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Original Article

Matrix-M adjuvanted virosomal H5N1 vaccine confers protection against lethal viral challenge in a murine model

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Background A candidate pandemic influenza H5N1 vaccine should provide rapid and long-lasting immunity against antigenically drifted viruses. As H5N1 viruses are poorly immunogenic, this may require a combination of immune potentiating strategies. An attractive approach is combining the intrinsic immunogenicity of virosomes with another promising adjuvant to further boost the immune response. As regulatory authorities have not yet approved a surrogate correlate of protection for H5N1 vaccines, it is important to test the protective efficacy of candidate H5N1 vaccines in a viral challenge study.

Objectives This study investigated in a murine model the protective efficacy of Matrix-M adjuvanted virosomal influenza H5N1 vaccine against highly pathogenic lethal viral challenge.

Methods Mice were vaccinated intranasally (IN) or intramuscularly (IM) with 7·5 μg and 30 μg HA of inactivated A/Vietnam/1194/2004 (H5N1) (NIBRG-14) virosomal adjuvanted vaccine formulated with or without 10 μg of Matrix-M adjuvant and challenged IN with the highly pathogenic A/Vietnam/1194/2004 (H5N1) virus.

Results and conclusions IM vaccination provided protection irrespective of dose and the presence of Matrix-M adjuvant, whilst the IN vaccine required adjuvant to protect against the challenge. The Matrix-M adjuvanted vaccine induced a strong and cross-reactive serum antibody response indicative of seroprotection after both IM and IN administration. In addition, the IM vaccine induced the highest frequencies of influenza specific CD4+ and CD8+ T-cells. The results confirm a high potential of Matrix-M adjuvanted virosomal vaccines and support the progress of this vaccine into a phase 1 clinical trial.

Keywords Influenza vaccine, ISCOM, Matrix-M, T-cells, viral challenge, virosomes.

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Introduction

The influenza A H5N1 virus has since 2003 been reported to infect nearly five hundred people causing serious illness with mortality in more than 60% of cases.[1] This has occurred through sporadic transmission from birds to man, but reassortment with other influenza A virus subtypes could lead to a more transmissible and deadly virus. Furthermore, H5N1 viruses have evolved into different clades and subclades, which are antigenically distinct. Therefore, an effective pre-pandemic vaccine that provides protection against distinct clades, in addition to drifted strains, is needed.

Vaccination is the best available method to limit the impact of an influenza pandemic. H5N1 vaccines are less immunogenic than both seasonal influenza and pH1N1 vaccines and an effective adjuvant is required to obtain protective immune responses (reviewed in Ref. 2). The immune stimulating complexes (ISCOMs) are cholesterol, phospholipid and saponin containing adjuvants produced from *Quillaja saponaria* Molina bark extract. The first generation of ISCOM based vaccines were produced by incorporating the antigen into the ISCOM particle.[3] These early ISCOMs contained a dozen different saponines, some of which had an inacceptable toxicology profile in rodents (reviewed in Ref. 4). It was later discovered that ISCOMs were also effective when mixed with antigen immediately prior to vaccination and these second generation ISCOMs were named Iscom-Matrix (reviewed in Ref. 5). The third generation ISCOM, Iscom-Matrix M (Matrix-M) was produced by incorporating two specific saponine fractions (A

and C) into separate matrices leading to an improved safety profile and comparable immunogenicity to the previous ISCOMs (unpublished data, K. Lövgren Bengtsson and B. Morein).

It is generally accepted that whole virus vaccines are more immunogenic than split vaccines in naïve populations but are also associated with higher incidence of adverse reactions.[6,7] One approach that combines the particulate nature and hence immunogenicity of whole virus vaccines, but in a more purified form is the virosomal vaccine (virosomes). Virosomes are produced by purifying the viral haemagglutinin and neuraminidase glycoproteins and incorporating these into a virus-like particle. As the envelope is reconstituted, virosomes have retained cell binding and membrane fusion capabilities, and thus virosomal vaccines have been reported to be more immunogenic than split and subunit vaccine formulations (reviewed in Ref. 8). Furthermore, virosomal vaccines contain a lower amount of residual egg proteins compared to whole vaccines and are therefore less prone to cause allergic reactions (reviewed in Ref. 9).

A needle-free intranasal influenza vaccine which may not only provide immunity at the portal of viral entry but also reduce viral transmission (reviewed in Ref. 10) is an attractive approach. Furthermore, intranasal vaccines may mediate an enhanced cross-protective response against antigenically drifted influenza viruses as mucosal IgA has a wider specificity than serum IgG.[11] However, intranasal administration of influenza antigen alone is poorly immunogenic in both humans and animals and novel mucosal adjuvants that enhance the immunogenicity of intranasal influenza vaccines are required (reviewed in Ref. 12). Promising mucosal adjuvant activities have been shown by cholera toxin B,[13] *Escherichia coli* heat-labile enterotoxin[14] and the bacterial second messenger bis(3,5)-cyclic dimeric guanosine and inosine monophosphates.[15,16] Previous generations of ISCOM have also shown promise as intranasal adjuvants for influenza vaccine formulation although requiring relatively high adjuvant doses (90–100 μg).[17,18]

Recent studies have found that candidate H5N1 pandemic vaccines can provide protection against highly pathogenic viral challenge in the absence of detectable HI antibodies.[19–21] In addition, serologic assays used as a surrogate correlate of protection for seasonal vaccines have shown limitations when used for evaluation of antibody responses towards influenza H5N1. To evaluate the protective efficacy it is therefore important to conduct a challenge study. In the study reported here, we evaluate the protective efficacy in mice of a Matrix-M adjuvanted virosomal H5N1 vaccine administered intranasally (IN) and intramuscularly (IM). Protective efficacy was compared with the kinetics of the humoural immune response assessed by ELISA and haemagglutination-inhibition (HI), the cytokine profiles and the qualitative and quantitative CD4+ and CD8+ T-cell responses. This is the first report of protection against challenge induced by a Matrix-M adjuvanted virosomal H5N1 vaccine, and our results support progression of the vaccine into a phase I human clinical trial.

Materials and methods

Vaccine and adjuvant

Inactivated influenza virosomal vaccine (Crucell, Leiden, the Netherlands) was produced as previously described.[7] The vaccine strain was the reverse genetics seed virus (NIBRG-14), derived from a reassortment between A/Vietnam/1194/2004 (H5N1) and A/Puerto Rico/8/34 (H1N1) produced by the National Institute for Biological Standards and Control (NIBSC), UK.[22] Matrix-M adjuvant (Isconova, Uppsala, Sweden/Crucell) was produced from purified *Quillaia* saponine extract and consists of mixtures of two separately formulated saponine fractions, Matrix A and Matrix C, mixed in proportions 91:9. The Matrix-M adjuvant and antigen were mixed directly before vaccination.

Mice

Female BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Kent, UK) and housed according to the appropriate national regulations (Norway and UK). The study was approved and conducted according to Norwegian Animal Welfare Acts and the Animal (Scientific Procedures) Act (UK).

Vaccination

Mice were IN or IM vaccinated with one or two doses (21 days apart) of virosomal adjuvanted influenza A H5N1 vaccine with or without Matrix-M adjuvant (10 μg). The study was divided into a protective efficacy and an immunogenicity study. In both studies, four groups of mice (10 per group) were IM administered 7·5 or 30 μg HA, with or without 10 μg Matrix-M, into the quadriceps muscles of the hind leg (50–100 μl). Two groups of mice (10 per group) were anaesthetized as previously described[23] and IN vaccinated with 7·5 μg HA (due to volume limitations only the low dose was given IN), alone or with Matrix-M, by a drop-wise administration of 5–5·5 μl vaccine per nostril twice at 5-minute intervals. Two other groups of 10 mice were used as controls and vaccinated with Matrix-M (10 μg) and/or PBS by IM or IN administration. An overview of vaccination and sampling schedules is found in Figure 1.

