DOI:10.1111/irv.12056
www.influenzajournal.com

Original Article

A study of Chitosan and c-di-GMP as mucosal adjuvants for intranasal influenza H5N1 vaccine

Signe C. Svindland,[a] Gabriel K. Pedersen,[a] Rishi D. Pathirana,[a] Geir Bredholt,[a] Jane K. Nøstbakken,[a] Åsne Jul-Larsen,[a] Carlos A. Guzmán,[b] Emanuele Montomoli,[c,d] Giulia Lapini,[c] Simona Piccirella,[d] Inderjit Jabbal-Gill,[e] Michael Hinchcliffe,[e] Rebecca J. Cox[a,f]

[a]Influenza Centre, The Gade Institute, University of Bergen, Bergen, Norway. [b]Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany. [c]University of Siena, Siena, Italy. [d]VisMederi, Siena, Italy. [e]Archimedes Development Ltd., Reading, UK. [f]Department of Research and Development, Haukeland University Hospital, Bergen, Norway.
Correspondence: Rebecca J. Cox, Influenza Centre, The Gade Institute, University of Bergen, N-5021 Bergen, Norway.
E-mail: rebecca.cox@gades.uib.no

Accepted 14 September 2012. Published Online 21 November 2012.

Background Highly pathogenic avian influenza A/H5N1 virus remains a potential pandemic threat, and it is essential to continue vaccine development against this subtype. A local mucosal immune response in the upper respiratory tract may stop influenza transmission. It is therefore important to develop effective intranasal pandemic influenza vaccines that induce mucosal immunity at the site of viral entry.

Objectives We evaluated the humoral and cellular immune responses of two promising mucosal adjuvants (Chitosan and c-di-GMP) for intranasal influenza H5N1 vaccine in a murine model. Furthermore, we evaluated the concept of co-adjuvanting an experimental adjuvant (c-di-GMP) with chitosan.

Methods BALB/c mice were intranasally immunised with two doses of subunit NIBRG-14 (H5N1) vaccine (7·5, 1·5 or 0·3 μg haemagglutinin (HA) adjuvanted with chitosan (CSN), c-di-GMP or both adjuvants.

Results All adjuvant formulations improved the serum and local antibody responses, with the highest responses observed in the 7·5 μg HA CSN and c-di-GMP-adjuvanted groups. The c-di-GMP provided dose sparing with protective single radial haemolysis (SRH), and haemagglutination inhibition (HI) antibody responses found in the 0·3 μg HA group. CSN elicited a Th2 response, whereas c-di-GMP induced higher frequencies of virus-specific $CD4^+$ T cells producing one or more Th1 cytokines (IFN-γ^+, IL-2^+, TNF-α^+). A combination of the two adjuvants demonstrated effectiveness at 7·5 μg HA and triggered a more balanced Th cytokine profile.

Conclusion These data show that combining adjuvants can modulate the Th response and in combination with ongoing studies of adjuvanted intranasal vaccines will dictate the way forward for optimal mucosal influenza vaccines.

Keywords Chitosan, c-di-GMP, H5N1, influenza, intranasal, Th17, vaccine.

Please cite this paper as: Svindland *et al.* (2012) A study of Chitosan and c-di-GMP as mucosal adjuvants for intranasal influenza H5N1 vaccine. Influenza Other Respiratory Viruses 7(6), 1181–1193.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 virus is now epizootic in large parts of South East Asia and remains a threat to human health. Although HPAI H5N1 viruses have not yet gained the ability for continuous human-to-human transmission, recent studies have found that only a few mutations are required for the viruses to become transmissible by the aerosol route in ferrets, which are commonly used to model human influenza infection.[1,2] Vaccination is the cornerstone of influenza prophylaxis. Pandemic influenza vaccines can limit viral spread, hospitalisations, serious complications of disease and ultimately death. During the influenza pandemic in 2009, the virus spread globally within 2 months, highlighting the importance of rapid deployment of vaccine. Developing countries had limited access to pandemic vaccines, and a needle free mucosal influenza vaccine would have allowed vaccination with limited numbers of public health providers.

Intranasally administered inactivated pandemic vaccines are poorly immunogenic requiring effective immunological adjuvants (reviewed in Ref. 3). The mucosal epithelium of the nose and upper respiratory tract is lined by mucosal associated lymphoid tissue (MALT) in which T cells, B cells and antigen-presenting cells (APCs) reside. Between the mucosal epithelial cells are microfold cells, which transport

luminal antigens into the MALT. APCs stimulate $CD4^+$ T cells, which further direct the B cells towards IgA production and affinity maturation either in the organised mucosal inductive site, for example, tonsils or the draining lymph nodes. $CD4^+$ T cells differentiate into distinct T helper (Th) subsets (reviewed in Ref. 4) of which Th1 cells produce IFN-γ, TNF-α and IL-2 and Th2 cells produce IL-4, IL-5, IL-10 and IL-13.[5–7] The Th17 subset is characterised by IL-17, IL-21 and IL-22 secretion and stimulated by different factors including TGF-ß and IL-23.[8–10] Influenza virus infection stimulates Th1 and Th17 responses.[10–13] Whereas some inactivated influenza vaccine formulations induce a Th2 polarisation, others elicit a more balanced response.[14–17] Adjuvants influence the Th responses, and it is well established that aluminium salts skew the Th response towards Th2. Interestingly, mice vaccinated with alum adjuvanted whole virus vaccine had more severe disease following influenza challenge than mice receiving whole virus vaccine alone, despite having higher haemagglutination inhibition (HI) titres.[18] In mice, distinct roles for IgG1 and IgG2a, associated with a Th2 and Th1 response, respectively, have been implicated in anti-influenza immunity,[19] suggesting a need for influenza vaccine formulations, which induce a balanced Th response.

Effective mucosal adjuvants reduce the amount of antigen needed to induce a protective immune response. Chitosan, being mucoadhesive, is thought to enhance the delivery of antigen by reducing clearance of the vaccine formulation from the nasal cavity thereby optimising its interaction with nasal immune tissue.[20,21] *In vitro* studies have also shown that chitosan may promote paracellular transport through a transient opening of intercellular tight junctions.[22] CSN is a safe mucosal adjuvant,[23] which augmented the immune response to intranasally administered influenza vaccine.[24] The bacterial second messenger (3′, 5′)-cyclic dimeric guanylic acid (c-di-GMP) has been identified in bacteria but not in higher eukaryotes (reviewed in Ref. 25), and several studies have emphasised its adjuvant potential.[26–29] The transmembrane protein stimulator of interferon genes (STING) was recently shown to function as a direct sensor for c-di-GMP and other cyclic dinucleotides.[30,31] A proposed mechanism for c-di-GMPs adjuvant properties is that STING ligation increases the production of type I interferons,[32] which in turn drives the adaptive immune response.

In this study, we have evaluated CSN, c-di-GMP and a combination of the two adjuvants in a dose response study of an intranasal subunit (SU) influenza H5N1 vaccine. The humoral and cellular immune responses were evaluated and compared between the different vaccine formulations. Both adjuvants augmented the immune response, but the Th profile differed with CSN eliciting a Th2-biased response, c-di-GMP a Th1-biased response and the adjuvant combination a more balanced Th profile. The c-di-GMP adjuvant was most effective at boosting local and systemic humoral immune responses and allowed significant dose sparing.

Materials and methods

Materials

Inactivated influenza subunit vaccine (NIBRG-14) and chitosan adjuvant (CSN, ChiSys®) were supplied by Archimedes Development Ltd., Reading, UK. The chitosan utilised in the study was chitosan glutamate 213 (manufactured by FMC BioPolymer AS, Drammen, Norway) which was 75–90% deacetylated and had a glutamate content of 35–50%. The bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) adjuvant was produced at the Helmholtz Centre for Infection Research as previously described.[28] The antigen was mixed with adjuvant immediately prior to vaccination.

Animals and vaccination

A dose-sparing study was conducted by intranasally immunising mice (twelve groups with five mice in each group) with two doses (21 days apart) of NIBRG-14 SU with or without CSN or c-di-GMP or a combination of the two adjuvants. The study was approved and conducted according to the Norwegian Animal Welfare Act.

