective cytokines (Fig. 4). A significant correlation was found for all the measured Th1 cytokines, ranging from  $r = 0.49$  for IL-2 to  $r = 0.63$  for IFN- $\gamma$  ( $P < 0.0004$ ).

#### Correlation of T cellular responses and serology

Previous studies found that vaccine-specific Th1 cells after the first vaccine dose are a good predictor of subsequent seroprotective antibody responses after the second dose<sup>25</sup> and later boost.<sup>21</sup> Multifunctional Th1 cells represent a distinct phenotype, producing more of each individual cytokine than single-cytokine producers. We evaluated the ability of multifunctional Th1 cells to predict seroprotection (Table 1). In addition to Th1 cytokines, Th2 cytokines may also predict seroprotection. We therefore compared Th1 vs. Th2 cytokines secreted in the supernatant of influenza-stimulated cells for correlation with previously reported serological responses in the HI, SRH, and virus MN assays. A kinetic analysis showed that 2 immunizations were required to elicit antibody titers above the protective threshold in all assays and peak antibody responses were found at day 35 (14 d after the second immunisation).<sup>24</sup> We plotted Th1 and Th2 cytokine concentrations from the supernatants of



**Figure 4.** A correlation was observed between cytokine responses measured by multiplex and intracellular cytokine staining. Four groups of 15 vaccinees were given 2 immunizations with 30  $\mu$ g HA of H5N1 NIBRG-14 virosomal vaccine alone (30  $\mu$ g-) or increasing doses of virosomal vaccine (1.5, 7.5, or 30  $\mu$ g HA) in combination with Matrix M<sup>TM</sup> adjuvant (+). PBMCs were isolated at day 21 into the study and stimulated in vitro with influenza H5N1 virosomes for 16 h for intracellular cytokine staining (ICS) or 72 h for Luminex analysis. ICS was performed on fresh cells, gating on CD3+CD4<sup>+</sup> T cells, while the supernatant was frozen at  $-80^{\circ}\text{C}$  and later thawed for analysis of IL-2, IFN- $\gamma$ , and TNF- $\alpha$ . A to C) Graphs show the correlation between CD4<sup>+</sup> T cells displaying the indicated cytokine after ICS (x-axis) plotted against cytokine concentrations measured by Luminex (y-axis). The correlation was tested by Spearman rank test and the correlation coefficient ( $r$ ) and  $P$  value is shown in the graphs.

influenza stimulated PBMCs 21 d after the first immunisation against peak day 35 and long-term day 180 antibody responses (Table 2). For the Th1 cytokines, there was a significant correlation between the concentrations of IL-2 at day 21 and MN ( $r =$

**Table 1.**

		Multifunctional Th1 cells
Day 35	HI	0.47**
	MN	0.41**
	SRH	0.50**
Day 180	HI	0.48**
	MN	0.43**
	SRH	0.02

The table shows the correlation between multifunctional Th1 cells, simultaneously secreting the cytokines IL-2, IFN- $\gamma$  and TNF- $\alpha$  measured at 21 d after the first vaccine dose by flow cytometry and haemagglutination inhibition (HI), microneutralisation (MN) and single radial hemolysis (SRH) titers measured at the peak of the serological response day 35 (14 d after the second vaccine dose) and at day 180 into the study. The Spearman correlation coefficients are shown in the table. \* and \*\* are significant correlations,  $P < 0.05$  and  $P < 0.01$ , respectively.

0.60), HI ( $r = 0.36$ ), and SRH ( $r = 0.42$ ) titers at day 35. A weaker correlation was found between the concentrations of IFN- $\gamma$  at day 21 and HI, MN, and SRH titers at day 35 (Table 2), while no association was observed between day 21 TNF- $\alpha$  concentrations and HI or SRH titers and only a weak correlation ( $r = 0.37$ ,  $P < 0.05$ ) between TNF- $\alpha$  and MN titers. The Th2 cytokines also correlated with serological responses. IL-10 and IL-13 secretion correlated with later HI and MN titers at day 35 ( $r = 0.35 - 0.44$ ,  $P < 0.05$ ), but not with SRH titers (Table 2). Cytokine responses at day 21 were not a good predictor for long-term antibody responses at day 180. Overall the concentrations of Th1 cytokines were a better predictor for subsequent seroprotection than Th2 cytokines in the in vitro stimulated PBMCs (Table 1).