Protective efficacy study

The protective efficacy study was performed within the biosafety level 4 facility of the NIBSC. All procedures were carried out according to the UK Home Office Licence

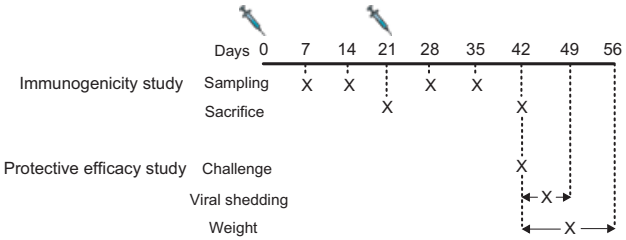

Figure 1. Overview of vaccination and sampling schedules The study was divided into a protective efficacy and an immunogenicity study. Groups of mice (10 per group) received intramuscular 7.5 or 30 μg HA, with or without 10 μg Matrix-M, into the quadriceps muscles of the hind leg (50–100 μl) or were intranasally vaccinated with 7.5 μg HA, alone or with Matrix-M (10 μg). Control groups consisted of mice receiving Matrix-M and/or PBS by intramuscular or intranasal administration. In the immunogenicity study five mice in each group were sacrificed at day 21 and the rest received a second vaccine dose. In the protective efficacy study all mice received two doses of vaccine and were challenged at day 41 with a lethal viral dose and observed for viral shedding and weight changes. Sampling refers to nasal washes and peripheral blood samples.

regulations and the NIBSC Ethical Review Process approved the study. The protective efficacy of the vaccine was studied by infecting mice with the highly pathogenic A/Vietnam/1194/2004 H5N1 parent strain of NIBRG-14. Three weeks after the second vaccination, each group of 10 mice was anaesthetized using ketamine (Vetalar; Pfizer Limited, Kent, UK) and challenged IN with 100 times the virus dose lethal to 50% of mice (MLD_{50}) in 20 μl. Following challenge, animals were weighed and observed for clinical signs of disease (ruffled fur, neurological signs and respiratory symptoms) for 14 days. Mice were euthanized following excessive weight loss (>20%) or poor general condition. Nasal wash samples [500 μl PBS/0·14% bovine serum albumin (BSA)] were collected at days 1–7 after challenge to monitor viral shedding.

Virus recovery from nasal washes

The presence of replicative virus in the nasal washes of challenged animals was quantified in confluent monolayers of MDCK cells as previously described.[19] Briefly, nasal wash samples were diluted and incubated on confluent MDCK cell monolayers. After 72 h incubation (35°C, 5% CO_2) a haemagglutination assay with 0·7% turkey red blood cells was used to detect replicative viruses in the supernatant. Wells were scored for the presence of virus and the titre expressed as the 50% tissue culture infective dose ($TCID_{50}$) per ml. Titres were calculated by the Spearman–Karber method.[24,25]

Immunogenicity study

The immunogenicity study was performed at The Influenza Centre, University of Bergen, Norway. After vaccination, sera and nasal washes (flushing twice with 350 μl PBS + 0·05% BSA) were collected weekly throughout the study and 21 days after first dose, five mice in each group were sacrificed and cardiac blood collected, whilst the remaining animals received a second dose of vaccine. Mice that received the second vaccination were sacrificed 21 days after the second dose and cardiac blood was collected. In addition, mononuclear cells were isolated from spleens using Lymphoprep™ (Axis-Shield, Oslo, Norway) as previously described[26] and resuspended in lymphocyte medium [RPMI 1640 containing l-glutamine and supplemented with 0·1 mm non-essential amino acids, 10 mm Hepes pH 7·4, 1 mm sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml fungizone and 10% foetal calf serum (FCS)] before use in the flow cytometry and bioplex cytokine assays.

Haemagglutination inhibition

Sera from day 21 after first and second dose were tested in HI assay by standard methods using a 0·7% (v/v) turkey erythrocyte suspension and a starting dilution of 1:8. The homologous HI responses were evaluated using NIBRG-14-virus (clade 1) and cross-reactive responses were evaluated against reverse genetics modified A/Anhui/1/05 (IBCDC-RG-6) (clade 2·3·4) and A/Cambodia/R0405050/2007 (NIBRG-88) (clade 1). The serum HI titre was expressed as the reciprocal of the highest dilution at which 50% haemagglutination was inhibited and negatives were assigned a value of 4 for calculation purposes.

Detection of influenza specific antibodies

The influenza-specific serum or nasal wash immunoglobulin classes and serum IgG subclasses (IgG1 and IgG2a) were quantified using an ELISA assay, as previously described[27,28] except that plates were coated with A/Vietnam/1194/2004 (H5N1) virosomes (2 μg/ml). The influenza-specific antibody concentrations were calculated using IgA, IgG, IgG1 and IgG2a standards and linear regression of the log-transformed readings.

Cytokine detection

Cytokine secretion was investigated 21 days after the second vaccination. Lymphocytes from spleens (10^6 cells/well) were incubated (37°C, 5% CO_2) for 72 h in 200 μl of lymphocyte medium containing 10 μg HA/ml of virosomal H5N1 influenza antigen or medium alone. After incubation, the supernatants were removed and stored at −80°C until used in the Bio-plex cytokine and IL-2 ELISA assays. The Bio-plex (Bio-rad, Hercules, CA, USA) and IL-2 ELISA (eBioscience, San Diego, CA, USA) cytokine kits were used according to the manufacturer's instructions to quantify cytokines of the T helper 1 (Th1) (IFN-γ, IL-2), Th2 (IL-4, IL-5 and IL-10) and Th17 (IL-17) subsets. The cytokine

concentrations for each individual mouse were calculated by subtracting the basal release (concentrations in supernatants from cells incubated with lymphocyte medium alone) from the concentrations in supernatants of cells stimulated with H5N1 influenza antigen.

CD4+ and CD8+ T-cell responses

The influenza specific CD4+ and CD8+ T-cell responses were measured by intracellular cytokine production. Live splenocytes (10^6 cells/well) were incubated (37°C, 5% CO_2) overnight in 200 μl lymphocyte medium containing 10 μg/ml HA of virosomal H5N1 influenza antigen (Crucell), 2 μg/ml anti-CD28 (Pharmingen, San Diego, CA, USA) and 10 μg/ml Brefeldin A (BD Biosciences, San Jose, CA, USA). Basal cytokine production was determined by incubating splenocytes from vaccinated mice in lymphocyte medium without antigen and the percentages of cytokine positive cells were subtracted from that of influenza-stimulated cells. As positive controls, cells were incubated in medium containing the mitogens phorbol myristate acetate (10 ng/ml) and ionomycin (250 ng/ml). 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 previously described.[29] Finally, cells (300 000 per sample) were resuspended in PBS containing 5% FCS and 0·1% sodium azide and light emission was measured by BD FACSCanto flow cytometer. Data were analysed using FlowJo v8·8·6 (Tree Star, Ashland, OR, USA), Pestle v1·6·2 and SPICE v5·0 (Mario Roederer; Vaccine Research Centre, NIH, Bethesda, MD, USA) and multifunctional T cells were identified as previously described.[28–30] T-cells were classified based on cytokine IFN-γ, IL-2 and TNF-α secretion as single producers (one cytokine), double producers (two cytokines) and triple producers (all three cytokines).

Statistical analysis

A two-tailed unpaired *t*-test [Prism v5·0a for Macintosh and SPICE v5·0 (Mario Roederer)] was used to analyse differences between groups and *P*-values <0·05 were considered significant.

Results

We have previously found that intramuscular administration of the virosomal Matrix-M adjuvanted H5N1 vaccine induces a promising quantitative and qualitative CD4+ T-cell response indicated by a high frequency of multifunctional CD4+ T-cells in mice.[28] Previous influenza H5N1 vaccines have been shown to be less immunogenic in humans, generally requiring two doses to elicit a sufficient immune response.[31] To address the regulatory requirements, we thus conducted a detailed protective efficacy and immunogenicity study with the proposed vaccine strengths to be used in a clinical trial (7·5 and 30 μg HA).

