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Killed *Bacillus subtilis* spores as a mucosal adjuvant for an H5N1 vaccine

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ABSTRACT

Heat killed spores of the Gram-positive bacterium *Bacillus subtilis* have been evaluated as a vaccine delivery system with mucosal adjuvant properties for influenza. Killed spores were able to bind H5N1 virions (NIBRG-14; clade 1) and, when intra-nasally administered to mice, resulting immune responses, both humoral and cell mediated, were enhanced compared to immunization with the virion alone. Levels of both systemic IgG and mucosal sIgA specific to the virion were elevated. Levels of IgG2a (a Th_1 antibody type) were strongly enhanced when the virion was co-administered with killed spores. Cytokine induction in stimulated splenocytes was also apparent indicating balanced T_h1 and T_h2 responses. Evidence of cross-neutralization of clade 2.2 viruses was shown. In a challenge experiment mice dosed two times with spores adsorbed with just 20 ng HA (hemagglutinin) of inactivated NIBRG-14 were fully protected against challenge with 20 LD_{50} of H5N2 virus. Interestingly, partial protection (60%) was observed in animals dosed only with killed spores. Mice dosed only with killed spores were shown to be fully protected against H5N2 (5 LD_{50}) infection indicating that innate immunity and its stimulation by spores may play an important role in protection. Supporting this killed spores were (i) shown to stimulate TLR-mediated expression of NF-κB, and (ii) able to recruit NK cells into lungs and induce maturation of DCs. This work demonstrates the potential and underlying mechanism for the use of bacterial spores as an adjuvant for H5N1 vaccination.

1. Introduction

Highly pathogenic H5N1 influenza infections are still relatively rare in humans yet the continued evolution of this virus makes it a potentially serious and looming public health threat [1]. A vaccine suitable for protection against a future pandemic caused by an H5N1 virus should provide cross-clade protective immunity since the evolution of H5N1 viruses in birds in south-east Asia is unprecedented while the strain that may cause a pandemic cannot be predicted and current parenteral seasonal influenza vaccines are all largely strain-specific [1]. In addition, such a vaccine should have three further attributes: be safe, require a low antigen content (antigen-sparing) to combat the low immunogenicity of H5N1 viruses and cater for production problems and finally, induce a long lasting immunity [2–4]. Regarding antigen-sparing, adjuvants such as AS03 or MF59, oil-in-water emulsions (based on squalene-like molecules) have been used parenterally in humans successfully [5]. A number of studies have shown that secretory IgA (sIgA) from the respiratory tract is more effective in cross-protection against heterologous influenza compared to IgG induced by parenteral vaccination [6,7]. Accordingly, mucosal adjuvants such as modified cholera toxin and the related *E. coli* heat labile enterotoxin (LT) have been shown effective at augmenting responses to inactivated influenza virus [8,9] but unfortunately, have been linked to adverse reactions including facial paralysis [10]. The nasal route of vaccination has been shown to induce more appropriate immune responses at the mucosal surface, the actual site of infection, and has been more effective to epidemics caused by a heterologous virus [11,12].

Spores of the Gram-positive bacterium, *Bacillus subtilis*, have been used successfully for antigen delivery. Using genetically engineered spores that express heterologous antigens protection has been achieved against a number of pathogens using animal models, notably *Clostridium perfringens* [13], *Clostridium difficile* [14], *Clostridium tetani* [15], and Rotavirus [16]. In all these examples

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spores have been delivered by a mucosal route where they have been shown to efficiently stimulate both systemic and localized immune responses. The attraction of using spores as a delivery system is 2-fold, first, their heat stability, and second, their existing use, worldwide as probiotic dietary supplements. With regard to stability, while native spores are intrinsically heat stable [17], this property has also been shown to be transferred to heterologous antigens expressed on the spore surface. In particular, the tetanus protective antigen TTFC (tetanus toxin fragment C) expressed on the spore surface has been validated in a challenge model after storage of lyophilized spores for 12 months [18]. As a probiotic, spores of a number of *Bacillus* species are used in human (*e.g.*, Enterogermina®) and animal feed products (*e.g.*, Gallipro®) and as live organisms for human consumption [19].

More recently, a non-GM approach to using spores has been developed making use of a novel attribute of the spore surface in binding heterologous antigens [20]. Using a combination of electrostatic and hydrophobic interactions proteins have been shown to bind to the spore surface. This approach enables the spore to serve as an antigen carrier without the need for genetic modification. Remarkably, using such an approach antigen presentation appears equally as efficient whether the spores are live or inactivated (heat killed). This approach then resembles other microparticulate adjuvants, which, by virtue of their size and multimeric presentation, mimic the pathogens the immune system has evolved to control, and facilitates uptake by antigen presenting cells [21,22]. Studies on the nature of antibody responses in mice have shown that spores co-administered with protein antigens at mucosal surfaces augment antigen-specific secretory IgA (sIgA) as well as inducing balanced T_h1/T_h2 responses [20,23].

Many animal viruses carry an envelope comprised of phospholipid embedded with lipoproteins and/or glycoproteins. Such viruses have been shown to efficiently adsorb to hydrophobic surfaces where binding increases as the pH declines [24]. This phenomenon should enable a virion to adsorb to the spore surface and was the rationale for the present study. In this work, we have shown that spores have unique characteristics as both an adjuvant and for antigen delivery. Mucosal delivery of a low dose of virion-adsorbed spores is shown to elicit protective immunity to influenza in a murine model of infection. We also show that spores by themselves act as a potential immune stimulant able to confer protection to influenza and we provide a basic mechanism for how this might occur.

2. Materials and methods

2.1. General methods

Methods for work with *B. subtilis* are described elsewhere [25]. *B. subtilis* strain PY79, is a standard, prototrophic, laboratory strain [26]. Sporulation was confirmed by phase-contrast microscopy and spore crops were harvested and purified as described elsewhere [27]. Spores were killed by autoclaving (121 °C, 15 p.s.i., 30 min).

2.2. Reagents

Inactivated H5N1 virus, A/Vietnam/1194/2004 (NIBRG-14; clade 1) and A/Turkey/1/2005 (clade 2.2) as well as reference antisera were obtained from the National Institute for Biological Standards and Control (NIBSC), UK.

2.3. Propagation, purification and titration of NIBRG-14

A live virus (NIBRG-14) suspension (50% Egg infective doses calculated for NIBRG-14 was 6.1 $\log_{10}$ EID_{50} per 0.2 ml) was diluted 10^{-3} in sterile PBS (0.01 M, pH 7.2) supplemented with 100 μg of gentamycin/ml and inoculated into 10-day old chicken embryos (Leghorn; 0.2 ml for each egg). After inoculation, eggs were incubated at 33.5 °C for 3 days. Eggs were refrigerated at 4 °C overnight and allantoic fluid was collected for haemagglutinin (HA) testing. The viral particles in the fluid were concentrated by ultracentrifugation (30,000 rpm, 2 h, Sorvall Ultra Pro80 using a T865 rotor). Pellets were resuspended in a small volume of PBS (2 ml) and laid on top of a discontinuous sucrose gradient (15, 30, 45 and 60% sucrose in PBS). The virus was banded by ultracentrifugation (25,000 rpm, 2 h, Swing-out Surespin-630 rotor) and dialyzed overnight in PBS at 4 °C to remove sucrose. The virus was inactivated with formalin at a final dilution of 1/4000 at 4–5 °C for 5 days. Formalin was neutralized by addition of 5 μl of 8.8% $NaHSO_3$ solution per ml of inactivated virus. HA concentration was determined by the SRD (single-radial immunodiffusion) assay [28] with an HA standard and specific sheep antiserum (NIBSC, UK). A suspension of NIBRG-14 at 60 μg/ml (HA) was prepared as a working stock and a representative SDS-PAGE gel of proteins extracted from NIBRG-14 is shown in Supplementary Fig. 1.