## Discussion

Influenza A H5N1 poses a pandemic threat and effective H5N1 vaccines are needed. The correlates of protection for seasonal influenza may not be directly extrapolated to highly pathogenic avian influenza strains, therefore candidate pandemic avian vaccines should undergo additional immunological testing. An important consideration is the vaccine-induced T helper cells. These orchestrate the anti-viral immune response and, based on the cytokines produced, can be defined as Th1 or Th2 skewed and/or involving one or more of the recently defined Th subsets, e.g., Th17. Th cells directly influence other arms of adaptive immunity, such as cytotoxic T lymphocytes and B cells. Furthermore, despite inducing functionally active influenza-specific antibodies, pure Th2 inducing vaccines may be inferior for protection against influenza, at least in mice.<sup>18</sup> The present study investigated the T cellular responses after influenza vaccination with H5N1 virosomes alone or in combination with the Matrix M<sup>TM</sup> adjuvant in man. We found that the vaccines elicited production of Th1, Th2, and Th17 cytokines and that the Matrix M<sup>TM</sup> adjuvant significantly augmented the cytokine concentrations. This finding in humans confirms the balanced T helper response we observed for these vaccines in murine studies.<sup>14,23,29</sup> In murine

studies of adjuvanted influenza vaccines, Matrix-M<sup>TM</sup>, induced significantly higher both Th1 and Th2 cytokine response than observed after AS03 or alum formulations.<sup>29</sup>

GM-CSF production was also elicited upon influenza-stimulation in all Matrix M<sup>TM</sup> adjuvanted groups, GM-CSF has adjuvant properties<sup>30</sup> and the adjuvant effect of Matrix M<sup>TM</sup> could partly rely on induction of GM-CSF. Similar to the frequencies of influenza-specific CD4<sup>+</sup> Th1 cells,<sup>25</sup> the concentrations of T helper cytokines in the supernatants from in vitro influenza stimulated cells peaked at day 21 and were not further increased by a second dose of vaccine. However, since we evaluated the cytokine responses at day 42, 3 weeks post the second dose, it is possible that these have peaked prior to the sampling point.

We found that the Matrix M<sup>TM</sup> adjuvant induced higher frequency of H5N1 specific CD4<sup>+</sup> T-cells compared with the unadjuvanted vaccine formulation. This has also previously been shown for oil-in-water adjuvants in previous human studies of H5N1.<sup>21,22,31</sup> Similarly, both specific CD4<sup>+</sup> T cells and Th1 and Th2 cytokines were increased after immunization with AS03 adjuvanted pandemic H1N1 vaccine.<sup>32-34</sup>

We also evaluated cytokine production upon stimulation in vitro with heterologous H5 clades (Anhui, clade 2.3.4, and BHG, clade 2.2). As expected, lower concentrations of most cytokines were produced after heterologous than homologous stimulation. The virosomal vaccine may induce T cells specific for epitopes within both the HA and NA, while only epitopes shared between the HA of NIBRG-14 and Anhui/BHG strains were measured in the cross-reactive responses. Surprisingly, 2-fold higher IL-10 concentrations were observed after BHG stimulation than NIBRG-14 stimulation. While BHG antigen may activate pre-existing memory T cells to a higher production of IL-10, it remains possible that the impurities in the BHG antigen preparation stimulates IL-10 production from other IL-10 producing cell types (reviewed in ref. 35). Notably, only a few vaccinees had CD8<sup>+</sup> T cell responses to the vaccine on any of the sampling points tested (days 21, 42, and 180). It is possible that the peak in CD8<sup>+</sup> T cells was missed, although significant frequencies of influenza-specific CD8<sup>+</sup> T cells were observed at 3 weeks after second vaccine dose (day 42) in the pre-clinical studies.<sup>14</sup> However, only low CD8<sup>+</sup> T cell frequencies reactive toward HA and NA have been observed in humans.<sup>22,36</sup> IL-2, IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF are produced by CD8<sup>+</sup> T cells as well as CD4<sup>+</sup> T cells, but the observed low frequencies of cytokine producing CD8<sup>+</sup> T cells suggest that most T cell cytokines measured in the supernatant of in vitro activated cells were Th derived.