Protection study

In this study, we have evaluated the protective efficacy of a Matrix-M adjuvanted virosomal vaccine. Groups of 10 mice were used as controls or vaccinated with two doses of 7·5 or 30 μg HA of virosomal adjuvanted influenza A H5N1 vaccine alone or further adjuvanted with Matrix-M and challenged 3 weeks after the second dose with 100 MLD_{50} of the highly pathogenic A/Vietnam/1194/2004 (H5N1). Following challenge the mice were evaluated for weight loss and clinical signs of disease for up to 14 days (Table 1). Nasal washings were also collected daily from each mouse up to day 7 after challenge to test for viral shedding. For non-vaccinated control groups, the group receiving IM PBS is described here, but similar results were found for two other groups of 10 mice receiving PBS and Matrix-M adjuvant IN and IM respectively.

All groups of mice displayed a temporary weight decrease 1 day after challenge, which is probably due to the anaesthetic, but the animals' weights increased again by day 2 (Figure 2A). Two days post-challenge, seven control and eight non-adjuvanted IN vaccinated mice had detectable viral shedding from the nasal cavity (mean of 10^2 $TCID_{50}$/ml for both groups) and from day 3 these mice started to lose weight dramatically (Figure 2A). These mice also had signs of influenza disease, including ruffled fur and reduced activity. By 6 days post-challenge, four control mice had to be euthanized due to excessive weight loss (>20%) and at day 7 the remaining mice in the control group were also euthanized due to clinical disease signs or reduction in weight (Figure 2B). Two mice in the IN non-adjuvanted group, died or were euthanized due to signs of severe disease at 6 days post-challenge. A further five mice in this group were euthanized at 7 and 8 days post-challenge and one mouse at 10 days post-challenge. Virus was recovered from the nasal cavity of all the unvaccinated mice and all except one of the mice vaccinated IN without adjuvant (Figure 2C). This and two other mice that were vaccinated IN with the non-adjuvanted vaccine started to gain weight and survived the challenge. Thus, the highly pathogenic A/Vietnam/1194/2004 (H5N1) was fatal to unvaccinated mice and also to mice vaccinated IN with the non-adjuvanted vaccine. In contrast, the IM vaccinated mice and the mice vaccinated IN with 7·5 μg HA formulated with adjuvant lost little weight and all survived the challenge. None of the IM vaccinated mice shed virus except for one mouse in the 7·5 μg HA non-adjuvanted group shedding $10^{1.6}$ $TCID_{50}$/ml at day 4. Thus, the vaccine protected the mice from disease and death following

Table 1. Protection against lethal viral challenge

Group	Parameter	Mouse 1	2	3	4	5	6	7	8	9	10
Control	Max weight loss (g)	5·5	5·3	3·4	4·6	4·0	4·5	4·6	3·4	3·8	4·7
	Viral shedding days	3/7	4/7	4/7	4/7	4/7	4/7	5/7	5/7	3/7	4/7
	Died+(day)	+(6)	+(7)	+(6)	+(6)	+(6)	+(7)	+(7)	+(7)	+(7)	+(7)
7·5 ug	Max weight loss (g)	1·3	4·6	4·2	2·6	6·2	5·4	4·6	2·9	5·3	4·6
IN-	Viral shedding days	1/7	3/7	1/7	–	1/7	1/7	1/7	2/7	2/7	3/7
	Died+(day)	–	+(8)	+(8)	–	+(10)	+(7)	+(7)	–	+(6)	+(6)
7·5 μg	Max weight loss (g)	0·8	0·7	0·7	0·8	–	1·1	1·1	0·1	–	1·7
IN +	Viral shedding days	No viral shedding									
	Died+(day)	All survived									
7·5 μg	Max weight loss (g)	–	0·3	0·4	0·7	1·2	0·4	1·1	0·6	1·3	0·6
IM-	Viral shedding days	–	–	1/7	–	–	–	–	–	–	–
	Died + (day)	All survived									
7·5 μg	Max weight loss (g)	1·3	0·4	1·3	0·8	0·3	0·9	0·4	–	0·4	–
IM+	Viral shedding days	No viral shedding									
	Died+ (day)	All survived									
30 μg	Max weight loss (g)	1·1	1·0	0·8	0·3	–	0·5	1·4	1·2	1·1	0·9
IM-	Viral shedding days	No viral shedding									
	Diedt(day)	All survived									
30 μg	Max weight loss (g)	0·6	–	0·5	1·1	1·2	1·4	1·0	1·0	0·9	0·3
IM +	Viral shedding days	No viral shedding									
	Died+(day)	All survived									

The table presents the outcome following highly pathogenic viral challenge. Mice were divided into groups of 10, which either received only PBS or PBS and Matrix-M adjuvant (control) or were intranasally (IN) or intramuscularly (IM) vaccinated with two doses of 7·5 or 30 μg of virosomal influenza H5N1 (RG-14) vaccine with (+) or without (-) Matrix-M adjuvant (10 μg). Maximum weight loss (max weight loss) is defined as the total drop in weight from pre-challenge values, irrespective of any weight gain, experienced by each individual mouse for 14 days post-challenge. Viral shedding was measured for 7 days and is presented as the number of days out of the seven that the individual mouse shed virus. The time point at which diseased mice died is shown in brackets.

viral challenge irrespective of adjuvant when administered IM, whereas formulation with Matrix-M adjuvant was required to protect the IN vaccinated mice.

Immunogenicity study

Haemagglutination inhibition antibody response

Despite the uncertainties in defining the correlates of protection for avian pandemic influenza vaccines, serum HI titres of 40 are generally accepted by regulators as surrogates of protection.[32] We have used a starting dilution of 1:4 and therefore an HI titre ≥32 endpoint as a surrogate correlate of protection in this study. One dose (7·5 or 30 μg HA) of the Matrix-M adjuvanted vaccine given IM induced HI titres ≥32 against the vaccinated H5N1 strain (Figure 3A). After two doses, the vaccine induced HI antibodies against the homologous strain in all vaccinated groups and HI titres of ≥32 were achieved in all vaccinated groups with the exception of two IN vaccinated mice that did not receive Matrix-M. The highest geometric mean titres (GMT) of 1024 were found in IM vaccinated mice receiving 7·5 or 30 μg HA formulated with Matrix-M adjuvant. The mice vaccinated IN with 7·5 μg HA had the lowest HI GMT, but after two doses of vaccine, the adjuvanted group obtained titres well above the protective threshold (GMT 256). In contrast, the virosomal vaccine alone IN group had a GMT of 16, which correlated with the protective efficacy study where the corresponding group suffered from disease and death following lethal viral challenge.

Cross-reactive antibody response

Pandemic vaccines should be able to induce protection against drifted influenza strains, and the geographically distinct H5N1 clades. We have therefore tested for cross-reactive serum HI antibody against the clade 2·3·4 virus IBCDC-RG-6 and the clade 1 virus NIBRG-88. After two vaccine doses an HI titre ≥32 was found against the cross-clade IBCDC-RG-6 in all IM vaccinated mice (Figure 3B), whilst only mice immunized with the Matrix-M adjuvanted vaccine had HI titres ≥32 against NIBRG-88 (Figure 3C). Generally, higher HI titres were found against IBCDC-RG-6 than NIBRG-88 (up to GMT 1024 and 128 respectively)