Six- to eight-week-old female BALB/c mice (Taconic M&B, Denmark) were housed at the Vivarium, University of Bergen at a temperature of 21°C with 12 hour light/dark cycles and food and water *ad libitum*. The mice were ear-marked, and each mouse was weighed before the first immunisation, and three and two weeks after the first and second immunisation, respectively. Prior to vaccination, the mice were anaesthetised by SC injection of 150 μl ketamine hydrochloride (10 mg/ml) (Ketalar; Pfizer, Kent, UK) and xylazine hydrochloride (1 mg/ml) (Rompun, Bayer, Germany). The anaesthetised mice were intranasally immunised with two doses (of a maximum of 5·5 μl per nostril at 5 minute intervals) of the appropriate NIBRG-14 SU vaccine formulation (7·5 1·5 or 0·3 μg HA). For each dose, groups of mice received the vaccine as antigen alone or formulated with CSN glutamate (82·5 μg), c-di-GMP (5 μg) or a combination of CSN glutamate (82·5 μg) and c-di-GMP (5 μg). Due to formulation issues (precipitation), the groups that were immunised with the adjuvant combination vaccine first received the antigen mixed with CSN and after 30 seconds received c-di-GMP as a pilot study. A control group received PBS alone.

Sample collection

Blood and nasal wash samples were collected at 2 and 3 weeks after the second immunisation and stored at −80°C. Spleens were collected at the time of sacrifice (3 weeks post-2nd vaccination).

Haemagglutination inhibition assay

The haemagglutination inhibition assay was performed at the University of Siena, Italy. Sera were treated with receptor-destroying enzyme (RDE) (one volume of serum to four volumes of RDE) and tested by a haemagglutination inhibition assay, using 0·7% turkey red blood cells (TRBC) and 8 HA units of whole inactivated H5N1 (NIBRG-14) virus.[24] The HI titre was expressed as the reciprocal of the highest dilution at which haemagglutination was inhibited, and titres <10 were assigned a value of 5 for calculation purposes.

Single radial haemolysis assay

Single radial haemolysis (SRH) was performed at the University of Siena, Italy, based on a reference method standardised by Schild and colleagues.[24,33] SRH plates were prepared using TRBC (10%), to which were added 2000 Haemagglutinin Units/ml of inactivated whole virus (WV). All samples were heat inactivated (56°C, 30 minutes), and 6 μl volumes of sera were added to duplicate sets of plates. The diameters of the haemolytic zones were measured using a Transidyne Calibrating Viewer (Transidyne General Corporation, Ann Arbor, MI, USA). A negative control sample and a positive control serum (sheep hyperimmune sera, National Institute for Biological Standards and Controls, UK) were included.

Virus neutralisation assay

Sera were tested for neutralising antibodies against the homologous NIBRG-14 strain and the heterologous clade 2·1 A/Indonesia/05/2005 (H5N1). All sera were heat inactivated at 56°C for 30 minutes and then serially diluted, using a starting dilution of 1:10. The diluted sera were incubated with 100 tissue culture infective dose 50 of virus for 1 hour, and the mixture was added to MDCK-SIAT1 cell layer (90% confluent). After 5 days of incubation, each well was scored for cytopathogenic effects (CPE). The neutralisation titre was defined as the serum dilution for which the cells were 50% protected from CPE and according to the Spearman–Kärber formula.[34,35]

Enzyme-linked immunosorbent assay

The influenza-specific serum and nasal wash immunoglobulin class (IgG and IgA) and subclass (IgG1 and IgG2a) were quantified using an ELISA assay, as previously described.[24,36] Enzyme-linked immunosorbent assay (ELISA) plates were coated with inactivated WV influenza (H5N1 (NIBRG-14 (Abbott, Hoofddorp, the Netherlands) or capture goat anti-mouse IgA, IgG, IgG1 or IgG2a (Southern Biotechnology, Birmingham, AL, USA). Dilutions of sera, nasal washes and antibody standards (mouse IgA, IgG, IgG1 or IgG2a) (Sigma, St. Louis, MO, USA), were added, and bound antibodies were detected with goat anti-mouse immunoglobulin class or subclass-specific biotin-conjugated antibody (Southern Biotechnology). The antibody concentrations (ng/ml) were calculated using the IgG, IgG1, IgG2a and IgA standards and linear regression of the log-transformed readings.

Isolation of splenocytes

Lymphocytes were isolated from the spleen as previously described[24,37] and resuspended in lymphocyte medium (RPMI 1640 with l-glutamine, 0·1 mm non-essential amino acids, 10 mm Hepes pH 7·4, 1 mm sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml fungizone and 10% foetal calf serum) at a density of $1{\cdot}0 \times 10^7$ cells/ml and used in the immunological assays described later.

Spleen cell proliferation assay

Splenocytes from individual mice were seeded in duplicate 96-well plates at $2{\cdot}5 \times 10^5$ cells per well with 0·125 μg/ml HA of NIBRG-14 subunit (HA and NA) antigen and medium only. Cells were cultured for 72 hours before 1 μCi ^{3}H-thymidine (Perkin Elmer, Boston, MA, USA) was added per well and cultured for 16 hours. Cultures were harvested onto filter plates (Perkin Elmer) using a cell harvester (Packard, Rockville, MD, USA). The filter plates were dried overnight, and 10 μl scintillation fluid (Perkin Elmer) was added. The plates were sealed, and incorporation of ^{3}H-thymidine was determined as counts per minute (cpm) using a β-scintillation counter (Packard). Background cpm (medium only) was subtracted from cpm of cells cultured with influenza antigen.

Bio-plex cytokine analysis

Splenocytes ($1{\cdot}0 \times 10^6$ cells) were incubated in lymphocyte medium containing virosomal influenza H5N1 antigen (containing HA and NA from NIBRG-14, kindly provided by Crucell, Leiden, the Netherlands) (1 μg HA/ml), mitogen (Phorbol myristate acetate (10 ng/ml) and ionomycin (250 ng/ml) (Sigma-Aldrich) (positive control) or medium alone (negative control) at 37°C and 5% CO_2 for 72 hours. The concentrations of cytokines secreted into the supernatants from stimulated spleen cells were evaluated using multiplex kits (Bio-rad, Hercules, CA, USA) to detect Th1 (IL-2, TNF-α and INF-γ), Th2 (IL-4, IL-5 and IL-10) and Th17 (IL-17F, IL-21, IL-22 and IL-23) cytokines according to the manufacturer's instructions.

Flow cytometry for multifunctional CD4$^+$ Th1 cells

Multifunctional T cells were detected as previously described.[14,36] Lymphocytes were incubated (37°C, 12 hours, 5% CO_2) with virosomal H5N1 influenza antigen (Crucell) (1 μg/ml), 2 μg/ml anti-CD-28 antibody (Pharmingen,

USA) and 10 μg/ml Brefeldin A (BD Biosciences, USA) in lymphocyte medium before intracellular cytokine staining. Splenocytes were also incubated in lymphocyte medium only (no antigen), and lymphocytes from non-immunised mice were incubated in medium with or without influenza antigen and used as controls. Subsequently, cells were stained for CD3, CD4, CD8, INF-γ, IL-2 and TNF-α (BD Biosciences) using the BD Cytofix/Cytoperm kit. Cells were acquired (≥300 000 cells per sample) using a BD FACSCanto flow cytometer. FlowJo v8.8.6 software (Tree Star, Ashland, OR, USA), Pestle and SPICE v5.21 (Mario Roederer; Vaccine Research Centre, NIH, Bethesda, MD, USA) were used to analyse data.[38]

Statistical analysis

The one-way anova test with Tukey's post hoc test (GraphPad Prism, La Jolla, CA, USA) was performed to analyse differences between groups, and a P value $< 0{\cdot}05$ was considered to be statistically significant. T-cell distributions were compared using the Wilcoxon Signed Rank test integrated in SPICE.[38]

Results

This study aimed to investigate the quality and magnitude of the B- and T-cell responses in mice after intranasal vaccination with an H5N1 subunit vaccine (NIBRG-14 SU). The effect of two different adjuvants (CSN and c-di-GMP) and a combination of the two adjuvants were evaluated. To assess the dose-sparing capabilities of the adjuvants, groups of mice were immunised with different doses (7·5, 1·5 or 0·3 μg HA) of NIBRG-14 SU alone or with one or both of the adjuvants.

Adjuvant augments the HI, SRH and VN antibody response.

The serum influenza-specific humoral immune responses are commonly measured by the HI, SRH and VN assays. An HI titre ≥40 or SRH zone area of ≥25 mm^2 has been associated with a 50% probability of being clinically protected against seasonal influenza,[39] and these cut-off values are used as a surrogate correlate of protection when evaluating candidate pandemic influenza vaccines. No correlate of protection has been established for VN, although titres of 20–80 have been suggested for H5N1 viruses. The post-vaccination HI, SRH and VN titres were measured in cardiac blood collected 3 weeks after the second immunisation (Figure 1A,B,C).