2.4. Image analysis of influenza virion adsorption

Purified inactivated H5N1 virions (NIBRG-14; 0.33 mg/ml, equivalent to 11 μM of HA) were directly labeled with 20 μM sucinimidyl tetramethyl rhodamine (TMR, Invitrogen) at room temperature (RT) for 45 min in phosphate buffered saline (PBS; 145 mM pH 7.4). The reaction was stopped by adding 10 mM Tris–HCl, and the unreacted dyes were carefully removed by sequential dialysis (4-times) in PBS. Labeled NIBRG-14-TMR was then centrifuged (10,000 rpm; 5 min) to remove precipitation that may have occurred during labeling and dialysis. The concentration of protein and TMR in the labeled sample was determined (using a NanoDrop) to be 0.27 mg/ml, equivalent to 9 μM of protein and 1.7 μM of TMR, indicating a protein to dye ratio of about 5:1. Inactivated PY79 spores (5×10^9) were directly labeled with 50 μM succinimidyl fluorescein isothiocyanate (FITC, Invitrogen) using the same labeling conditions as those used for NIBRG-14. The labeled PY79-FITC spores were removed from unincorporated dyes by sequential washing with a 100-fold volume of 10× PBS (1-times) followed by 1X PBS (2-times) by centrifugation (12,000 rpm, 3 min). The labeled PY79-FITC spores were observed by light and epifluorescence microscopy to confirm that all (100%) spores were labeled. PY79-FITC spores (1×10^8) were incubated (with gentle agitation) with 6 ml of NIBRG-14-TMR for 30 min at RT. Virion-labeled spores were harvested by centrifugation (12,000 rpm, 3 min) and washed 3-times with PBS (100-fold). Labeled spores were observed under laser confocal fluorescence microscope using a Carl Zeiss LSM510.

2.5. Adsorption of virions to spores for immunization

Autoclaved spores were suspended in 10X PBS (0.1 M, pH 7.2) at RT for 30 min, at a concentration of 5×10^9 spores ml^{-1} buffer. Spores were then washed 3 times with 1× PBS (0.1 M, pH 7.2) by centrifugation and pellets re-suspended in the same buffer. Inactivated NIBRG-14 virions were added and the mixture shaken gently at RT for 30 min. After adsorption, the mixture was incubated on ice until use.

2.6. Animals

Animals used in this work were BALB/c or C57BL/6 mice (Charles River or The Jackson Laboratory) for antibody production, NK cell recruitment and for analysis of immune responses. In all cases females, aged 6–8 weeks were used. Animal procedures in the UK were performed under the Home Office project license PPL 70/6126. In S. Korea animal experiments were performed in accordance with

international guidelines and approved by the International Vaccine Institute Institutional Animal Care and Use Committee.

2.7. Analysis of immune responses

Groups of mice ($n = 6$; BALB/c) were immunized intra-nasally (i.n.) using three-doses on days 1, 15 and 36. Immunogens were (i) inactivated NIBRG-14 (1.2 μg HA/dose) and (ii) PY79^K spores (1×10^9/dose) adsorbed with inactivated NIBRG-14 (1.2 μg HA). In all experiments control groups included naïve (PBS) and a group receiving only PY79^K spores (1×10^9). Blood samples were collected before each dose, and splenocyte and final bleeds were collected three weeks after the last immunization. Serum was inactivated at 56 °C (30 min) and stored at −80 °C till use. Saliva was taken seven days after the last dose using saliva collection swabs (Probact Transport Swabs, Tech. Service Ltd., Heywood, UK). Swabs were applied for 5 min till completely wet and then incubated in protease inhibitor (1 mM PMSF in PBS, 0.2 ml) for 3 h on ice. The extractions were stored at −80 °C till use.

2.8. Challenge studies

The animal and viral experiments using H5N2 virus were conducted in animal and clinical biosafety level 3 containment facilities, and all personnel wore personal protective equipment, including Tyvek suits and N95 or HEPA-filtered air respirators (3 M). Groups of mice (BALB/c) were anesthetized by intra-peritoneal (i.p.) injection of ketamine (100 mg kg^{-1} body weight)/xylazin hydrochloride (10 mg kg^{-1} body weight). For challenge with inactivated influenza virus (Section 3.4), a two-dose regimen was used; animals were i.n. primed on day 1 with whole inactivated H5N1(NIBRG-14) virus (containing either 0.02 μg or 0.5 μg of HA in 20 μl of PBS) in the presence or absence of 2×10^9 CFU of autoclaved spores (PY79^K) and were boosted with the same antigen at day 14. Control groups were animals dosed with either spores or NIBRG-14 only. For virus challenge, an H5N2 virus, A/Aquatic Bird/Korea (H5N2), was used. A/Aquatic Bird/Korea was used because the highly pathogenic H5N1 virus was not available at the time of challenge. The HA protein of A/Aquatic Bird/Korea is 92% and 93% conserved with the HA proteins from NIBRG-14 and A/Turkey/1/2005, respectively. Anesthetized mice were challenged i.n. four weeks after the second immunization with a 20LD$_{50}$ (50% mouse lethal doses) of A/Aquatic Bird/Korea (H5N2) as outlined in the results section. Mice were monitored daily for clinical signs of influenza infection and body weight recorded daily. Mice that lost greater than 25% of body weight were euthanized. The two dose regimen was chosen based on extensive preliminary challenge trials where we determined (i) the lowest i.n. dose of NIBRG-14 (0.02 μg) that did not confer protection in mice using different dosing regimens, and (ii) whether adsorbed spores could enhance NIBRG-14-specific IgG responses. A similar strategy was used for evaluation of protection conferred by escalating doses of PY79^K spores (Section 3.5) but a final challenge dose of 5LD$_{50}$ was used 27 days after the last dose of spores.

2.9. Determination of antibody responses by indirect ELISA

The ELISA methods for measuring IgG, IgG1, IgG2a or IgA were made using the same format. Plates were coated with 50 μl of inactivated NIBRG-14 (20 haemagglutination units (HAU) in carbonate–bicarbonate buffer, pH 9) per well and incubated at 4 °C overnight. After blocking with 2% BSA in PBS at RT for 1 h, serum samples were applied as a 2-fold dilution series, starting with a 1/100 dilution in assay diluent buffer (10 mM PBS [pH 7.4], 1% [wt/vol] BSA, 0.05% [vol/vol] Tween 20). For each plate, a pooled serum from the naïve group (1/100) was included in replicated wells. Plates were incubated for 2 h at RT before the addition of anti-mouse horseradish peroxidase conjugates for serum samples (all obtained from Serotec, UK). Plates were incubated for one additional hour at RT and then developed with the substrate TMB (3,3′,5,5′-tetramethyl-benzidine; Sigma). Reactions were stopped with 2 N H_2SO_4. Dilution curves were drawn for each sample and end-point titers were calculated as the dilutions producing the same optical density as the 1/100 dilution of a pre-immune serum.