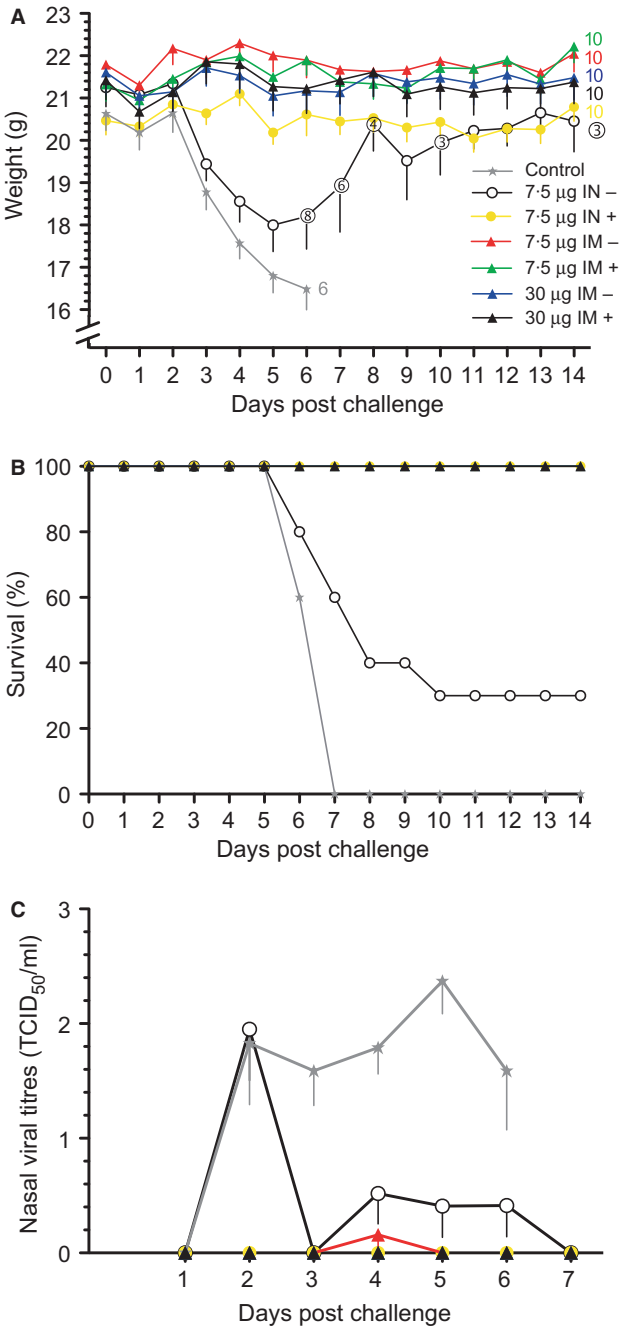

Figure 2. Survival and weight loss following challenge Groups of ten mice were either used as a control group or vaccinated with virosomal influenza H5N1 (RG-14) vaccine, with (+) or without (–) Matrix-M adjuvant, by the intranasal (IN) or the intramuscular (IM) route. Three weeks after the second dose the mice were challenged with 100 MLD_{50} of highly pathogenic A/Vietnam/1194/2004 (H5N1) virus. (A) The mice were weighed for 14 days and observed for clinical parameters of disease. The curve numbers state remaining mice in the respective groups on the specific days (survival) and the mean weight is based upon remaining mice. (B) Survival curve. (C) Nasal wash (NW) samples were collected daily up to seven days post challenge and analysed for presence of replicative virus (log10 TCID50/ml) using MDCK cells. Errorbars indicate SEM.

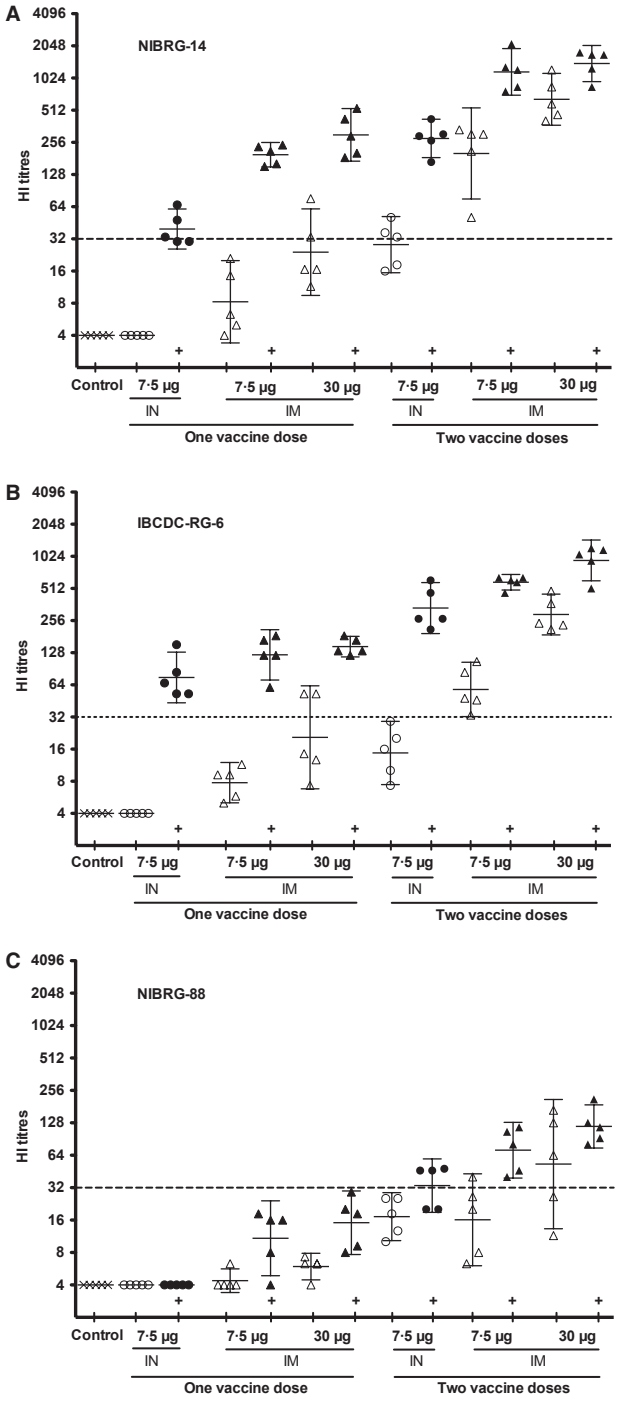

Figure 3. Haemagglutination inhibition response Mice were intranasally (circles) or intramuscularly (triangles) vaccinated with one or two doses (three weeks apart) of virosomal vaccine (7,5 or 30 μg HA/dose) alone or with 10 μg of Matrix-M adjuvant (filled symbols). The serum HI response was determined three weeks after first and second dose respectively towards NIBRG-14 (A), IBCDC-RG-6 (B) and NIBRG-88 (C). The data show the geometric mean titre ±95% confidence interval obtained from 5 mice in each group and is based on geometric mean titre of three independent experiments. The dotted line represents an HI titre of 32.

and interestingly, only one dose of Matrix-M adjuvanted vaccine was necessary to confer protective HI levels (GMT 64) towards IBCDC-RG-6 irrespective of the route of administration. The results thus show that the vaccine induced a better cross reactivity towards the clade 2·3·4 virus IBCDC-RG-6 than the clade 1 virus NIBRG-88.

Influenza specific antibody response

The vaccine induced high levels of influenza specific IgG antibodies, especially after the second dose (Figure 4A and B). The IgG antibody responses were higher in the IM vaccinated (up to 1500 μg/ml), compared with the IN vaccinated (up to 320 μg/ml) groups. From 2 weeks after the first dose and throughout the study, regardless of administration route, significantly higher antibody concentrations ($P < 0{\cdot}05$) were found in animals vaccinated with the Matrix-M adjuvanted vaccine compared to the non-adjuvanted groups. For the IgG subclasses (IgG1 and IgG2a), the IM adjuvanted vaccine induced a mixed T-helper response (Figure 4C) indicated by production of both IgG1 (up to 1000 μg/ml) and IgG2a (up to 450 μg/ml) (data not shown). By contrast, the virosomal alone IM and the IN vaccinated groups had a bias towards IgG1. A Th2 polarized response was thus induced in the non-adjuvanted, but also the IN adjuvanted, vaccine groups.