No HI antibody was observed in the control group and in the antigen alone groups, except in 2 mice in the 7·5 μg HA antigen alone group (HI titres 10 and 20) (Figure 1A). HI antibody responses were elicited in all the mice in the 7·5 μg HA-adjuvanted groups, with the highest titres detected in the groups receiving the 7·5 μg HA with the adjuvant combination (significantly higher ($P < 0{\cdot}05$) than all the other 7·5 μg HA and the 1·5 and 0·3 μg HA combination groups). Also mice in the 1·5 and 0·3 μg HA c-di-GMP-adjuvanted groups had detectable HI titres, with 3 of 5 mice having protective HI titres in each group. In the 7·5 μg HA CSN-adjuvanted group, all mice had detectable titres, and in 3 of 5 mice, the titres were above 40.

SRH antibody was not detected in the control animals or 1·5 μg and 0·3 μg HA antigen alone groups, except in 1 mouse in the control group that had very low SRH titres (Figure 1B).

SRH titres <25 mm^2 were detected in the 7·5 μg HA antigen alone mice. All the 7·5 μg HA-adjuvanted vaccine groups had significantly higher SRH titres than the antigen alone group. The highest titres were observed in the 7·5 μg HA c-di-GMP, and lower, albeit protective, titres were observed in the two lower dose (1·5 and 0·3 μg HA) c-di-GMP-adjuvanted groups. All mice in the 7·5 μg HA CSN-adjuvanted group had an SRH zone area >25 mm^2. Thus, in summary, mice receiving 7·5 μg HA formulated with c-di-GMP, CSN or a combination of the adjuvants had SRH titres ≥25 mm^2.

To evaluate the capability of the serum antibodies to neutralise virus, we performed a virus neutralisation assay. Serum from the non-adjuvanted groups failed to neutralise virus, although one mouse in the 7·5 μg group had a VN titre of 15. In contrast, all mice in each of the 7·5 μg HA-adjuvanted groups were seropositive in the assay (Figure 1C). Furthermore, VN titres in all the 7·5 μg HA-adjuvanted groups were significantly ($P < 0{\cdot}05$) higher than in the non-adjuvanted group. The c-di-GMP adjuvant also provided dose sparing with all mice seropositive in the 1·5 and 0·3 μg HA-adjuvanted groups. The adjuvant combination was only effective at the 7·5 μg HA dose.

Cross-reactive responses

The high mutation rate of influenza H5N1 viruses necessitates effective strategies to elicit cross-reactive vaccine responses. We therefore continued to evaluate the cross-clade neutralising antibodies against the antigenically distinct clade 2·1 virus A/Indonesia/05/2005 (H5N1) (Figure 1D). No neutralising antibody directed against the A/Indonesia/05/2005 (H5N1) virus was observed in the antigen alone groups, whilst 4 of 5 mice in each of the 7·5 μg-adjuvanted groups were seropositive. In addition, 4 of 5 mice in the 1·5 μg c-di-GMP-adjuvanted group and 2 of 5 mice in the 0·3 μg c-di-GMP-adjuvanted group had cross-clade neutralising antibodies.

Adjuvant augments the local IgA response

Effective mucosal adjuvants should boost the concentrations of nasal influenza-specific IgA, which can neutralise influenza at the site of viral entry. Figure 2A shows the

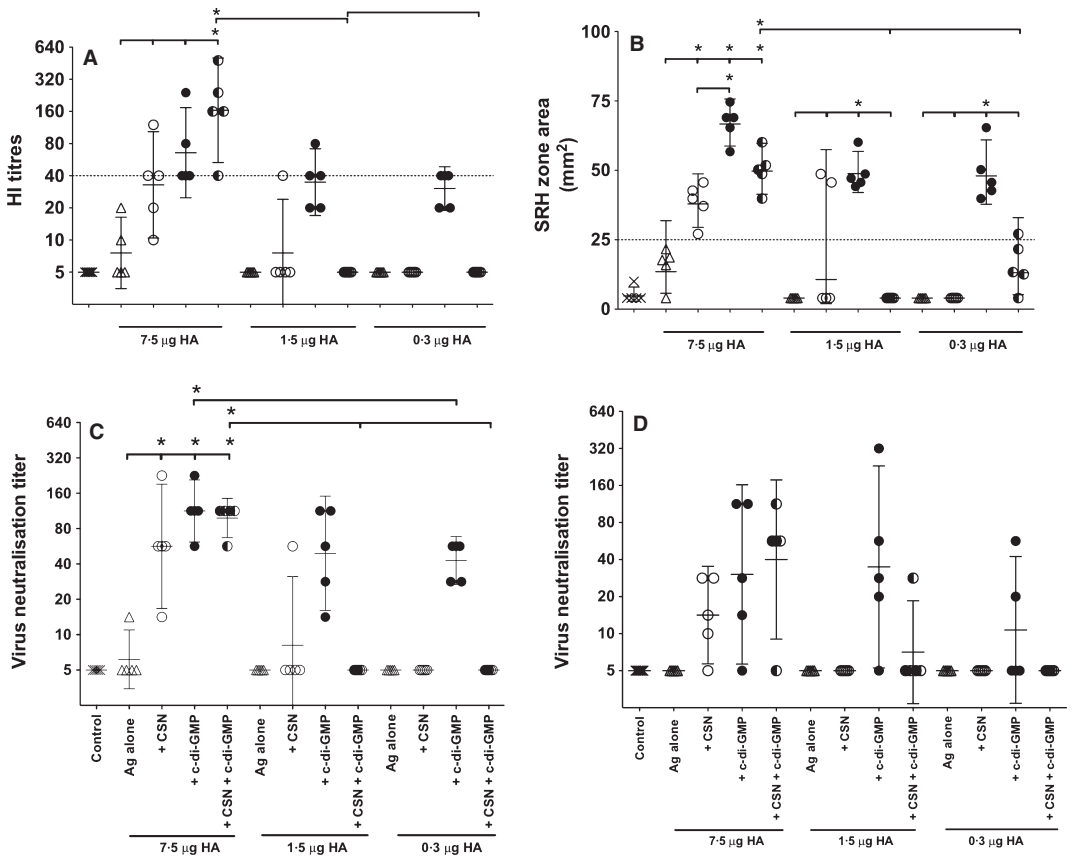

Figure 1. The serological antibody response induced after vaccination. Groups of 5 mice were immunised with two doses (21 days apart) of H5N1 NIBRG-14 subunit vaccine [7·5, 1·5 or 0·3 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (CSN) (82·5 μg), c-di-GMP (5 μg) or a combination of the two adjuvants. Control mice were mock immunised with PBS. (A) serum haemagglutination inhibition (HI) titres, (B) single radial haemolysis (SRH) zone areas (mm^2) and (C) virus neutralisation titres were measured at 3 weeks after the second vaccination against the homologous strain. (D) cross-clade neutralising antibody responses against A/Indonesia/05/2005 (H5N1). The lines represent the geometric mean titre (GMT) ± 95% confidence interval, and each symbol represents one animal. The limit of detection of the HI and VN assays was 10 and negative titres were assigned an arbitrary value of 5. The dotted lines represent the protective HI titre (40) and SRH (25 mm2) zone areas. *Indicates statistical significant difference ($P < 0·05$) measured by the anova with Tukeys post hoc test.

local IgA concentration measured in nasal wash samples collected 2 and 3 weeks after the second dose. The highest response was generally observed 2 weeks after the second dose. The 7·5 μg HA c-di-GMP and 7·5 μg HA CSN + c-di-GMP-adjuvanted groups had significantly higher IgA concentrations ($P < 0·05$) than the corresponding antigen alone group. At 3 weeks after the second dose, the highest responses were observed in the adjuvanted groups, with 7·5 μg HA c-di-GMP and c-di-GMP + CSN-adjuvanted groups having significantly higher ($P < 0·05$) IgA concentrations than corresponding 1·5 and 0·3 μg HA groups.