2.10. Cytokine determinations

Splenocytes (5×10^5) were seeded in 96-well cell culture plates in complete medium (DMEM medium supplemented with 10% fetal Bovine Serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin). Cells were stimulated with 200 HAU ml^{-1} of inactivated NIBRG-14, 1 μg/ml ConA as a positive control or with medium only at 37 °C in 5% CO_2. Supernatants were harvested from cultures after 48 h of incubation. Cytokines (IFN-γ, IL-2 and Il-6) were detected using Ready-Set-Go ELISA kits (eBioscience, UK) and according to the manufacturers procedures and standards provided. In brief, microtitre plates (96 well; Maxisorb; Nunc, UK) were coated with purified capture anti-mouse antibody in 10 mM PBS (pH 7.4) overnight at 4 °C. Free binding sites were blocked with assay diluent at RT for 1 h. Culture supernatants were incubated at RT for 2 h. Before detection, biotinylated anti-mouse antibody was added and incubated at RT for 1 h. Streptavidin-horseradish peroxidase conjugate was added and incubated at RT for 30 min. Recombinant mouse cytokines were used as a standard and cytokines quantified by interpolation from a standard curve.

2.11. Haemagglutination inhibition (HAI) assay

One volume of immunized mouse serum was incubated with five volumes of cholera filtrate (Sigma) at 37 °C for approximately 16 h, followed by 1 h of incubation at 56 °C. To 50 μl of the 2-fold dilution series of serum (in PBS), 25 μl of PBS containing 4 HAU of inactivated virus (A/Vietnam/1194/2004 or A/Turkey/1/2005) was added and incubated for 30 min at 37 °C. Next, 25 μl of 1% chicken erythrocyte suspension in PBS was added followed by 1 h incubation at 4 °C. Subsequent haemagglutination patterns were examined and expressed as the value of the highest serum dilution which can completely inhibit haemagglutination.

2.12. TLR expression

The TLR reporter macrophage cell line (RAW Blue, Invivogen, France) was used for assessment of TLR signaling. Raw Blue cells were cultured in 10% FBS DMEM (Sigma) supplemented with 4.5 g/l glucose, 2 mM L-glutamine, 100 μg/ml Normocin™ and 200 μg/ml Zeocin™ at 37 °C and 5% CO_2. For the TLR stimulation assay, cells were seeded to 96 well plate at 5.5×10^5/ml with 180 μl cell suspension per well (~100,000 cells/well). Wells already contained 20 μl of ligand suspension (heat killed *B. subtilis* PY79 spores). Controls used were 1.1 μm diameter latex beads (Sigma), *B. subtilis* PY79 vegetative cells, LPS (*E. coli*; Sigma K325) and media only. Cells were incubated for 23 h at 37 °C after which supernatants were added to the QuantiBlue™ SEAP detection assay (Invivogen). TLR stimulation was made by measurement of OD_{655} readings after 1.5 h of incubation and the assay was repeated twice.

2.13. Maturation of bone marrow derived dendritic cells (BM-DC) by spore in vitro

BM-DCs were generated from murine bone marrow cells. Briefly, bone marrow was flushed from the tibiae and femurs of BALB/c mice and was depleted of red blood cells (RBC)

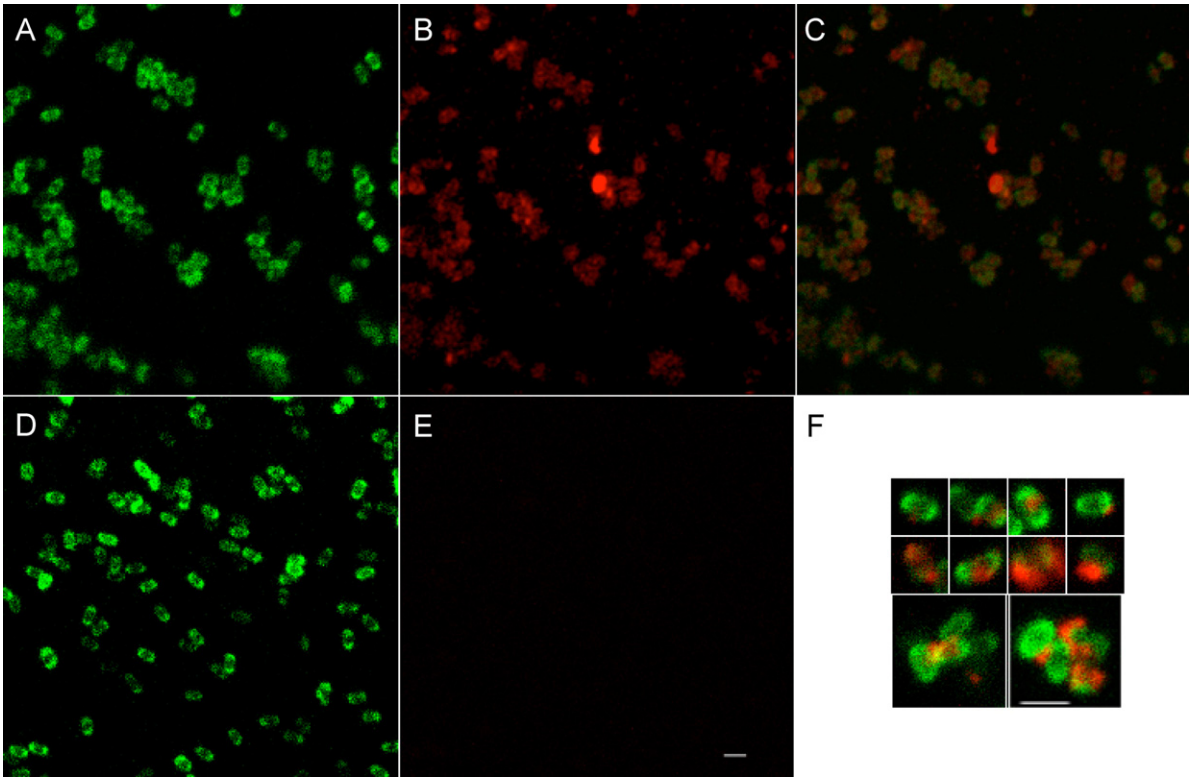

Fig. 1. Adsorption of H5N1 onto spores. Laser scanning confocal micrographs showing individual fluorescent-labeled PY79-FITC spores (panel A, green signal) bound with fluorescent-labeled virus particles NIBRG-14-TMR (panel B, red signal) on dual excitations of 488 nm and 543 nm, respectively. Panel C is a merged image of panels A and B. Panels D and E are images of a control sample in which PY79-FITC spores were incubated with only TMR dye at a 4-fold higher concentration than that of NIBRG-14-TMR. Panel D the green signal of individual fluorescent-labeled PY79-FITC spores, while Panel E is a dark image without any observable red signal of NIBRG-14-TMR. Panel F shows magnified representative images (taken from panel C and other observations) of clusters of NIBRG-14-TMR bound on PY79-FITC spores. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

with ACK buffer. Cells were plated in six-well culture plates (1×10^6 cells/well) in RPMI1640 medium (Invitrogen, USA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FBS, and 20 ng/ml recombinant mouse GM-CSF and 10 ng/ml recombinant mouse IL-4 and then incubated at 37 °C and 5% CO_2 for 5 days. BM-DCs were stimulated with 2 μg/ml of Cholera toxin (CT) and 1, 10, and 100 MOI of spores for 24 h. Cells were stained with CD11c plus CD80, CD86 or MHC class II for 20 min at 4 °C. Stained cells were analyzed by flow cytometry, FACS Calibur. All cytometric data were expressed as mean fluorescence intensity (MFI) and analyzed using FlowJo software. The assay was repeated twice.