Local IgA can prevent viral infection at the portal of entry in the nasal cavity and is thought to be more cross-reactive than IgG.[33–35] We therefore measured influenza specific IgA in the serum and nasal washes from immunized mice (Table 2). No IgA was found in the IM vaccinated mice. However, for the IN vaccinated mice, IgA levels in the serum were significantly higher ($P < 0{\cdot}05$) in the Matrix-M adjuvanted compared to the non-adjuvanted animals. The nasal wash IgA levels were also higher in mice that had received adjuvant compared to the non-adjuvanted group, although the difference was only significant ($P < 0{\cdot}05$) at 1 week after the second dose. In the protective efficacy study, the IN Matrix-M adjuvanted group was protected against challenge in contrast to the non-adjuvanted group.

Cytokine response

The Th1 (IL-2 and IFN-γ), Th2 (IL-4, IL-5 and IL-10) and Th17 (IL-17) cytokine profiles of the vaccinated mice were determined in *ex vivo* stimulated splenocytes isolated 3 weeks after the second dose (Figure 5). All groups produced the INF-γ upon stimulation and in the IM vaccinated mice, the inclusion of Matrix-M significantly ($P < 0{\cdot}05$) increased the production of IFN-γ compared to their non-adjuvanted counterparts. However, Matrix-M did not augment the IFN-γ response in the IN vaccinated mice (2 ng/ml produced in both groups). The Th2 cytokines IL-4, IL-5 and IL-10 were detected in all vaccinated animals

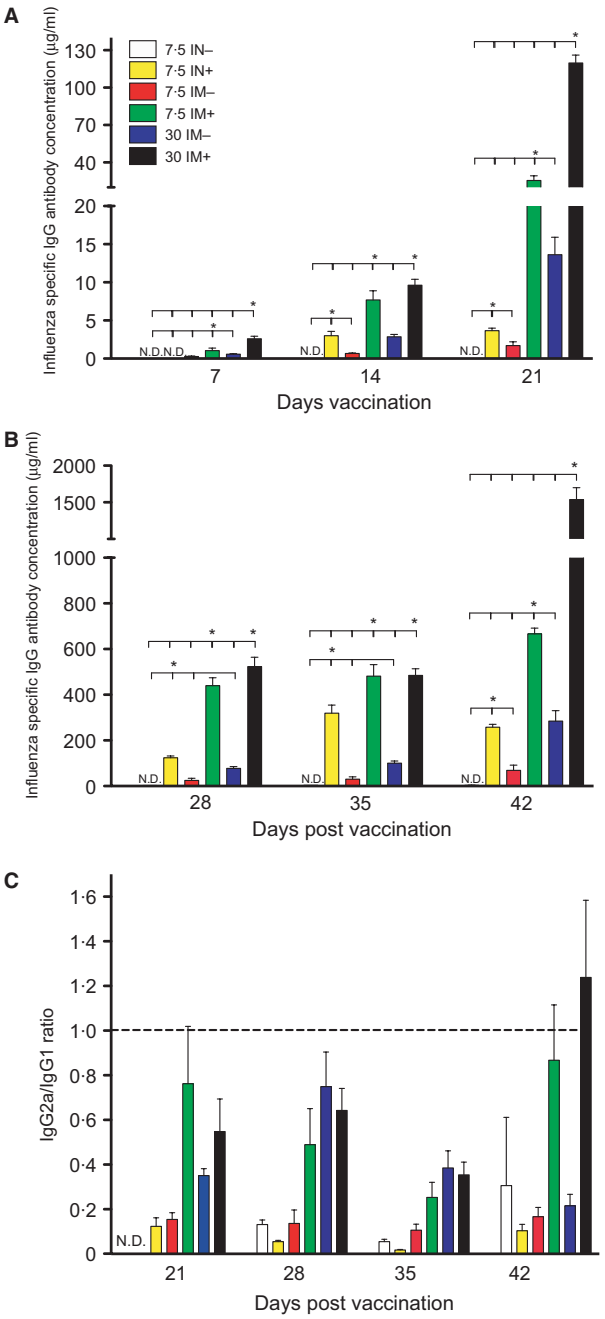

Figure 4. Influenza specific IgG and IgG subclass antibody response Mice were intranasally (IN) or intramuscularly (IM) vaccinated with virosomal influenza H5N1 (RG-14) and Matrix-M adjuvant (+) or antigen alone (–). The serum influenza specific IgG, IgG1 and IgG2a concentrations induced after vaccination was measured by ELISA. The IgG concentration was measured at 7, 14 and 21 days (A) and 28, 35 and 42 days (B), whilst the IgG2a/IgG1 ratio was determined at day 21, 28, 35 and 42 days (C) post vaccination. Each column represents mean IgG antibody concentration (μg/ml) or mean IgG2a/IgG1 ratio (a ratio below 1.0 indicates a bias towards IgG1) from five vaccinated mice ± SEM. * Indicates groups with significantly higher IgG concentrations as compared to the other vaccine groups and/or the control group (Two-tailed unpaired T-test, $P < 0.05$). N.D., not detected.

Table 2. IgA response induced after vaccination

		Days post first dose			Days post second dose		
	Group	**7**	**14**	**21**	**7**	**14**	**21**
Serum	IN–	N.D.	M.D.	N.D.	138 (25)*	153 (26)*	170 (40)*
	IN+	N.D.	293 (86)	275 (72)	5490 (1025)	3231 (598)	3111 (501)
	IM+/–	N.D.					
	Control						
NW	IN–	N.D.	N.D.	N.D.	60 (11)*	25 (5)	9 (3)
	IN+	N.D.	N.D.	N.D.	142 (16)	74 (26)	14 (3)
	IM+/–	N.D.					
	Control						

The table presents the local and systemic influenza specific IgA response. 7·5 μg HA of virosomal influenza H5N1 (RG-14) vaccine was administered intranasally (IN) or intramuscularly (IM) with (+) or without (-) Matrix-M adjuvant. Serum and nasal wash (NW) samples were collected 7, 14 and 21 days after the first and second dose and IgA concentration measured by ELISA. The IgA concentration is presented in ng/ml and brackets indicate SEM. *Statistically different from the adjuvanted group ($P < 0{\cdot}05$, two-tailed unpaired *t*-test). N.D.: not detected.

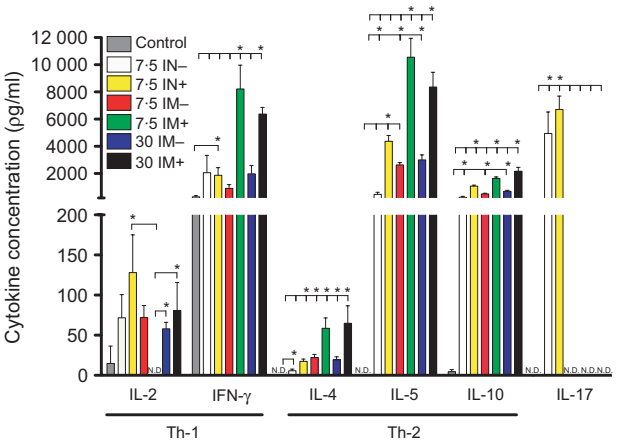

Figure 5. Cytokine production from in vitro activated lymphocytes Groups of ten mice were either used as control or vaccinated with virosomal influenza H5N1 (RG- 14) vaccine, with (+) or without (–) Matrix-M adjuvant, by the intransal (IN) or the intramuscular (IM) route. Splenocytes were isolated three weeks after second dose and incubated for 72 hours with 10 μg/ml virosomal H5N1 RG-14 before analysis by ELISA (IL-2) or Bioplex. Each row represents mean values from five mice ±SEM. * Indicates groups with significantly higher cytokine concentrations as compared to the other vaccine groups and/or the control group (Two-tailed unpaired T-test, $P < 0.05$).

and the highest concentrations (60 pg/ml IL-4, 10 ng/ml IL-5 and 1 ng/ml IL-10) were found in mice vaccinated with adjuvanted 7·5 or 30 μg HA vaccine by the IM route. Matrix-M adjuvanted vaccine induced the highest Th2 cytokine concentrations, which were significantly ($P < 0{\cdot}05$) higher for IL-5 and IL-10 cytokines compared to the virosomal alone groups. Thus, vaccination induced all the analysed Th2 cytokines and high concentrations of IFN-γ particularly from the adjuvanted IM groups and the cytokine profile agrees with the mixed Th1/Th2 profile which was observed for the IgG antibody subclasses. Interestingly, the Th17 cytokine IL-17 was only produced from splenocytes isolated from IN vaccinated mice, supporting the induction of a different type of immune response when using this route of administration compared with the IM route.