Serum IgA and IgG response

The influenza-specific serum IgA and IgG antibodies were measured in the ELISA assay 2 and 3 weeks after the 2nd dose (Figure 2B,C). All adjuvants augmented the serum IgA and IgG responses with only low concentrations of IgG detected in the antigen alone groups. The 7·5 μg HA c-di-GMP-adjuvanted group had the highest serum IgA response, and this response was significantly higher than that found in the chitosan-adjuvanted group and the antigen alone group ($P < 0·05$). The CSN + c-di-GMP adjuvant combination also boosted serum IgA in the 7·5 and 1·5 μg HA groups but not at the lowest dose of 0·3 μg HA. The 7·5 μg HA CSN + c-di-GMP-adjuvanted group had the highest IgG concentrations, which were significantly higher ($P < 0·05$) than those observed in the c-di-GMP, CSN-adjuvanted groups and antigen alone groups at 2 weeks after the second dose. We further analysed the IgG response by investigating the IgG1 and IgG2a subclasses by ELISA (Figure 3A–D). IgG1 is indicative of a Th2 and IgG2a of a Th1 response. The CSN-adjuvanted vaccine induced high levels of IgG1, particularly at the high dose 7·5 μg HA (≥100 μg/ml), but low IgG2a concentrations (<4 μg/ml) at all doses and time points tested, thus indicating a Th2-biased response. The c-di-GMP-adjuvanted vaccine induced

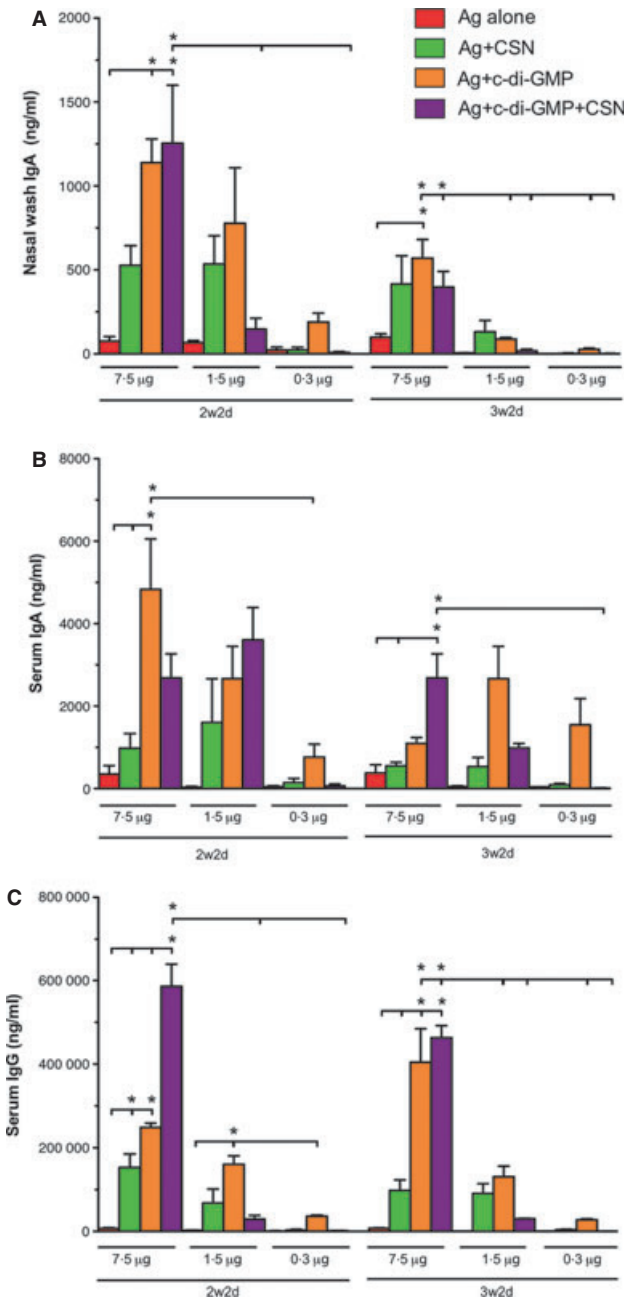

Figure 2. The local and systemic antibody responses elicted after vaccination. Groups of 5 mice were immunised at 21 days interval with two doses of H5N1 NIBRG-14 subunit vaccine [7·5, 1·5 or 0·3 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (CSN) (82·5 μg), c-di-GMP (5 μg) or a combination of the adjuvants. (A) nasal wash IgA and serum (B) IgG and (C) IgA were measured by ELISA at two (2w2d) and three (3w2d) weeks post second vaccination. The bar reflects the mean antibody concentration (ng/ml) ± the standard error of the mean. *Indicates statistical significant difference ($P < 0·05$) measured by the anova with Tukeys post hoc test.

a higher production of IgG2a and an IgG2a/IgG1 ratio ≥1, supporting a more balanced or Th1 type of response. The adjuvant combination induced high levels of both IgG1 and IgG2a, but the concentrations of IgG1 were highest, thus indicating a more Th2-biased response.

In summary, the c-di-GMP or combination CSN + c-di-GMP groups had the highest humoral responses followed by the CSN group. Although the c-di-GMP adjuvant provided significant dose sparing, lower concentrations of influenza-specific antibodies were generally found in the 1·5 and 0·3 μg HA groups than in the 7·5 μg HA group.

Adjuvant increases the proliferation of spleen cells

Proliferation of splenocytes was evaluated 3 weeks after the second immunisation. Both CSN and c-di-GMP effectively enhanced proliferation as compared to antigen alone (Figure 4). Significantly higher counts per minute were observed in all c-di-GMP-adjuvanted groups than in the corresponding CSN-adjuvanted groups. The combination of c-di-GMP with CSN offered no further boost in responses as compared to the groups receiving only c-di-GMP and antigen (Figure 4). No antigen dose response in the proliferative response was observed neither in the adjuvanted nor the non-adjuvanted groups.

The adjuvant affects the T helper profile

Cytokines produced by spleen cells collected 3 weeks after the second immunisation were measured in the Bio-plex cytokine assay to evaluate the concentrations of cytokines typically produced by Th1 (IL-2, IFN-γ and TNF-α) and Th2 (IL-4, IL-5 and IL-10) cells. Splenocytes from the c-di-GMP-adjuvanted groups produced higher concentrations of Th1 cytokines than cells from the non-adjuvanted or CSN-adjuvanted groups (Figure 5A,B,C). This difference was statistically significant ($P < 0·05$) for all the measured Th1 cytokines (IL-2, IFN-γ and TNF-α) at antigen concentrations of both 7·5 and 1·5 μg HA. In contrast, only low Th1 cytokine concentrations were observed in the CSN-adjuvanted and antigen alone groups. For the Th2 cytokines, there was a tendency towards higher concentrations in the CSN than the c-di-GMP-adjuvanted groups, although significant differences were only observed for IL-5 in the 0·3 μg HA groups (Figure 5D,E,F).

The groups receiving H5N1 vaccine with the adjuvant combination had lower IL-2 and IFN-γ concentrations than the c-di-GMP-adjuvanted groups, but similar Th2 cytokine concentrations to those observed in the CSN-adjuvanted groups. When analysing the dose-sparing capabilities of the two adjuvants, we observed that in the c-di-GMP-adjuvanted groups, cytokine responses of similar magnitude were observed between the 7·5 μg and 1·5 μg HA doses, whilst significantly lower concentrations ($P < 0·05$) of Th1 cytokines were found in the groups receiving the 0·3 μg HA dose. For the CSN-adjuvanted and antigen alone groups, no clear dose response was observed.

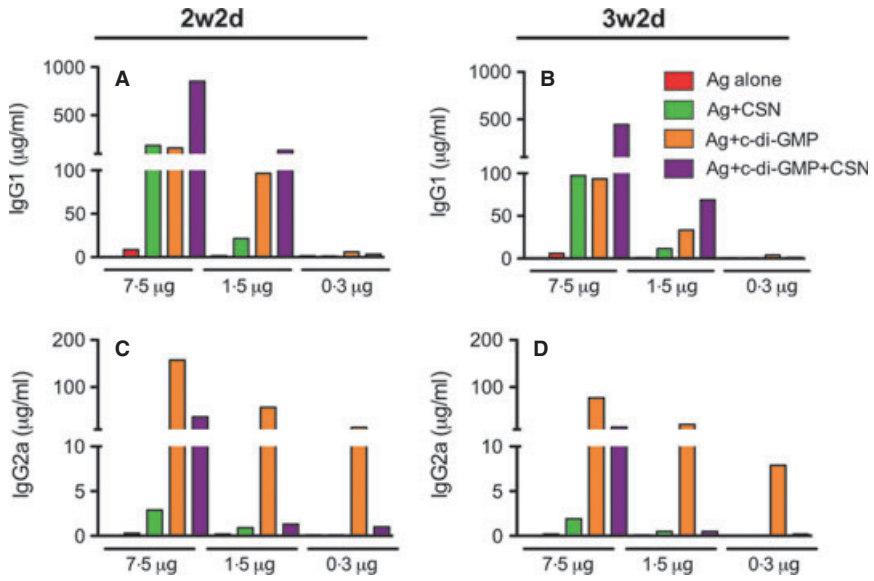

Figure 3. The ELISA subclass responses. Groups of 5 mice were immunised with two doses (21 days apart) of H5N1 NIBRG-14 subunit vaccine [7·5, 1·5 or 0·3 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (CSN) (82·5 μg), c-di-GMP (5 μg) or a combination of the two adjuvants. Control mice were mock immunised with PBS. Serum IgG subclasses were measured by ELISA at two (2w2d) and three (3w2d) weeks after the second vaccine dose. (A) IgG1 2w2d, (B) IgG1 3w2d, (C) IgG2a 2w2d and (D) IgG2a 3w2d. The assay was performed on pooled sera from 5 mice in each group.