2.14. IL-12p40 enzyme-linked immunosorbent assay

To identify the maturation of dendritic cell, IL-12p40 was measured using Ready-set-go ELISA kit and performed according to the manufacturers' instruction (eBioscience, Inc.).

2.15. Preparation of lung single-cell suspensions

To obtain single-cell suspensions, lungs from both BALB/c and C57BL/6 were minced and incubated in RPMI-1640 containing 10% fetal bovine serum (FBS and with 2 mg/ml of collagenase D (Roche) and 0.1 mg/ml of DNase I (Sigma) at 37 °C for 45 min. A single-cell suspension was prepared after RBC lysis using ACK buffer, filtered through a 100-nm cell strainer (BD), and pelleted by centrifugation. Samples were treated with Fc block (BD Pharmingen) before staining. Cells were stained with anti-CD3-FITC plus anti-NK1.1-PerCP Cy5.5, and anti-CD3-Allophycocyanin (APC) plus anti-DX5-PE. Stained cells were analyzed by flow cytometry, FACS Calibur. All cytometric data was analyzed using FlowJo software. The assay was repeated twice.

2.16. Statistics

All data were analyzed with GraphPad Prism version 5 (GraphPad software, SanDiego, CA). A P value of >0.05 was considered non-significant.

3. Results

3.1. Adsorption of virus to spores

Purified H5N1 (NIBRG-14) virions were labeled with TMR and mixed with a suspension of pure spores of *B. subtilis* strain PY79 that had been labeled with FITC. Spores were harvested, washed repeatedly and examined by confocal imaging (Fig. 1). FITC-labeled spores were found to be coated with TMR-labeled virions (Fig. 1B and C). Generally, NIBRG-14 was uniformly labeled on the spore surface although some clustering was apparent (visible as intense red patches, see Fig. 1B, C and F) which is presumably due to virion aggregates. To confirm that the spore was binding to the virion and not TMR we incubated PY79-FITC-labelled spores with TMR only at a 4-fold higher concentration than that used with NIBRG-14-TMR. Following this, only green labeling of spores was observed (Fig. 1D) and no red fluorescence due to TMR binding to the spore surface was detectable (Fig. 1E). To verify that spores bound to NIBRG-14 virions a competitive binding experiment was performed by incubation of PY79-FITC with a mixture of non-labeled H5N1/labeled H5N1-TMR at a ratio of 10:1. Under these conditions we could not observe a red signal due to H5N1-TMR binding to PY79-FITC spores

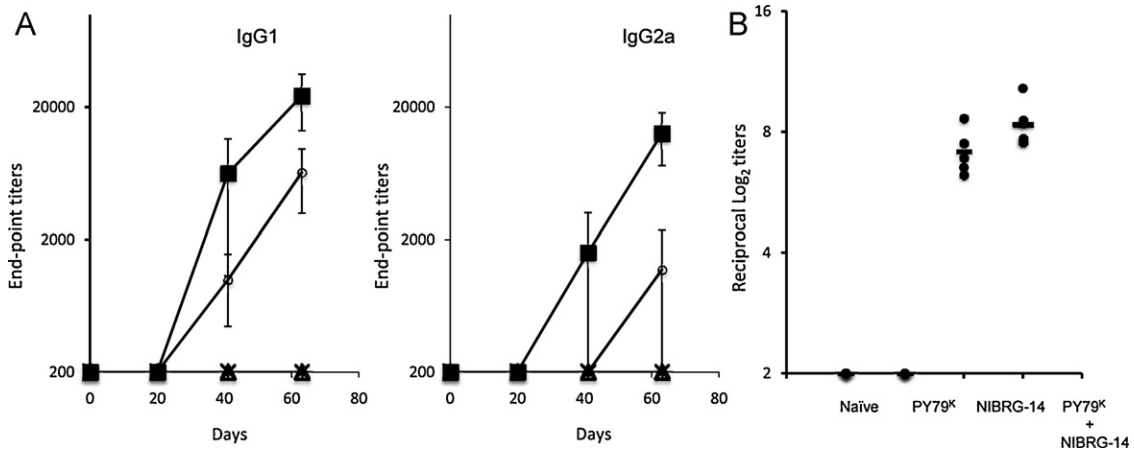

Fig. 2. NIBRG-14-specific humoral responses. Panel A, mice were immunized i.n. with three doses (days 1, 21 and 42) of PY79^K spores pre-adsorbed with inactivated NIBRG-14 1.2 μg HA (■) and serum IgG1 and IgG2a titers determined. Controls included naïve mice (×), mice immunized with PY79^K spores alone (△) or with inactivated NIBRG-14 (1.2 μg HA) alone (○). Bleeds were taken on days 0, 20, 41 and 63. Data are presented as arithmetic means and error bars are standard deviation. Panel B, salivary IgA titers from the same study as panel A. Samples were taken on days 0, 20, 41 and 63. Data shows individual mouse (●) and arithmetic means (–).

(data not shown). These results demonstrate that an H5N1 virion can bind to the surface of *B. subtilis* spores. It is not possible at this stage to accurately determine the number of H5N1 virions that bind to a single spore. However, we estimate the number to be at least eight virions per spore based on the following calculation: (i) the total surface area of an ellipsoidal spore having a length of 1.38 μm and diameter of 0.63 μm was calculated as ~2.60 μm^2; (ii) a single fluorophore emits a spot with a diameter equal to the wavelength of emission light, in the case of TMR this is 572 nm. As the size of a single virion is about 50 nm, which is much smaller than the size of a single fluorescent spot, a single virion conjugated with either single or multiple TMR fluorophores would therefore also emit a spot with a diameter of about 572 nm. Thus, the emission area of a single virion was calculated to be 0.32 μm^2. Assuming a uniform intensity of TMR on individual spores, there would be at least 8 virions (2.60 μm^2/0.32 μm^2 = 8.1) bound per spore.

3.2. Heat-killed spores enhance humoral and cellular immune responses to NIBRG-14

Virus neutralising antibodies including both local and systemic play an important role in immunity to influenza infection [29–31]. Specifically, HA-specific antibodies neutralize the virus and prevent infection [32]. We therefore evaluated the antibody responses specific to H5N1 in mice dosed three-times intra-nasally (i.n.) with spores adsorbed with inactivated NIBRG-14. Groups of mice (n = 6) were immunized i.n. with PY79 spores that had been killed by autoclaving (PY79^K) and adsorbed with NIBRG-14 (1.2 μg HA). Control groups included animals dosed with only killed spores or NIBRG-14. NIBRG-14-specific IgG responses were measured by indirect ELISA in serum samples (Fig. 2). Analysis of the IgG1 and IgG2a subclasses (Fig. 2A) revealed that heat-killed spores induced virion-specific IgG1 and IgG2a in the group where spores were adsorbed with NIBRG-14 and that IgG2a responses were both earlier and higher (10-fold) than those to inactivated virus delivered alone. The value of the IgG1:Ig2a ratios (Supplementary Fig. 2) of the adsorbed spore-NIBRG-14 group were lower (~1.8) than the value of NIBRG-14 alone (~5), indicating a T_h1 bias induced by killed spores. Secretory IgA (sIgA) was also determined in saliva samples (Fig. 2B). NIBRG-14 dosed alone induced virus-specific sIgA, while levels detected in animals dosed with spores adsorbed with NIBRG-14 were higher, albeit not significantly (P = 0.2240).