CD4<sup>+</sup> Th1 cytokines were measured both in the supernatant by the Luminex assay and intracellularly by flow cytometry. There was a moderate correlation between the 2 methods ( $r = 0.49-0.60$ ,  $P < 0.0004$ ) as reported previously.<sup>37,38</sup> The discrepancy may be explained by the difference in stimulation time (16 h for ICS and 72 h for Luminex) and the ICS potentially measuring non-secreted cytokines. Conversely, cytokines in the supernatant may be utilized by other cells and thus not detected in the Luminex assay. ICS can only be used for a limited number of analytes at a time, while the Luminex assay can evaluate many analytes in one setup and is easily standardisable, allowing for



high-throughput analysis of vaccine responses.<sup>39</sup> It is therefore advantageous to study vaccine-induced cell-mediated responses using both methods.

There is an interest in early identification of vaccine non-responders who could be offered other preventive measures or revaccination in due time. CD4<sup>+</sup> Th1 cells detected after one dose of H5N1 vaccine may predict subsequent seroprotection after the second dose,<sup>25</sup> a later booster dose<sup>21</sup> or revaccination.<sup>32</sup> The previous studies did not exclude the possibility that also Th2 cytokine responses would correlate with seroprotection. We compared the Th1 vs. Th2 cytokine responses found after the first immunization to predict serological responses after the second vaccine dose. We found only a moderate correlation between day 21 Th1 or Th2 cytokines and serological MN, HI and SRH responses after the second immunization, but Th1 cytokines showed the strongest correlation. Particularly, day 21 IL-2 concentrations correlated significantly with MN, HI and SRH responses ( $r = 0.36-0.60$ ,  $P < 0.05$ ) at day 35 confirming our previous observation where IL-2 responses were associated with antibody responses to a cell-grown H7N1 vaccine.<sup>40</sup> However, the relatively modest correlation coefficients imply that more robust early predictors for vaccine response are needed.

We conclude that the virosomal H5N1 vaccine adjuvanted with Matrix M<sup>TM</sup> adjuvant induces a balanced T helper response with production of both Th1 and Th2 cytokines in humans. The vaccine also induced T cells cross-reactive toward the HA of heterologous H5N1 virus clades, thus emphasizing the promise for this vaccine against divergent influenza H5N1 viruses.

## Materials and Methods

### Study design

The open label phase I clinical trial (ClinicalTrials.gov NCT00868218) was conducted in accordance with the Helsinki declaration. Study details are described elsewhere.<sup>24</sup> Sixty healthy volunteers were vaccinated intramuscularly with either 30 µg HA of virosomal H5N1 vaccine alone or 1.5, 7.5, or 30 µg HA of vaccine with 50 µg of Matrix M<sup>TM</sup> adjuvant. The vaccine was administered twice, at days 0 and 21 ( $\pm 1$ ) and blood collected immediately prior to vaccination. The volunteers provided blood samples at days 0, 3, 7, 14, 21, 24, 28, 35, 42, and 180. Peripheral blood mononuclear cells (PBMCs) were isolated from CPT<sup>41</sup> and resuspended in lymphocyte medium (RPMI 1640 with L-glutamine, 0.1mM non-essential amino acids, 10 mM Hepes, 1 mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL fungizone and 10% FBS) prior to use in the multiplex and intracellular cytokine staining (ICS) assays.