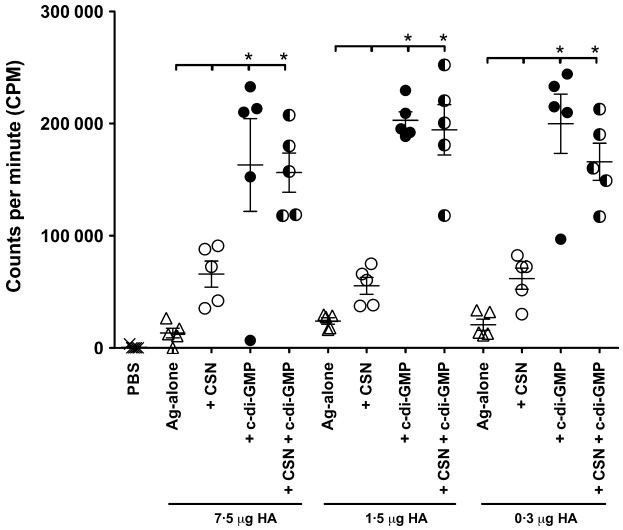

Figure 4. The proliferative response elicited after vaccination. Groups of 5 mice were immunised with two doses (21 days apart) of H5N1 NIBRG-14 subunit vaccine [7·5, 1·5 or 0·3 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (CSN) (82·5 μg), c-di-GMP (5 μg) or a combination of the two adjuvants. Control mice were mock immunised with PBS. The proliferative response was measured in lymphocytes isolated from the spleen 3 weeks after the second dose by stimulation with homologous virosomal H5N1 antigen. Each symbol represents one animal and the bar shows the mean proliferative responses (cpm) ± standard error of the mean. *Indicates statistical significant different ($P < 0{\cdot}05$) measured by the anova with Tukeys post hoc test.

Th17 responses may be induced by influenza infection[11] and are generally observed following intranasal administration of vaccines.[40] We found that the subunit vaccine alone elicited only low levels of the Th17 cytokines IL-17F, IL-21, IL-22 and IL-23 (Figure 6A–D). In contrast, both the adjuvants boosted Th17 cytokine production, with c-di-GMP being the most potent adjuvant. Furthermore, similar magnitudes of all Th17 cytokines, except IL-23 were observed in the adjuvant combination and c-di-GMP-adjuvanted groups. In summary, the c-di-GMP-adjuvanted subunit H5N1 vaccine produced a Th1/Th17-biased response, whilst the CSN-adjuvanted and subunit vaccine alone groups had a cytokine profile indicating a more balanced or Th2/Th17 skewed response. The subunit H5N1 vaccine administered with the adjuvant combination induced significantly lower concentrations of Th1 cytokines, and a tendency towards higher concentrations of Th2 cytokines, than found in the c-di-GMP-adjuvanted group.

The adjuvants boosted the multifunctional Th1 cell frequencies

In this study, we have investigated the capability of the CSN and c-di-GMP adjuvants to boost the frequency of influenza-specific $CD3^+$ $CD4^+$ T cells producing one or more of the Th1 cytokines IL-2, IFN-γ and TNF-α. The subunit vaccine alone failed to induce Th1 cells, except for a low cell frequency (<0·1%) in the 1·5 μg HA group (Figure 7A). When the vaccine was administered in combination with one or both of the adjuvants, higher frequencies of influenza-specific Th1 cells were observed. The CSN-adjuvanted vaccine groups had a mean of 0·2–0·4% cells producing one or more of the measured cytokines, whilst significantly higher ($P < 0{\cdot}05$) frequencies (mean of 3–7%) were observed in the c-di-GMP-adjuvanted groups. The response in the c-di-GMP adjvanted groups was also significantly higher than that found in the groups receiving the adjuvant combination, in which a mean of 1·5–2% influenza-specific Th1 cells were detected. The frequency of influenza-specific Th1 cells did not follow an antigen dose response.

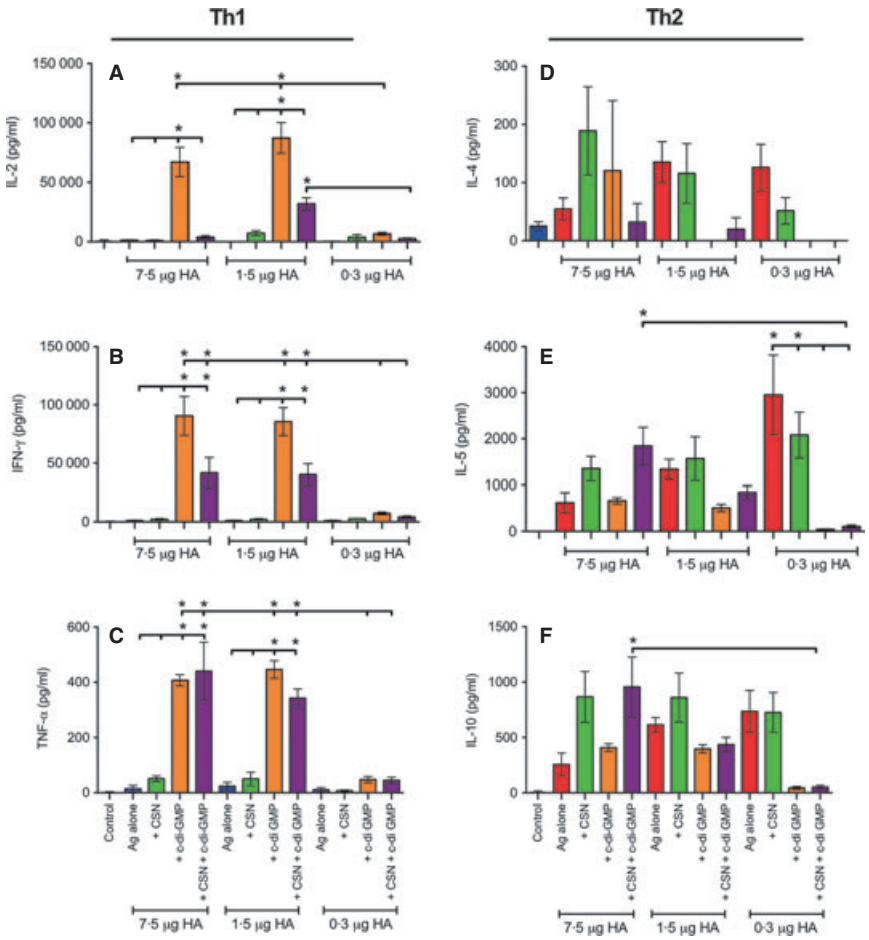

Figure 5. The cytokine response induced in the spleen after influenza vaccination. Mice were immunised with two doses at 21 days interval of H5N1 NIBRG-14 subunit vaccine [7·5, 1·5 or 0·3 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (CSN) (82·5 μg), c-di-GMP (5 μg) or a combination of two the adjuvants. Lymphocytes were isolated from the spleens 3 weeks after the second vaccine dose and were activated *in vitro* with homologous virosomal H5N1 antigen for 72 hours. Supernatants were used to measure the concentration (pg/ml) of influenza-induced Th1 (IL-2, IFN-γ and TNF-α, (A–C), and Th2 (IL-4, IL-5 and IL-10, (D–F) cytokines by Bio-Plex cytokine assay. The data are presented as the mean cytokine concentration (pg/ml) ± standard error of the mean. *Indicates significant difference ($P < 0{\cdot}05$) measured by the anova with Tukeys post hoc test.