Although cell mediated immunity does not prevent infection it does play an important role in the recovery of influenza-associated complications. Potent T-helper responses play an important role in stimulating antibody production and the proliferation of cytotoxic T lymphocytes (CTL) and by the production of cytokines [33]. IL-2 and IFN-γ produced by T_h1 cells and IL-6, a pro-inflammatory

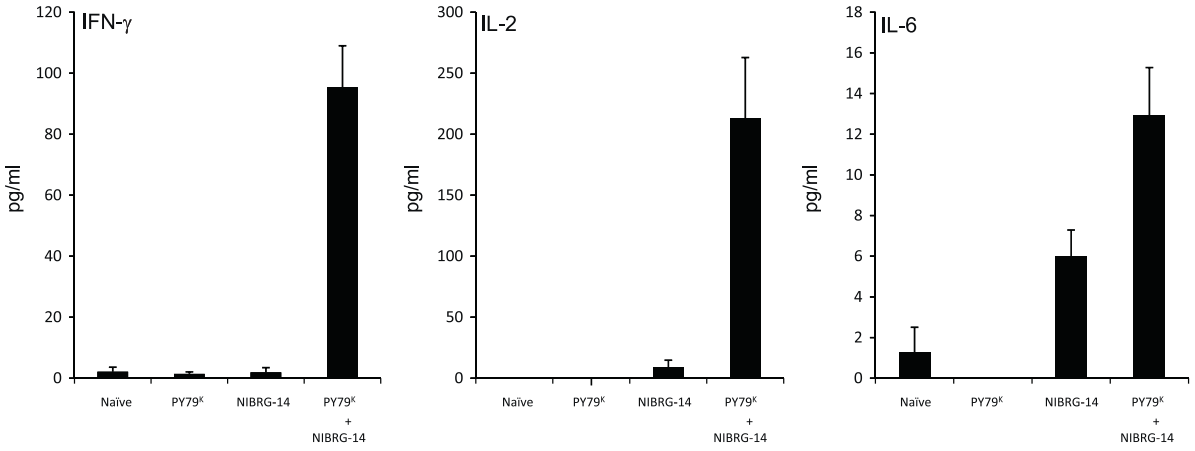

Fig. 3. NIBRG-14 cytokine responses. Cytokine levels were determined from antigen (NIBRG-14) stimulated splenocytes by ELISA. Spleens were taken from mice immunized using a three-dose i.n. regime. Groups were naïve, autoclaved spores (PY79^K), NIBRG-14 (1.2 μg HA) and PY79^K spores adsorbed with NIBRG-14 (1.2 μg HA).

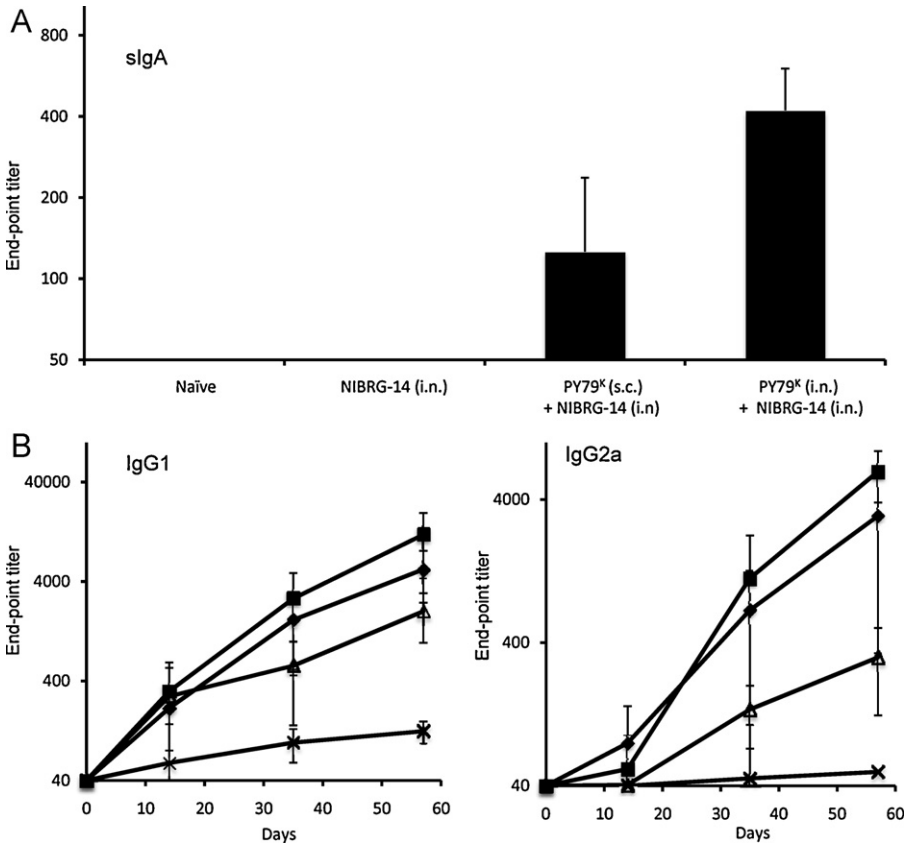

Fig. 4. Route of administration. Salivary sIgA (panel A) and IgG1 and IgG2a titers (panel B) from mice immunized i.n. with three doses (days 1, 15 and 36) of PY79^{K} spores (1×10^9) pre-adsorbed with inactivated NIBRG-14 (1.2 μg HA, ■), or, 1×10^9 PY79^{K} spores administered by the sub-cutaneous (s.c.) route and NIBRG-14 (1.2 μg HA) by the i.n. route (♦). Controls included naïve mice (×) and mice immunized i.n. with inactivated NIBRG-14 alone (1.2 μg HA, △). Bleeds were taken on days 0, 14, 35 and 57. Data are presented as arithmetic means and error bars are standard deviation.

cytokine produced by T_h2 cells, were measured in NIBRG-14 stimulated splenocytes (Fig. 3). In each case significantly higher responses were found in animals dosed with heat-killed spores mixed with NIBRG-14 than to the inactivated NIBRG-14 alone (IL-2, $P = 0.0005$; IFN-γ, $P < 0.0001$; IL-6, $P = 0.0001$). These results show that heat-killed virion adsorbed spores could elicit both T_h1- and T_h2-specific cell mediated and antibody responses.

Serum anti-HA antibody responses were also further confirmed by the hemagglutination inhibition (HAI) assay, using 1% chicken erythrocytes and inactivated NIBRG-14. Table 1 shows that HAI titers of mice dosed with PY79^{K}-NIBRG-14 were significantly higher ($P = 0.0185$) than to those dosed with NIBRG-14 alone (187 ± 65 *vs.* 88 ± 44) and with all mice having an endpoint titer higher than 160 compared to just one mouse responding when dosed with NIBRG-14 alone ($P = 0.0189$). Importantly, with inactivated A/Turkey/1/2005, a clade 2.2 H5N1 virus, 5 out of 6 mice responded with HAI titers greater than 160 (200 ± 98) compared to no mice dosed with NIBRG-14 alone.