The CD4+ and CD8+ T-cell responses

The ability of CD4+ T-cells to simultaneously secrete more than one type of the cytokines TNF-α, IFN-γ and IL-2 can predict the degree of protection against *Leishmania major*[29] and may be related to a good clinical outcome following HIV-2 infection.[30,36] These multifunctional cells may also play an important role in protective efficacy of influenza vaccines. Therefore, we have evaluated the ability of CD4+ Th1 cells from the vaccinated mice to secrete one or more of the cytokines TNF-α, IFN-γ and IL-2 (Figure 6A). All three cytokines were produced and all IM vaccinated groups had significantly higher ($P < 0{\cdot}02$) frequencies of cytokine producing cells as compared to the control group. The highest percentages of cells producing each individual cytokine were found in the Matrix-M adjuvanted IM groups. These groups had significantly more ($P < 0{\cdot}05$) TNF-α, IFN-γ and IL-2 cytokine positive T-cells as compared to all other groups. For the IN vaccinated groups, a lower number of cytokine producing cells was elicited, but inclusion of Matrix-M led to a significantly higher ($P < 0{\cdot}05$) percentage of IFN-γ and IL-2 cytokine producing cells compared to the group receiving only virosomes. The percentages of cytokine producing CD8+ T-cells were generally lower than those of CD4+ T-cells (Figure 6B).

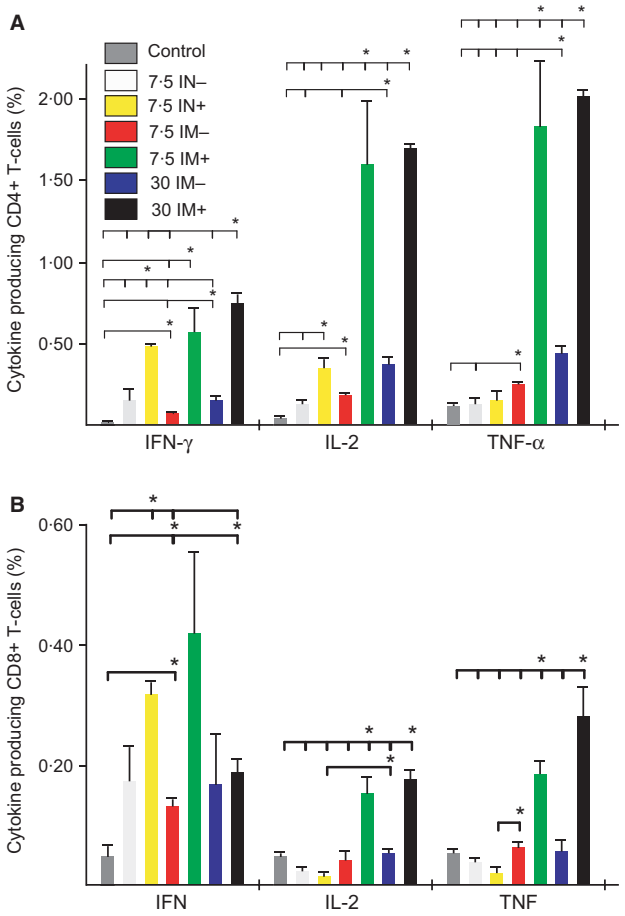

Figure 6. The intracellular CD4+ and CD8+ T-cell cytokine response Mice were vaccinated intramuscularly (IM) or intranasally (IN) with 7.5 or 30 μg HA of virosomal influenza H5N1 (RG-14) vaccine formulated with (+) or without (–) Matrix-M adjuvant. Splenocytes were isolated three weeks after second dose and in vitro activated with 10 μg/ml virosomal H5N1 RG-14 for 72 hours before fixation, intracellular cytokine staining for CD3, CD4, CD8, IFN-γ, IL-2 and TNF-α and analysis by flow cytometry. Data is presented as the percentage of cytokine producing (A) CD4+ cells and (B) CD8+ cells. *Indicates groups with significantly higher percentages of cytokine producing CD4+ or CD8+ T-cells as compared to the other vaccine groups and/or the control group (Two-tailed unpaired T-test, $P < 0.05$).

The highest numbers of IFN-γ producing CD8+ cells were found in the adjuvanted 7·5 μg IM and IN groups. Also IL-2 and TNF-α producing cells were more represented in the Matrix-M adjuvanted IM groups compared with all other groups (significantly higher, $P < 0{\cdot}05$).

Importantly, when administered IM, the vaccine induced CD4+ T-cells to simultaneously produce two or three cytokine types (Figure 7A). This was also most prominent for the Matrix-M adjuvanted groups and the percentage of cells simultaneously producing all three cytokines, or any combination of two cytokines, was significantly higher ($P < 0{\cdot}05$) for the group receiving the 30 μg adjuvanted dose as compared to the group receiving virosomes alone. In contrast to the IM groups, only a small percentage of cells from the IN vaccinated and the control mice produced more than one cytokine. The control and IN vaccinated mice produced almost exclusively one cytokine type, whilst the IM administered vaccine induced a CD4+ T-cell multifunctionality, with cells simultaneously secreting two or more cytokines. Interestingly, the median fluorescence intensity for each cytokine was higher for cells producing two or three cytokines simultaneously (Figure 7B). Thus, cells from the 30 μg adjuvanted group producing all three cytokines simultaneously had a significantly higher ($P < 0{\cdot}05$) MFI for each individual cytokine compared with cells producing only one cytokine. In addition to producing more than one type of cytokine, the multifunctional cells are thus also able to produce higher concentrations of each individual cytokine illustrating that these cells may serve an important effector function.

Discussion

Before the spring of 2009 it was speculated that the cause of the next pandemic would be H5N1 influenza transmitted from birds to humans. This was not the case, but the risk of an H5N1 pandemic has probably never been higher due to the increased possibility of reassortment between bird and swine influenza viruses.[37,38] Pandemic avian candidate vaccines have thus far been poorly immunogenic, requiring two immunizations and adjuvants to elicit an adequate immune response. Effective immune potentiating strategies are therefore needed. Our results show that the Matrix-M adjuvanted virosomal H5N1 vaccine induces a strong protective and cross-reactive immune response both by IN and IM administration and thus, the vaccine is an attractive human pandemic vaccine candidate. The vaccine induced protection against lethal challenge in the group receiving adjuvanted vaccine by the IN route, whilst mice in the virosomal alone IN group suffered from severe disease and seven out of ten mice died. Thus, the ability of IN administered Matrix-M adjuvanted virosomal vaccine to protect against lethal viral challenge confirms a high effectiveness of Matrix-M as an IN adjuvant. This is in agreement with previous studies using higher doses of ISCOM (90 μg) and Iscom-Matrix (100 μg).[17,18] Also, in this study we found an augmentation of the Matrix-M mucosal adjuvant properties by using a higher antigen dose (7·5 μg HA) compared with the dose (5 μg HA) we have previously studied.[28] All IM vaccinated groups were also protected against challenge with the highly pathogenic parent virus. This suggests a sufficient intrinsic immunogenicity of the virosomal H5N1 vaccine when used IM and thus, it was not possible to observe the Matrix-M adjuvant effect on protection from viral challenge in the IM groups. This