### Vaccine and adjuvant

The virosomal subunit vaccine was produced by Crucell, The Netherlands; Berna Biotech, Switzerland<sup>24</sup> from the NIBRG-14 virus with the haemagglutinin and neuraminidase of A/Vietnam/1194/2004 (H5N1) on the A/Puerto Rico/8/34

**Table 2.**

IL-2		T helper 1 cytokines			T helper 2 cytokines			
		IFN- $\gamma$	TNF- $\alpha$	IL-4	IL-5	IL-10	IL-13	
Day 35	HI	0.36*	0.32*	0.27	0.26	0.03	0.42**	0.35*
	MN	0.60**	0.44**	0.37*	0.36*	0.04	0.44**	0.38**
	SRH	0.42**	0.33*	0.22	0.16	0.07	0.21	0.12
Day 180	HI	0.03	0.31*	0.24	0.16	0.07	0.21	0.12
	MN	0.30*	0.39**	0.19	0.35*	0.04	0.35*	0.33*
	SRH	0.06	0.03	0.03	0.01	0.15	0.10	0.16

The table shows the correlation between cytokines measured at 21 d after the first vaccine dose in the supernatant of in vitro stimulated PBMCs and haemagglutination inhibition (HI), microneutralisation (MN) and single radial hemolysis (SRH) titers measured at the peak of the serological response day 35 (14 d after the second vaccine dose) and at day 180 into the study. Cytokine concentrations were plotted against serological responses and the Spearman correlation coefficients are shown in the table. \* and \*\* are significant correlations,  $P < 0.05$  and  $P < 0.01$ , respectively.

(H1N1) backbone and was standardized based on HA content. The Matrix M<sup>TM</sup> adjuvant was manufactured by Isconova AB, Sweden.<sup>24</sup> The adjuvanted vaccine containing 50 µg of Matrix M<sup>TM</sup> was supplied in pre-filled syringes.

### Multiplex cytokine assay

The PBMCs were stimulated ( $10^6$  cells/200 µL of lymphocyte medium for 72 h) with 10 µg/mL HA of the influenza H5N1 virosomes or HA from A/Anhui/1/05 or A/Bar-headed Goose/Qinghai/1A/05 (produced in tobacco plants by Fraunhofer, a kind gift from Dr Vidadi Yusibov). The supernatant was assessed for GM-CSF, IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-4, IL-5, IL-10, IL-12, IL-13, and IL-17 by a Bio-plex 200 (Bio-Rad) according to the manufacturer's instructions. Values for unstimulated samples (medium alone) were subtracted for data analysis.

### Intracellular cytokine staining

T cells were intracellularly stained for cytokines as described previously.<sup>41</sup> Fresh PBMCs were stimulated for 16 h with 10 µg/mL HA of influenza H5N1 NIBRG-14 virosomes or HA from A/Anhui/1/05 or A/Bar-headed Goose/Qinghai/1A/05 in lymphocyte medium containing anti-CD28 (1 µg/mL), anti-CD49d (1 µg/mL) (PharMingen), Brefeldin A (1 µg/mL) and Monensin (0.7 µg/mL) (BD). Cells were stained for CD3, CD4, CD8, IFN- $\gamma$ , IL-2, and TNF- $\alpha$  using the Cytofix/Cytoperm kit (BD) and analyzed by a BD FACSCanto flow cytometer. The basal cytokine production (non-stimulated cells) was subtracted for data analysis.

### Serological assays

The MN assay and a modified HI assay, using horse erythrocytes were performed by the Health Protection Agency (HPA), UK as previously described.<sup>42-44</sup> The SRH assay was performed by Istituto Superiore di Sanità (ISS), Italy.<sup>45</sup>

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Materials

Supplemental materials may be found here:  
[www.landesbioscience.com/journals/vaccines/article/29583/](http://www.landesbioscience.com/journals/vaccines/article/29583/)

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