We next evaluated the dose of spores required to generate an immune response using the three-dose i.n. regimen. NIBRG-14 (1.2 μg of HA/dose) was adsorbed with escalating concentrations of heat-killed spores (PY79^{K} at 1×10^7, 1×10^8 and 1×10^9). We calculated that the highest dose of spores would have adsorbed 1 μg of NIBRG-14, 1×10^8 100 ng and 1×10^7 10 ng. NIBRG-14-specific IgG (serum) and sIgA (saliva) responses were determined by indirect ELISA (Supplementary Fig 3). Levels of IgG in groups dosed with spores adsorbed with NIBRG-14 (titre = >2000) were, in all cases, significantly higher ($P < 0.05$) than in control groups (titre = <1000; naive, mice dosed with 1×10^9 PY79^{K} or with NIBRG-14 alone) (Supplementary Fig. 3A). Although the IgG titers were highest using 1×10^9 spores adsorbed with NIBRG-14 no significant differences ($P > 0.2$) were found with responses obtained using the lower spore concentrations. A similar profile of sIgA responses was observed (Supplementary Fig. 3B) with higher antibody titers (correlating with higher spore doses) but these were not significantly different ($P > 0.08$). Interestingly, using the three-dose regimen it was clear that administration of NIBRG-14 alone produced no detectable sIgA responses while spores adsorbed with NIBRG-14 produced substantial levels (n.b., this contrasts with the previous experiment where low levels of NIBRG-14-specific sIgA were observed in mice dosed with NIBRG-14 alone).

3.3. *Killed spores can augment immune responses without virion adsorption*

In order to investigate the adjuvant property of spores we measured the immune responses to spores adsorbed with NIBRG-14 that had been delivered by one route (i.n.) *vs.* administration of spores and virion by separate routes (sub-cutaneous and i.n. respectively). Groups of mice ($n = 6$) were dosed on days 1, 15 and 36 using two, 3-dose, regimens. Either, 1×10^9 PY79^{K} spores mixed with inactive NIBRG-14 (1.2 μg HA) using the i.n. route, or, 1×10^9 PY79^{K} spores by the sub-cutaneous (s.c.) route and 1.2 μg of NIBRG-14 by the i.n. route (administered on the same day). NIBRG-14-specific sIgA (saliva) and serum IgG1 and IgG2a levels were determined (Fig. 4). Virion-specific sIgA responses (Fig. 4A) showed a significant increase ($P = 0.0003$) when spores and virion were administered by the same route. Interestingly though, dosing of spores and virions by separate routes did show a clear induction ($P = 0.0485$) of virion-specific sIgA whereas nasal administration of NIBRG-14 generated

Table 1
Hemagglutination inhibition.

Group[a]	A/Vietnam/1194/04 (NIBRG-14, clade 1, H5N1)		A/Turkey/1/05 (clade 2.2, H5N1)	
	No. of mice[b]	GMT[c] of mice	No. of mice[b]	GMT[c] of mice
Naive	0/6	0 ± 0	0/6	0 ± 0
$PY79^K$ spores[d]	0/6	0 ± 0	0/6	0 ± 0
NIBRG-14	1/6	88 ± 44	0/6	72 ± 18
$PY79^K$ spores[d] + NIBRG-14	6/6	187 ± 65	5/6	200 ± 98

[a] Serum from immunized or unimmunized (naïve) mice.
[b] Mice that responded with final HI titer >160.
[c] Geometric mean titer.
[d] Autoclaved spores.

no local antibody responses (note that in an earlier experiment shown in Fig. 2 we did observe low levels of virion-specific sIgA when animals were dosed with virion alone). The same profile of serum antibody responses (Fig. 4B) were observed with spores showing the greatest augmentation of antibody responses when administered using the same route. Although our data does reflect any possible changes in production of saliva resulting from intranasal delivery these results show firstly, that the greatest adjuvant effect is seen when spores are adsorbed with virion. Second, that spores can augment immune responses to NIBRG-14 when administered by a separate route. We interpret this as evidence that spores, by themselves have natural adjuvant properties.

3.4. Antigen co-delivered with spores protects mice from a lethal dose of influenza

Groups of mice ($n = 5$) were immunized i.n. with $PY79^K$ spores adsorbed with NIBRG-14 (either 0.02 μg or 0.5 μg HA per dose) using two doses and then challenged with 20 times the 50% lethal dose of A/Aquatic Bird/Korea (H5N2) virus (Fig. 5). The rationale for the choice of a two-dose regimen is explained in Section 2. All mice dosed with virion-adsorbed spores (0.02 μg or 0.5 μg of HA) were fully protected to lethal challenge. Animals dosed with spores adsorbed with 0.02 μg NIBRG-14 did though show a transient drop in body weight but went on to recover. All mice in the naïve control group as well as those dosed with 0.02 μg of NIBRG-14 lost weight rapidly and had to be euthanized. Animals immunized i.n. with NIBRG-14 (at 0.5 μg HA) alone were afforded some protection (4/5 mice) but most interestingly, heat-killed spores administered without adsorbed antigen were also able to provide protection in 3 out of 5 animals. In a repeat experiment using a similar dosing regimen the same results were found (Supplementary Fig. 4). Therefore these results show that heat-killed spores mixed with as little as 0.02 μg of antigen serve as an adjuvant being able to provide full protection to a lethal dose of H5N2.

3.5. Administration of heat-killed spores alone is protective

Before the challenge reported above, NIBRG-14 antibody titers (IgG) from immunized mice were measured (Fig. 6). This revealed that anti-NIBRG-14 IgG titers from the group dosed with 0.5 μg (HA) of NIBRG-14 alone were higher than the group dosed with spores adsorbed with 0.02 μg (HA) NIBRG-14, yet the resulting protection was lower (100% *vs.* 80%). This indicates that antibodies to A/Aquatic Bird/Korea (H5N2) alone are not sufficient for protection and may imply a more direct role of the spore in augmenting immune responses and protection. In support of this it is apparent that spores themselves, without delivery of virion, are able to confer some level of protection (60%; Fig. 5). Further, when administered by separate routes spores were still able to enhance antibody responses to the virus when delivered by a different route. One potential explanation is that spores themselves induce an innate immune response, which, although short-lived could provide protection. To address this we immunized groups of mice ($n = 4$) with escalating doses of heat-killed spores (ranging from 1×10^7 to 2×10^9) and the same two-dose regimen as that used in the vaccination experiments described above. Twenty-seven days after the 2nd dose mice were challenged with $5LD_{50}$ of H5N2 (Fig. 7). Mice dosed with 5×10^8 or 2×10^9 spores were fully protected against H5N2 challenge for a minimum duration of 14 days.