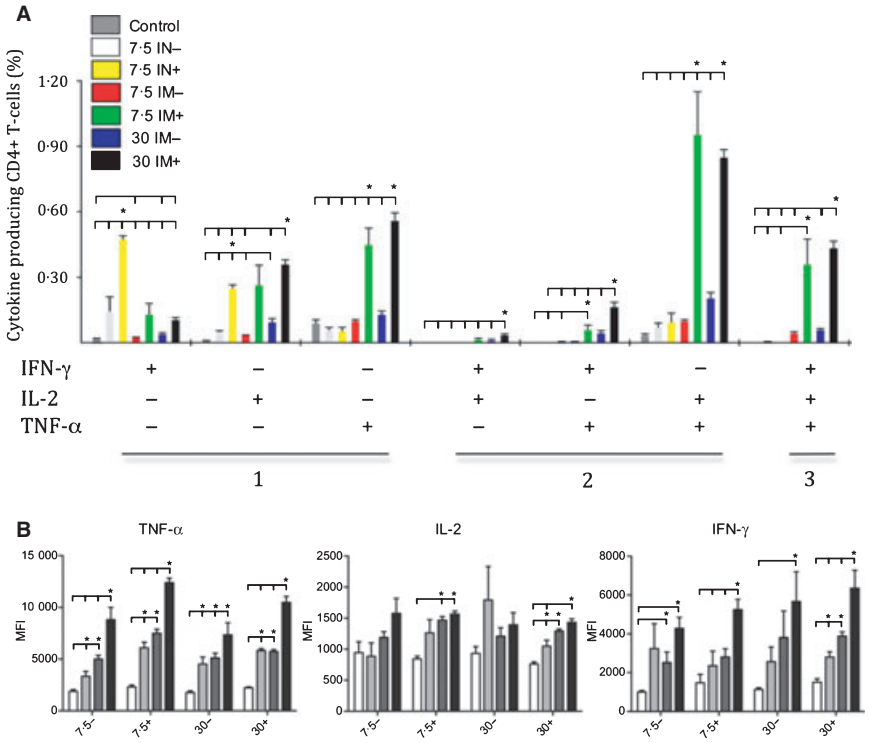

Figure 7. The polyfunctional CD4+ T-cell cytokine response after vaccination Mice were vaccinated intramuscularly (IM) or intranasally (IN) with 7.5 or 30 μg HA of virosomal influenza H5N1 (RG-14) vaccine formulated with (+) or without (–) Matrix-M adjuvant. Splenocytes were isolated three weeks after second dose and in vitro activated with 10 μg/ml virosomal H5N1 RG-14 for 72 hours before fixation, intracellular cytokine staining for IFN-γ, IL-2 and TNF-α and flow cytometric analysis. (A) The columns show, for each vaccine group, the frequency of CD4+ T-cells (%) producing the different possible combinations of the cytokines IFN-γ, TNF-α and IL-2. *Indicates groups with significantly higher percentages of cytokine producing CD4+ T-cells as compared to the other vaccine groups and/or the control group (Two-tailed unpaired T-test, $P < 0.05$). (B) In addition to producing more than one cytokine type, the polyfunctional T-cells also had the highest production of the individual cytokines as indicated by median fluorescence intensity (MFI). The MFI of the fluorophore conjugated antibodies for TNF-α, IL-2 and IFN-γ were measured for cells that produced one cytokine type (white columns), two cytokine types (light and dark grey columns) or all three cytokine types (black columns). MFI data is only presented for intramuscularly vaccinated mice due to the low frequency of polyfunctional T-cells in the intranasal and control groups. *Indicates significantly higher MFI for triple and/or double cytokine producing CD4+ T-cells as compared to single producers (Two-tailed unpaired T-test, $P < 0.05$).

finding has prompted ongoing pilot murine challenge studies using lower doses of the virosomal vaccine. In preliminary experiments, we analysed the effect of low antigen doses of virosomal vaccine to provide a platform for future analysis of adjuvants. Strikingly, the virosomal vaccine alone protected mice from death at doses as low as 0·04 μg HA (Table 3), although all these mice had virus in the lower respiratory tract, which was not observed with the higher doses (1 and 5 μg HA). A further group of mice receiving a single dose of 1 μg virosomal vaccine alone also experienced transient weight loss, but all the mice survived challenge (data not shown). Interestingly, we found that a single dose of Matrix-M adjuvanted virosomal vaccine (7·5 μg HA) elicited similar HI antibody titres (GMT 196) as found in the protected virosomal alone IM 7·5 μg HA group, suggesting that a single dose of Matrix-M adjuvanted vaccine would also provide protection. Furthermore, the

Table 3. Virosomal vaccine dose reduction

	Control	0·04	0·2	1	5
Survival	×	√	√	√	√
Weight loss	√	×	×	×	×
Lung titres	√	√	√	×	×
Nasal titres	√	√	√	√	√

The table presents the dose reduction studies of the virosomal vaccine. Groups of 20 mice were vaccinated IM with two doses 21 days apart of the virosomal vaccine (0·04, 0·2, 1 or 5 μg HA) and challenged 3 weeks later with 100 MLD_{50} of A/Vietnam/1194/2004 (H5N1). Ten mice in each group were sacrificed on different days to test for the presence of virus in the lung and nasal tissue, whilst the remaining mice were followed for 15 days to monitor for changes in weight and clinical signs of disease.

levels of cross-reactive HI antibodies against different strains (GMT 128 for IBCDC-RG-6) suggest that the vaccine could be used for pre-pandemic vaccination. The cross-protective capability of the vaccine against H5N1 viruses from different clades should be investigated in a future murine challenge study.

A vaccine response consisting of both Th1 cells and Th2 cells is important for elimination of viral pathogens.[39,40] The Matrix-M adjuvanted virosomal H5N1 vaccine elicited a balanced Th response indicated by production of both IgG1 and IgG2a antibodies in addition to Th1 (IFN-γ) and Th2 (IL-4, IL-5 and IL-10) cytokines. This finding is in agreement with our previous results[28] and to that observed with the similar Iscom-Matrix adjuvant.[5] But, in contrast, results obtained with an IM administered Matrix-M adjuvanted H9N2 vaccine showed that Matrix-M induced a Th1 skewed type of immunity in mice.[41] The contradictory findings can be explained by a possible variation between the studies in the fraction A and C proportions of the Matrix-M adjuvant. Although, a more likely explanation is that adjuvants cannot independently determine the Th response, but that the type and formulation of viral antigen also affects the response. The Th17 cytokine IL-17 was only produced by lymphocytes isolated from IN vaccinated mice and no significant difference in IL-17 concentration was observed between the Matrix-M adjuvanted and virosomal alone groups. IN vaccination has earlier been described as eliciting IL-17[42] and it has also been shown that IL-17 is produced upon IN influenza viral challenge.[43,44] In our study IL-17 production did not coincide with protection against lethal viral challenge and it remains to be elucidated whether the Th17 cells have an important function in protection against influenza viral disease.

We have previously shown that IM administration of the Matrix-M adjuvanted virosomal vaccine elicits high frequencies of multifunctional CD4+ T-cells simultaneously producing INF-γ, IL-2 and TNF-α.[20] In this study, we have confirmed and extended our previous results by showing that the multifunctional CD4+ T-cells also had a higher cytokine production than the single producers as indicated by MFI. We further found that the Matrix-M adjuvanted vaccine induced a high percentage of CD8+ T-cells when administered IM and importantly, it provided protection from lethal viral challenge after both IN and IM administration. A multifunctional T-cell response has previously been associated with protection or an improved outcome following disease.[27,28,33] In this study the IN vaccinated mice had the lowest multifunctional CD4+ T-cell response irrespective of adjuvant use, but only the virosomal alone IN vaccinated mice died from lethal viral challenge. A clear association between the vaccines ability to elicit multifunctional CD4+ T-cell responses and induce protection against challenge was thus not found in this study. But, the mice receiving vaccine formulated without adjuvant by the IN route had a low percentage of cytokine producing T-cells and the lowest levels of influenza specific IgG and haemagglutination inhibition antibody titres. From this study it was therefore not possible to point out one single endpoint as a surrogate correlate of protection and protection appeared to be multifaceted illustrating the importance of using a broad range of immunological methods combined with a protective efficacy study when evaluating candidate pandemic vaccines.