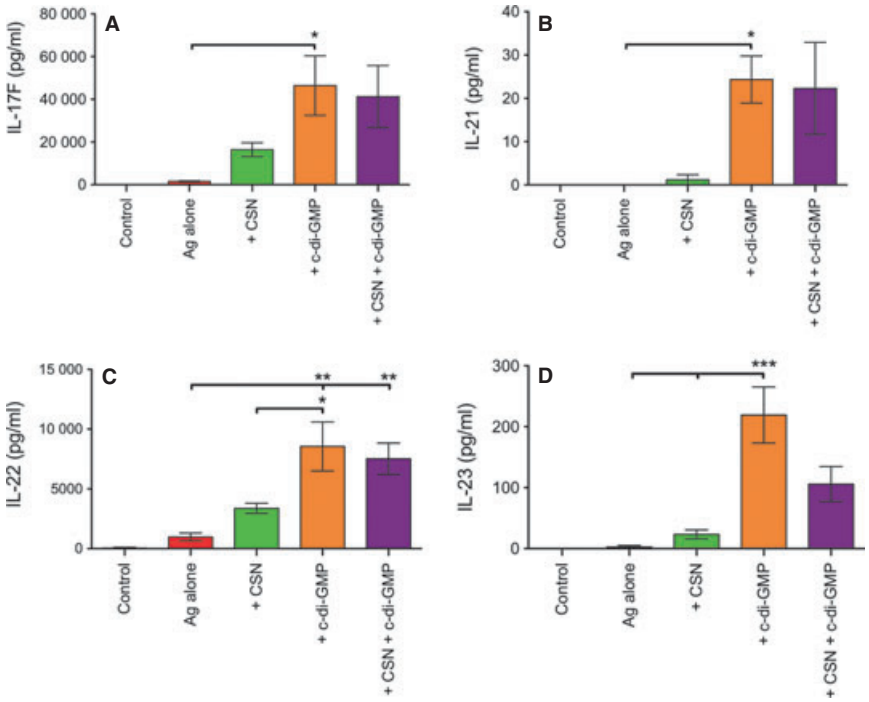

Figure 6. The Th17 cytokine response induced in the spleen after influenza H5N1 vaccination. Mice were immunised with two doses at 21 days interval of H5N1 NIBRG-14 subunit vaccine [7·5 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (CSN) (82·5 μg), c-di-GMP (5 μg) or a combination of two the adjuvants. Lymphocytes were isolated from the spleens 3 weeks after the second immunisation and were activated *in vitro* with homologous virosomal H5N1 antigen for 72 hours. Supernatants were used to measure the concentration of (A) IL-17F, (B) IL-21, (C) IL-22, and (D) IL-23 cytokines by Bio-Plex cytokine assay. The data are presented as the mean cytokine concentration (pg/ml) ± standard error of the mean. *Indicates significant difference ($P < 0{\cdot}05$) measured by the anova with Tukeys post hoc test.

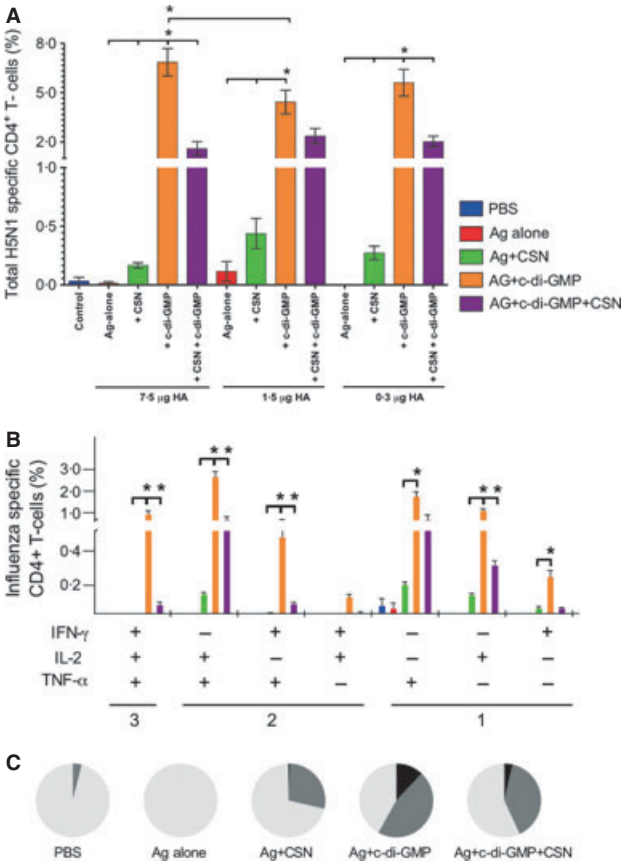

Figure 7. The CD4$^+$ Th1 cytokine response induced in the spleen after influenza H5N1 vaccination. The influenza H5N1 specific CD4$^+$ Th1 cytokine-producing response induced in the spleen after influenza vaccination. Groups of 5 mice were immunised with two doses at 21 days interval of H5N1 vaccine subunit [7·5, 1·5 or 0·3 μg haemagglutinin (HA)] alone or adjuvanted with chitosan (82·5 μg), c-di-GMP (5 μg) or a combination of the adjuvants. Control mice were mock immunised with PBS. Three weeks after the second vaccine dose, splenocytes were activated *in vitro* with H5N1 NIBRG-14 virosomes and intracellularly stained for the Th1 cytokines IFN-γ, IL-2 and TNF-α. Subsequently, the cells were analysed flow cytometric analysis. (A) All CD4$^+$ T cells producing one or more of the measured cytokines were summed to quantify the total frequency of influenza-specific Th1 cells from each mouse with Tukeys post hoc test. *Indicates statistical significant difference ($P < 0{\cdot}05$) measured by the anova. (B) The data show the mean frequencies of CD4$^+$ cells expressing each of the seven possible combinations of IFN-γ, IL-2 and TNF-α for each individual animal in the 7·5 μg groups and lines represent the mean ± standard error of the mean. *Indicates statistical significant difference ($P < 0{\cdot}05$) measured by Wilcoxon signed-rank test. (C) The pie charts show the proportion of single (light grey), double (dark grey) or triple cytokine (black) producing CD4$^+$ Th1 cells in each of the 7·5 μg vaccine groups.

When analysing the influenza-specific CD4$^+$ Th1 cell response in more detail, we found that it consisted predominantly of cells producing one or two cytokines simultaneously, whilst few triple cytokine producers were observed in all vaccine groups. The single producers were primarily IL-2 or TNF-α, whilst the double cytokine producers were either IL-2$^+$TNF-α^+ or IFN-γ^+TNF-α^+. Triple IL-2$^+$ IFN-γ^+TNF-α^+ cytokine producing CD4$^+$ Th1 cells were mainly observed in the c-di-GMP and the adjuvant combination groups, where they constituted approximately 10% and 3% of the influenza-specific CD4$^+$ T cells, respectively (Figure 7B,C). In contrast, only 1% of CD4$^+$ Th1 cells in the CSN-adjuvanted group were triple producers, and no triple cytokine producing CD4$^+$ T cells were observed in the subunit vaccine alone group. The quality of the Th1 cell response for the groups receiving the lower (1·5 and 0·3 μg) vaccine doses was similar to that of the 7·5 μg antigen dose (Data not shown).

Discussion

The zoonotic influenza A/H5N1 virus is a pandemic threat, particularly in view of the recent data on the few mutations required for this virus subtype to gain the ability of airborne transmission between ferrets, the standard animal model for human influenza infection.[1,2,41] Clearly, this highlights a need for continued research into the optimal pandemic vaccine formulation. Recent meta-analyses and systemic reviews have found that intramuscularly administered seasonal influenza vaccines (containing non-adjuvanted subunit or split virus antigens) are only moderately effective at preventing disease caused by a homologous strain[42,43] and ineffective at preventing disease caused by heterologous strains.[42] Mucosal vaccines can protect against initial influenza infection of the upper respiratory tract and can be administered without the use of needles. Intranasal live-attenuated influenza vaccines have been safely administered for decades, but have so far proved ineffective against influenza H5N1 strains in clinical trials.[44] Mucosal adjuvants enhance immune responses to mucosally administered inactivated vaccines, but the only licensed human-adjuvanted intranasal influenza vaccine, containing the *E. coli* heat labile toxin (LT), was withdrawn from the market due to a significant association with facial paralysis (Bell's Palsy).[45] Novel safe and effective mucosal adjuvants are therefore needed, and studies aimed at directly comparing different adjuvants using the same antigen doses and assay parameters are crucial in this regard.