3.6. Toll-like receptor (TLR) signalling

Induction of TLRs is one principal mechanism by which innate immunity can be activated and where interaction of a ligand with a TLR leads, *via* transcription of NF-κB target genes, to regulation of the function of antigen-presenting cells (APCs) and upregulation of co-stimulatory molecules such as CD80 and CD86 [34]. For

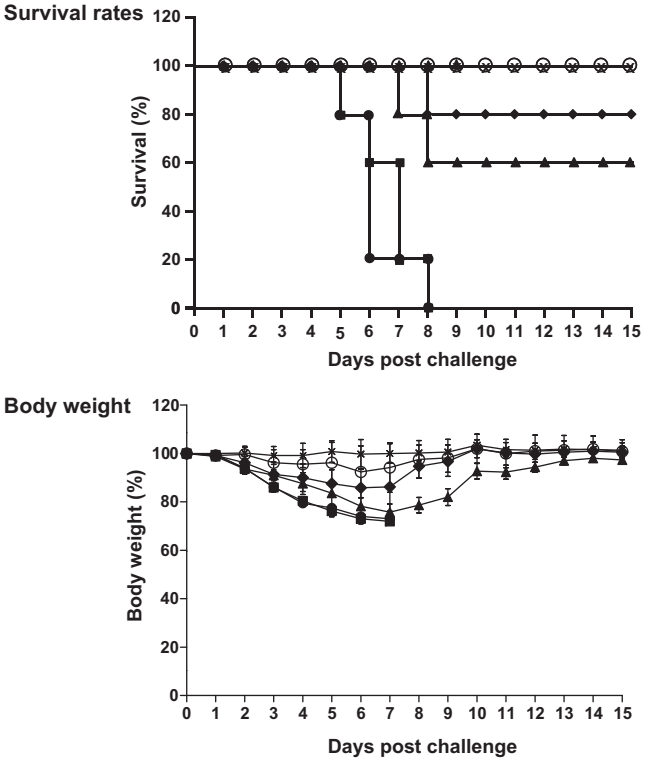

Fig. 5. Protection against H5N2 challenge. BALB/c mice ($n = 5$ per group) were immunized as described in materials and methods on days 1 and 14. Four weeks after the second immunization (day 42), mice were i.n. challenged with $20LD_{50}$ of a mouse-adapted strain of the H5N2 virus. Mice were then monitored daily to determine survival and body weight changes. Groups were mice dosed with $PY79^K$ spores (2×10^9) adsorbed with 0.02 μg of inactive NIBRG-14 (○), 0.02 μg of inactive NIBRG-14 (■), $PY79^K$ (2×10^9) adsorbed with 0.5 μg of inactive NIBRG-14 (X), 0.5 μg inactive NIBRG-14 (♦). Control groups were naïve (●) and animals dosed with $PY79^K$ (2×10^9) only (▲). ***$P < 0.001$ *vs.* control group.

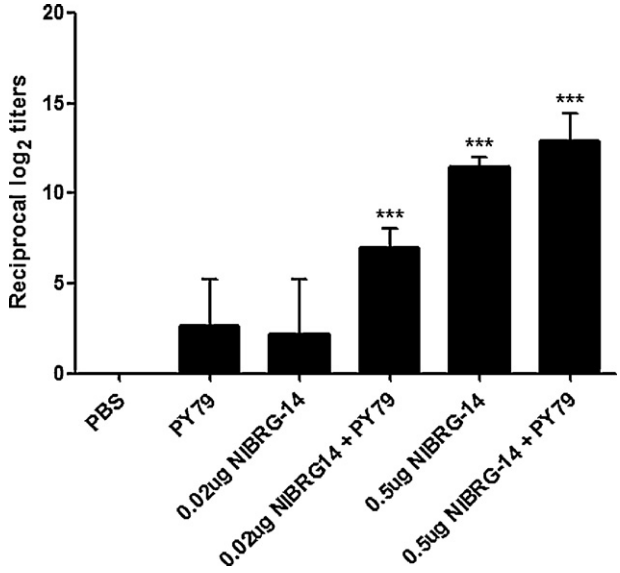

Fig. 6. Pre-challenge humoral responses. Groups of mice ($n = 5$) were immunized i.n. two times (days 1 and 15) with whole inactivated H5N1 virus (NIBRG-14) that contained 0.02 μg or 0.5 μg of HA in the presence or absence of 2×10^9 autoclaved PY79 spores (S). Sera was collected 1 week after the last immunization and tested individually for the presence of NIBRG-14-specific antibodies by indirect ELISA. Naïve animals received PBS only. Results are expressed as the reciprocal $\log_2$ titers and error bars indicate the standard deviation. $^{***}P < 0.001$ *vs.* PBS group.

analysis we used the RAW Blue murine macrophage cell line that carries all TLR genes (with the exception of TLR5) and has been engineered to express an NF-κB-inducible gene expressing a protein, SEAP, that is measurable colorimetrically. Incubation of heat-killed PY79 spores with RAW Blue cells produced high levels of SEAP activity in a dose-dependent manner (Fig. 8). As positive controls *E. coli* LPS was also shown to induce SEAP expression as well as

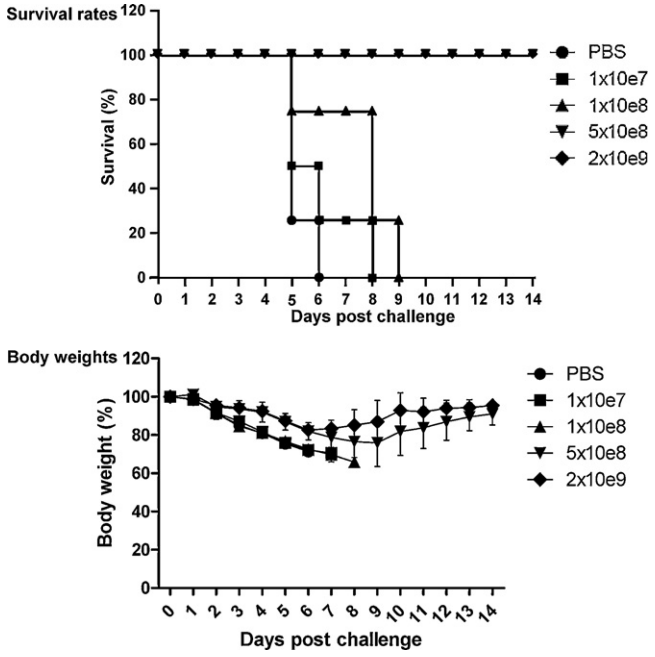

Fig. 7. Protection mediated by spores alone. BALB/c mice ($n = 4$ per group) were immunized (i.n.) with different doses of killed (autoclaved) PY79 spores on days 1 and 14. Twenty-seven days after the second immunization (day 42), mice were i.n. challenged with $5LD_{50}$ of a mouse-adapted strain of the H5N2 virus. Mice were then monitored daily to determine survival and body weight changes. Statistical comparisons between groups were performed with the unpaired *t*-test.