This is the first publication on the protective efficacy of a virosomal H5N1 vaccine further adjuvanted with Matrix-M. We conclude that both IM and IN administration of the vaccine formulation is promising in terms of protection against viral challenge and the studied immunogenicity markers.

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References

1 WHO. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO [cited 2010 0511]; Available from: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_11_19/en/index.html, 2010.
2 Manzoli L, Salanti G, De Vito C, Boccia A, Ioannidis JP, Villari P. Immunogenicity and adverse events of avian influenza A H5N1 vaccine in healthy adults: multiple-treatments meta-analysis. Lancet Infect Dis 2009; 9:482–492.
3 Morein B, Sundquist B, Hoglund S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature 1984; 308:457–460.
4 Sjolander A, Cox JC, Barr IG. ISCOMs: an adjuvant with multiple functions. J Leukoc Biol 1998; 64:713–723.
5 Sanders MT, Brown LE, Deliyannis G, Pearse MJ. ISCOM-based vaccines: the second decade. Immunol Cell Biol 2005; 83:119–128.
6 Barry DW, Mayner RE, Staton E *et al.* Comparative trial of influenza vaccines. I. Immunogenicity of whole virus and split product vaccines in man. Am J Epidemiol 1976; 104:34–46.
7 Mischler R, Metcalfe IC. Inflexal V a trivalent virosome subunit influenza vaccine: production. Vaccine 2002; 20(Suppl 5):B17–B23.
8 Huckriede A, Bungener L, Daemen T, Wilschut J. Influenza virosomes in vaccine development. Methods Enzymol 2003; 373:74–91.
9 Herzog C, Hartmann K, Kunzi V *et al.* Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine. Vaccine 2009; 27:4381–4387.
10 Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11:S45–S53.

11 Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. Vaccine 2007; 25:5467–5484.

12 Hasegawa H, Ichinohe T, Ainai A, Tamura S, Kurata T. Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses. Ther Clin Risk Manag 2009; 5:125–132.

13 Tamura S, Ito Y, Asanuma H *et al.* Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. J Immunol 1992; 149:981–988.

14 Barackman JD, Ott G, O'Hagan DT. Intranasal immunization of mice with influenza vaccine in combination with the adjuvant LT-R72 induces potent mucosal and serum immunity which is stronger than that with traditional intramuscular immunization. Infect Immun 1999; 67:4276–4279.

15 Ebensen T, Schulze K, Riese P, Morr M, Guzman CA. The bacterial second messenger cdiGMP exhibits promising activity as a mucosal adjuvant. Clin Vaccine Immunol 2007; 14:952–958.

16 Libanova R, Ebensen T, Schulze K *et al.* The member of the cyclic di-nucleotide family bis-(3',5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. Vaccine 2010; 28:2249–2258.

17 Sjolander S, Drane D, Davis R, Beezum L, Pearse M, Cox J. Intranasal immunization with influenza-ISCOM induces strong mucosal as well as systemic antibody and cytotoxic T-lymphocyte responses. Vaccine 2001; 19:4072–4080.

18 Coulter A, Harris R, Davis R *et al.* Intranasal vaccination with IS-COMATRIX adjuvanted influenza vaccine. Vaccine 2003; 21:946–949.

19 Cox RJ, Major D, Hauge S *et al.* A cell-based H7N1 split influenza virion vaccine confers protection in mouse and ferret challenge models. Influenza Other Respi Viruses 2009; 3:107–117.

20 Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine 2007; 25:3554–3560.

21 Suguitan AL Jr, McAuliffe J, Mills KL *et al.* Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. PLoS Med 2006; 3:e360.

22 Wood JM, Robertson JS. From lethal virus to life-saving vaccine: developing inactivated vaccines for pandemic influenza. Nat Rev Microbiol 2004; 2:842–847.

23 Visweswaraiah A, Novotny LA, Hjemdahl-Monsen EJ, Bakaletz LO, Thanavala Y. Tracking the tissue distribution of marker dye following intranasal delivery in mice and chinchillas: a multifactorial analysis of parameters affecting nasal retention. Vaccine 2002; 20:3209–3220.

24 Spearman C. The method of 'right and wrong cases' ('constant stimuli') without Gauss's formula. Br J Psychol 1908; 2:227–242.

25 Karber G. A contribution to the collective treatment of a pharmacological experimental series. Arch Exp Path Pharmak 1931; 162:480–487.

26 Cox RJ, Mykkeltvedt E, Robertson J, Haaheim LR. Non-lethal viral challenge of influenza haemagglutinin and nucleoprotein DNA vaccinated mice results in reduced viral replication. Scand J Immunol 2002; 55:14–23.

27 Hovden AO, Cox RJ, Haaheim LR. Whole influenza virus vaccine is more immunogenic than split influenza virus vaccine and induces primarily an IgG2a response in BALB/c mice. Scand J Immunol 2005; 62:36–44.

28 Madhun AS, Haaheim LR, Nilsen MV, Cox RJ. Intramuscular Matrix-M-adjuvanted virosomal H5N1 vaccine induces high frequencies of multifunctional Th1 CD4+ cells and strong antibody responses in mice. Vaccine 2009; 27:7367–7376.

29 Darrah PA, Patel DT, De Luca PM *et al.* Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med 2007; 13:843–850.

30 Duvall MG, Precopio ML, Ambrozak DA *et al.* Polyfunctional T cell responses are a hallmark of HIV-2 infection. Eur J Immunol 2008; 38:350–363.

31 Prieto-Lara E, Llanos-Mendez A. Safety and immunogenicity of prepandemic H5N1 influenza vaccines: a systematic review of the literature. Vaccine 2010; 28:4328–4334.

32 CHMP. Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application, 2008. http://www.ema.europa.eu/pdfs/human/vwp/471703en.pdf [database on the Internet].

33 Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur J Immunol 1984; 14:350–356.

34 Waldman RH, Wigley FMSPA Jr. Specificity of respiratory secretion antibody against influenza virus. J Immunol 1970; 105:1477–1483.

35 Shvartsman YS, Agranovskaya EN, Zykov MP. Formation of secretory and circulating antibodies after immunization with live and inactivated influenza virus vaccines. J Infect Dis 1977; 135:697–705.

36 Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008; 8:247–258.

37 Boni MF, Manh BH, Thai PQ *et al.* Modelling the progression of pandemic influenza A (H1N1) in Vietnam and the opportunities for reassortment with other influenza viruses. BMC Med 2009; 7:43.

38 Zhang Y, Lin X, Wang G *et al.* Neuraminidase and hemagglutinin matching patterns of a highly pathogenic avian and two pandemic H1N1 influenza A viruses. PLoS ONE 2010; 5:e9167.

39 Huber VC, McKeon RM, Brackin MN *et al.* Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza. Clin Vaccine Immunol 2006; 13:981–990.

40 Kistner O, Crowe BA, Wodal W *et al.* A whole virus pandemic influenza H1N1 vaccine is highly immunogenic and protective in active immunization and passive protection mouse models. PLoS ONE 2010; 5:e9349.

41 Radosevic K, Rodriguez A, Mintardjo R *et al.* Antibody and T-cell responses to a virosomal adjuvanted H9N2 avian influenza vaccine: impact of distinct additional adjuvants. Vaccine 2008; 26:3640–3646.

42 Zygmunt BM, Rharbaoui F, Groebe L, Guzman CA. Intranasal immunization promotes th17 immune responses. J Immunol 2009; 183:6933–6938.

43 Crowe CR, Chen K, Pociask DA *et al.* Critical role of IL-17RA in immunopathology of influenza infection. J Immunol 2009; 183:5301–5310.

44 Hamada H, Garcia-Hernandez Mde L, Reome JB *et al.* Tc17, a unique subset of CD8 T cells that can protect against lethal influenza challenge. J Immunol 2009; 182:3469–3481.