In the present work, we have conducted a dose-sparing study and evaluated two promising mucosal adjuvants, CSN and c-di-GMP for boosting influenza H5N1 vaccine responses after intranasal immunisation. Furthermore, we have conducted preliminary studies of combining the two adjuvants. Retention of vaccine components on mucosal surfaces by polymer systems such as CSN has been shown to boost vaccine responses after mucosal administration.[24,46,47] The adjuvant property of CSN relies on its mucoadhesive

properties, activation of the NLRP3 inflammasome pathway,[48] and also possibly its ability to increase the permeability of epithelial cell tight junctions, thereby enhancing antigen uptake.[22] The grade of CSN utilised in this study (chitosan glutamate 213) was shown in previous studies to be an effective adjuvant for an intranasally administered dry powder diphtheria vaccine[49] and a liquid influenza vaccine.[50] c-di-GMP functions as a bacterial second messenger and regulates bacterial motility and cell-to-cell signalling.[51] The adjuvant effect of c-di-GMP is based on immune cells sensing c-di-GMP as a danger signal[52] probably involving stimulator of interferon genes (STING) receptor activation,[30] leading to a significant enhancement of vaccine-specific humoral and cell-mediated immune responses.[26,27,53] Safety is important for vaccines intended for intranasal administration. CSN is a promising adjuvant due to the vast amount of animal and human safety data supporting the use of this biopolymer.[23] The toxicology of the c-di-GMP adjuvant has not been reported so comprehensively, although preliminary investigations have shown that exposure of normal rat kidney cells and human neuroblastoma cells to c-di-GMP at biologically relevant concentrations (0–100 μm) does not impair cell viability.[52] Ultimately, additional toxicity data would be needed on the c-di-GMP adjuvant (and any combination adjuvant system). We did not directly evaluate the safety of the CSN and c-di-GMP adjuvant in the present study. As a measure of acute toxicity, we weighed all mice and observed a tendency towards lower weights in mice receiving the 7·5 μg HA with the adjuvant combination as compared to all other groups of mice. No significant differences in weights were found between adjuvanted, non-adjuvanted or control (PBS) groups (Figure S1).

The limited influenza vaccine manufacturing capacity makes dose-sparing adjuvants attractive in a pandemic scenario. We evaluated the dose-sparing capabilities of the CSN and c-di-GMP adjuvants using antigen doses of 1·5 and 0·3 μg HA and found that the c-di-GMP adjuvant was superior to CSN. Although the c-di-GMP adjuvant provided dose sparing, a clear reduction in humoral responses with decreasing antigen doses was observed. Interestingly, the same was only partially true for the T-cellular responses, for which the all c-di-GMP-adjuvanted groups, irrespective of antigen dose, had similar proliferative responses and frequencies of IL-2, IFN-γ and TNF-α producing $CD4^+$ T cells measured by intracellular cytokine staining. However, the same cytokines, as measured in the supernatant of influenza-stimulated cells, were significantly lower in the 0·3 μg HA, than in the 1·5 and 7·5 μg HA, c-di-GMP-adjuvanted groups.

The safety and biodegradability of CSN makes it possible to combine CSN with other potentially more potent adjuvants with the possibility of directing the immune responses towards a specific Th phenotype or obtain additive effects on the magnitude of vaccine-specific immune responses. Indeed, in combination with monophosphoryl lipid A or cholera toxin, CSN was found to further augment antigen-specific immune responses.[54,55] In the present study, we examined the quality and magnitude of responses following vaccination with a combination of CSN and c-di-GMP adjuvant. The adjuvant combination was as effective at boosting the humoral immune responses for the 7·5 μg antigen dose as c-di-GMP, but gave rise to lower Th1 (IL-2 and IFN-γ) and higher Th2 (IL-5 and IL-10) cytokine concentrations than c-di-GMP-adjuvanted vaccine. Thus, in terms of the cytokine profiles, the combination of the Th2-inducing CSN and the Th1-inducing c-di-GMP provided similar humoral responses as the c-di-GMP adjuvant, but a more balanced Th response. This may be beneficial because a balanced Th1/Th2 response has been found important for protection against influenza in mice.[19] It should be noted, however, that the vaccine containing the adjuvant combination induced both IgG1 and IgG2a, but a Th2-biased response in terms of IgG2a/IgG1 ratio and was ineffective at the lower doses tested (1·5 and 0·3 μg HA). The latter could be due to the study design, where antigen and CSN were first administered together, followed by the c-di-GMP adjuvant. But, chitosan being a mucoadhesive is unlikely to undergo significant clearance over the 30-second interval prior to the administration of c-di-GMP. Furthermore, *in vivo* 'pulse-chase' studies in anaesthetised rats have shown that chitosan solution can be pre-applied to the nasal mucosa up to 30 minutes without compromising absorption enhancement of a nasally applied insulin solution.[56] More studies on optimally formulated CSN and c-di-GMP, followed by challenge studies, should be conducted before any conclusions can be drawn in terms of quantitative benefits of combining the two adjuvants. Clearly, compatibility and stability issues would need to be fully resolved in order to facilitate a commercial product.

Th17 cells are particularly important in terms of mucosal immunity and can up-regulate polymeric Ig receptor and thus transport of secretory IgA into the lumen.[57] Furthermore, the mucosal adjuvant activity of cholera toxin requires intact Th17 responses.[58] We report here that both the CSN and c-di-GMP and the adjuvant combination boost Th17 responses as indicated by increased production of Th17 cytokines as compared to antigen alone.

We have previously reported that immunisation with influenza virosomes in combination with c-di-GMP stimulates high frequencies of influenza-specific $CD4^+$ T cells simultaneously producing IL-2 and TNF-α,[26] a phenotype, which has been suggested to indicate T-cell memory potential (reviewed in Ref. 59). Both CSN and c-di-GMP

boosted the frequencies of influenza-specific $CD4^+$ T cells more than antigen alone. However, c-di-GMP induced significantly higher frequencies of both double ($IL\text{-}2^+$ $TNF\text{-}\alpha^+$) and triple ($IL\text{-}2^+$ $TNF\text{-}\alpha^+IFN\text{-}\gamma^+$) cytokine producing $CD4^+$ Th1 cells than CSN, thus confirming the Th1 profile for the c-di-GMP adjuvant. Furthermore, the quality of the $CD4^+$ Th1 cell response was markedly different between mice receiving the CSN and the c-di-GMP adjuvant. Approximately 1% of $CD4^+$ T cells were triple cytokine producers in groups receiving the CSN-adjuvanted vaccine, whilst 10% triple cytokine producing $CD4^+$ T cells were observed in the c-di-GMP-adjuvanted groups. A $CD4^+$ Th1 cell multifunctional profile has been shown to correlate with protection from a number of infectious diseases caused by intracellular pathogens (reviewed in Ref. 59), and such cells have also been found to be functionally superior to single cytokine producers (displaying higher median fluorescence intensities of each of the measured cytokines).[14,60] The present study illustrates how two different mucosal adjuvants can direct the $CD4^+$ T-cellular response in different directions, which could be important in the design of future vaccines. Furthermore, by combining two adjuvants, that is, CSN and c-di-GMP, the Th bias, as observed for each of the adjuvants separately, may be rendered more balanced. It should be noted that although c-di-GMP boosted Th1 cell responses significantly more than CSN, there are as yet no reports suggesting that c-di-GMP would be more protective against influenza disease. Initial studies demonstrate that the CSN-adjuvanted vaccine protects ferrets against intranasal challenge with highly pathogenic influenza H5N1 (manuscript in preparation).

This study has provided a thorough evaluation of two mucosal adjuvants for boosting immune responses to an influenza H5N1 antigen. We found that both CSN and c-di-GMP boosted functional antibody responses. Furthermore, the c-di-GMP adjuvant provided significant dose sparing with three of five mice having seroprotective HI antibody titres and all mice having seroprotective SRH titres following vaccination with the lowest dose of 0·3 μg HA. Combining c-di-GMP and CSN enhanced HI titres and IgG concentrations for the 7·5 μg HA dose more than c-di-GMP alone. In contrast, the adjuvant combination did not significantly enhance the magnitude of the antigen-specific immune responses for the 1·5 and 0·3 μg HA doses, which may be due to formulation compatibility issues. However, the adjuvant combination skewed the cytokine responses towards a more balanced Th profile than either of the adjuvants alone. This study highlights the importance of assessing the detailed humoral and cellular immune responses following immunisation to find the optimal mucosal influenza vaccine formulation.

Acknowledgements

This study was supported by the EU FP7 funded project NASPANVAC, the Norwegian Research Counsel Globvac (185441) and intramurally by the Influenza Centre, University of Bergen. We thank, Ewa Szyszko and Turid Helen Felli Lunde for help with performing experiments and the staff at the animal facilities at the University of Bergen for their help with handling and taking care of the animals.

Addendum

Signe C. Svindland designed the experiments, performed immunisations, ELISA, proliferation assay and wrote the manuscript. Gabriel Kristian Pedersen performed immunisations and flow cytometry and wrote the manuscript. Rishi Pathirana performed and analysed flow cytometry. Geir Bredholt performed and analysed proliferation assay, Jane Kristin Nøstbakken performed data analysis and ELISA. Åsne Jul-Larsen vaccinated and sampled mice. Carlos Guzman provided the c-di-GMP adjuvant and corrected the manuscript. Emanuele Montomoli performed the haemagglutination inhibition, virus neutralisation and single radial haemolysis assays and corrected the manuscript. Giulia Lapini performed the haemagglutination inhibition assay. Simona Piccirella performed the virus neutralisation assay. Inderjit Jabbal-Gill and Michael Hinchcliffe provided the chitosan adjuvant and corrected the manuscript. Rebecca J. Cox designed the experiments and wrote the manuscript. All authors have read and approved the manuscript.

Conflicts of interest

Inderjit Jabbal-Gill and Michael Hinchcliffe work for Archimedes Development Ltd, UK. Carlos Guzman owns a patent of the c-di-GMP adjuvant.

References

1 Imai M, Watanabe T, Hatta M *et al.* Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature 2012; 486:420–428.
2 Herfst S, Schrauwen EJ, Linster M *et al.* Airborne transmission of influenza A/H5N1 virus between ferrets. Science 2012; 336:1534–1541.
3 Amorij JP, Hinrichs W, Frijlink HW, Wilschut JC, Huckriede A. Needle-free influenza vaccination. Lancet Infect Dis 2010; 10:699–711.
4 Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nat Immunol 2011; 12:509–517.
5 Bucy RP, Panoskaltsis-Mortari A, Huang GQ *et al.* Heterogeneity of single cell cytokine gene expression in clonal T cell populations. J Exp Med 1994; 180:1251–1262.
6 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.

7 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 1989; 7:145–173.

8 McGeachy MJ, Bak-Jensen KS, Chen Y *et al.* TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. Nat Immunol 2007; 8:1390–1397.

9 Aggarwal S, Ghilardi N, Xie MH, De Sauvage FJ, Gurney AL. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003; 278:1910–1914.

10 Esplugues E, Huber S, Gagliani N *et al.* Control of TH17 cells occurs in the small intestine. Nature 2011; 475:514–518.

11 Bermejo-Martin JF, Ortiz de Lejarazu R, Pumarola T *et al.* Th1 and Th17 hypercytokinemia as early host response signature in severe pandemic influenza. Crit Care 2009; 13:R201.

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

13 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.

14 Pedersen G, Major D, Roseby S, Wood J, Madhun AS, Cox RJ. Matrix-M adjuvanted virosomal H5N1 vaccine confers protection against lethal viral challenge in a murine model. Influenza Other Respir Viruses 2011; 5:426–437.

15 Moran TM, Park H, Fernandez-Sesma A, Schulman JL. Th2 responses to inactivated influenza virus can Be converted to Th1 responses and facilitate recovery from heterosubtypic virus infection. J Infect Dis 1999; 180:579–585.

16 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.

17 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.

18 Bungener L, Geeraedts F, Ter Veer W, Medema J, Wilschut J, Huckriede A. Alum boosts TH2-type antibody responses to whole-inactivated virus influenza vaccine in mice but does not confer superior protection. Vaccine 2008; 26:2350–2359.

19 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.

20 Soane RJ, Hinchcliffe M, Davis SS, Illum L. Clearance characteristics of chitosan based formulations in the sheep nasal cavity. Int J Pharm 2001; 217:183–191.

21 Soane RJ, Frier M, Perkins AC, Jones NS, Davis SS, Illum L. Evaluation of the clearance characteristics of bioadhesive systems in humans. Int J Pharm 1999; 178:55–65.

22 Smith J, Wood E, Dornish M. Effect of chitosan on epithelial cell tight junctions. Pharm Res 2004; 21:43–49.

23 Baldrick P. The safety of chitosan as a pharmaceutical excipient. Regul Toxicol Pharmacol 2010; 56:290–299.

24 Svindland SC, Jul-Larsen A, Pathirana R *et al.* The mucosal and systemic immune responses elicited by a chitosan-adjuvanted intranasal influenza H5N1 vaccine. Influenza Other Respi Viruses 2012; 6:90–100.

25 Burdette DL, Monroe KM, Sotelo-Troha K *et al.* STING is a direct innate immune sensor of cyclic di-GMP. Nature 2011; 478:515–518.

26 Pedersen GK, Ebensen T, Gjeraker IH *et al.* Evaluation of the Sublingual Route for Administration of Influenza H5N1 virosomes in combination with the bacterial second messenger c-di-GMP. PLoS ONE 2012; 6:90–100.

27 Madhun AS, Haaheim LR, Nostbakken JK *et al.* Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional Th1 CD4 + cells and strong mucosal and systemic antibody responses in mice. Vaccine 2011; 29:4973–4982.

28 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.

29 Karaolis DK, Means TK, Yang D *et al.* Bacterial c-di-GMP is an immunostimulatory molecule. J Immunol 2007; 178:2171–2181.

30 Burdette DL, Monroe KM, Sotelo-Troha K *et al.* STING is a direct innate immune sensor of cyclic di-GMP. Nature 2011; 478:515–518.

31 Shang G, Zhu D, Li N *et al.* Crystal structures of STING protein reveal basis for recognition of cyclic di-GMP. Nat Struct Mol Biol 2012; 19:725–727.

32 Ishikawa H, Ma Z, Barber GN. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 2009; 461:788–792.

33 Schild GC, Pereira MS, Chakraverty P. Single-radial-hemolysis: a new method for the assay of antibody to influenza haemagglutinin. Applications for diagnosis and seroepidemiologic surveillance of influenza. Bull World Health Organ 1975; 52:43–50.

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

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

36 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.

37 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.

38 Roederer M, Nozzi JL, Nason MX. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A 2011; 79:167–174.

39 Nauta JJ, Beyer WE, Osterhaus AD. On the relationship between mean antibody level, seroprotection and clinical protection from influenza. Biologicals 2009; 37:216–221.

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

41 Chen LM, Blixt O, Stevens J *et al. In vitro* evolution of H5N1 avian influenza virus toward human-type receptor specificity. Virology 2011; 422:105–133.

42 Jefferson T, Di Pietrantonj C, Rivetti A, Bawazeer GA, Al-Ansary LA, Ferroni E. Vaccines for preventing influenza in healthy adults. Cochrane Database Syst Rev 2010; CD001269.

43 Osterholm MT, Kelley NS, Sommer A, Belongia EA. Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis. Lancet Infect Dis 2011; 12:36–44.

44 Karron RA, Talaat K, Luke C *et al.* Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. Vaccine 2009; 27:4953–4960.

45 Mutsch M, Zhou W, Rhodes P *et al.* Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004; 350:896–903.

46 Mannila J, Jarvinen K, Holappa J, Matilainen L, Auriola S, Jarho P. Cyclodextrins and chitosan derivatives in sublingual delivery of low

solubility peptides: a study using cyclosporin A, alpha-cyclodextrin and quaternary chitosan N-betainate. Int J Pharm 2009; 381:19–24.
47 Saint-Lu N, Tourdot S, Razafindratsita A *et al.* Targeting the allergen to oral dendritic cells with mucoadhesive chitosan particles enhances tolerance induction. Allergy 2009; 64:1003–1013.
48 Li H, Willingham SB, Ting JP, Re F. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J Immunol 2008; 181:17–21.
49 Mills KH, Cosgrove C, McNeela EA *et al.* Protective levels of diphtheria-neutralizing antibody induced in healthy volunteers by unilateral priming-boosting intranasal immunization associated with restricted ipsilateral mucosal secretory immunoglobulin a. Infect Immun 2003; 71:726–732.
50 Read RC, Naylor SC, Potter CW *et al.* Effective nasal influenza vaccine delivery using chitosan. Vaccine 2005; 23:4367–4374.
51 Tamayo R, Pratt JT, Camilli A. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu Rev Microbiol 2007; 61:131–148.
52 Karaolis DK, Cheng K, Lipsky M *et al.* 3′,5′-Cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. Biochem Biophys Res Commun 2005; 329:40–45.
53 Ebensen T, Libanova R, Schulze K, Yevsa T, Morr M, Guzman CA. Bis-(3′,5′)-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. Vaccine 2011; 29:5210–5220.
54 Baudner BC, Giuliani MM, Verhoef JC, Rappuoli R, Junginger HE, Giudice GD. The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines. Vaccine 2003; 21:3837–3844.
55 Wimer-Mackin S, Hinchcliffe M, Petrie CR *et al.* An intranasal vaccine targeting both the Bacillus anthracis toxin and bacterium provides protection against aerosol spore challenge in rabbits. Vaccine 2006; 24:3953–3963.
56 Illum L, Farraj NF, Davis SS. Chitosan as a novel nasal delivery system for peptide drugs. Pharm Res 1994; 11:1186–1189.
57 Jaffar Z, Ferrini ME, Herritt LA, Roberts K. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. J Immunol 2009; 182:4507–4511.
58 Datta SK, Sabet M, Nguyen KP *et al.* Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax. Proc Natl Acad Sci USA 2010; 107:10638–10643.
59 Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 2008; 8:247–258.
60 Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL, Amara RR. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. J Virol 2007; 81:8468–8476.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The weight of the mice after influenza H5N1 vaccination.