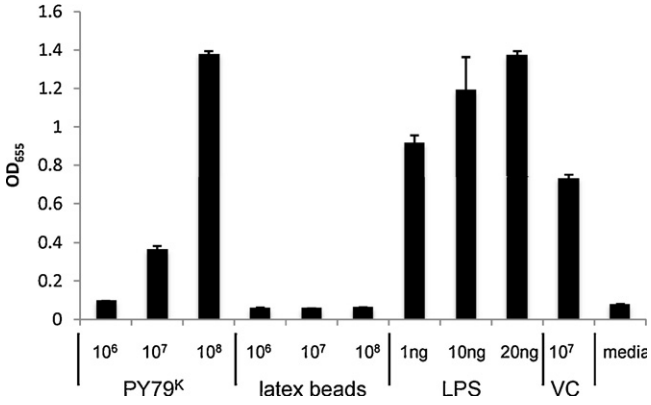

Fig. 8. TLR induction. RAW blue murine macrophage cells containing the NF-κB inducible SEAP gene were incubated with autoclaved PY79 spores, latex beads, *E. coli* LPS or PY79 live vegetative cells (VC, 1×10^7 CFU) for 23 h. SEAP activity was detected using the QuantiBlue SEAP detection kit. Media is the background level from RAW Blue culture. Three sets of readings were taken and error bars (denoting 1 SD) are shown. The entire experiment was repeated with similar results.

live vegetative cells of PY79 which have been shown elsewhere to stimulate TLR2 expression presumably due to their peptidoglycan content [35]. One further control we used was 1.1 μm latex beads that are of equivalent size to bacterial spores. These failed to induce SEAP expression and demonstrate that TLR activation by heat-killed spores must arise from a specific ligand presented on the surface of PY79 spores.

3.7. Spores promote DC maturation and recruitment of NK cells

To further identify the impact of spores on innate cell activation, we evaluated the co-stimulatory molecules of bone-marrow derived dendritic cells (BM-DCs) and migration of natural killer (NK) cells in airways by flow cytometric analysis. It is well known that DC is crucial for the effective induction of adaptive immune responses by increasing the MHC class molecules, B-7 family molecules and releasing cytokines such as IL-12p40 [36,37]. Thus, we confirmed the IL-12p40 secretion in BM-DC treated with different spore MOIs. As expected, spores induced IL-12p40 in a dose-dependent manner ((1–100, spores to DCs) (Fig. 9).

Next, we investigated the recruitment of NK cells into the airways. To identify migration of NK cells, two strains of mice, BALB/c and C57BL/6 were administered 1×10^9 CFU of killed spores ($n = 5$) or 2 μg of CT by the i.n. route and sacrificed after 24 h. Strikingly, lungs following spore administration showed much higher recruitment of both $NK1.1^+$ NK cells ($CD3^-NK1.1^+$) and $DX5^+$ NK cells ($CD3^-DX5^+$) cells than those of the PBS and CT groups (Fig. 10). These results demonstrate that the presence of NK cells is required for spore mediated cell recruitment into the lungs and suggests that these NK cells may play an important role in the induction of the innate immune responses.

4. Discussion

Resistance to influenza virus infection and disease relies upon mucosal and systemic immunity with sIgA active primarily in the upper respiratory tract and serum IgG in the lower tract [4]. Cell-mediated immune responses, while important, are more focused on clearing virus-infected cells than in prevention. Parenteral influenza vaccines can induce neutralizing IgG but fail to produce sIgA with this being one of the major drawbacks with current vaccination strategies [2,4,38]. In this work we have explored the use of bacterial spores as both an antigen delivery vehicle and as an adjuvant using H5N1 as an example. Unlike other studies where

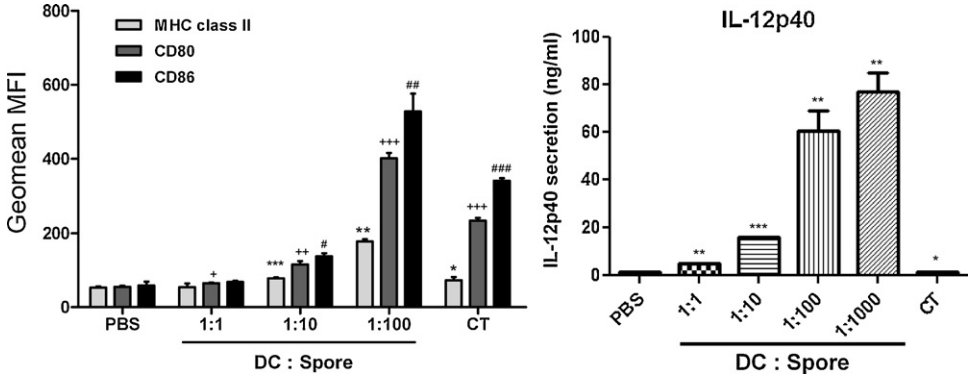

Fig. 9. Killed spores induce DC maturation and IL-12p40 production. Immature BM-DCs were treated with PBS, different MOIs of spores or CT. After incubation for 24 h, BM-DCs were stained with CD11c-FITC plus CD80-PE, CD86-PE and MHC class II-PerCP-eFluor710. Stained cells were analyzed by flow cytometry, FACS Calibur. All cytometric data were expressed as mean fluorescence intensity (MFI) and analyzed by using FlowJo software. At the end of 24 h, supernatants from BM-DC was collected for measurement of Il-12p40 by ELISA. The results are shown as mean IL-12p40 in ng/ml/5×10^5 cells BM-DC ± SD averaged from triplicate cultures. Significant differences by paired *t*-test between maturation from adjuvant and PBS treated DCs by MHC class II ($^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$), CD80 ($^{+}P<0.05$; $^{++}P<0.01$; $^{+++}P<0.001$), and CD86 ($^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$), respectively.

the *Bacillus* spore has been genetically manipulated, here, we have simply used the spore in an inactive, killed, form. The rationale for this approach is that *B. subtilis* spores, approximately 1 μm in length, have been shown to carry natural adjuvant properties with the capacity to adsorb protein antigens [20,23]. Our studies are encouraging and demonstrate that spores can efficiently bind to H5N1 virions, and when administered nasally induce virion-specific serum and mucosal antibody responses greater than if the virion were administered alone. Just two doses of spores mixed with 0.02 μg (HA) of H5N1 were sufficient to confer complete protection in a murine model of infection. These findings then compare favorably with other adjuvant and vaccines currently under development. For example, VLP (virus like particles) vaccines carrying recombinant HA (0.6 μg) also were found to confer protection to avian influenza following i.n. (2 doses) administration but requiring considerably more (30-times) immunogen than that of spores [39]. Interestingly, inactivated cells (not spores) of *Bacillus firmus* (referred to as dilapidated *B. firmus* or DBFs) have also been shown to carry a natural adjuvant property and can induce both protective and cross-protective immunity against influenza virus A H1N1 [40,41].

The HAI assay is considered the gold standard for predicting protection (50%) against a seasonal influenza strain (*i.e.*, HAI titers > 40) [42] but this was not the case using avian H5N1. We found titers of >40 in serum from animals dosed with virus alone yet these animals were not protected. This observation supports that of others [39] and indicates that new correlates of protection for H5N1 infection are needed. If efficacy is proven in humans the use of *B. subtilis* spores could stretch the limited supply of stockpiled vaccines enabling a simplified adjuvant for influenza. Evidence for balanced cell-mediated responses was also observed including the production of cytokines correlating with activated T_h1 and T_h2 cells although evidence for a T_h1-bias was observed based on the ratio of IgG isotypes. An important consideration of future influenza adjuvants is homotypic immunity and cross-clade protection. Clade 2 viruses are genetically and antigenically distinct from clade 1 isolates and have emerged recently so it is encouraging that serum from mice immunized with spores adsorbed with a clade 1 virus is potentially capable of neutralizing a virus from one of the most diverged subclades (clade 2.2) [2]. Presumably, this cross-clade protection arises from sIgA which is known to be cross protective against variant strains of influenza [43,44].

One of the most interesting findings from this work is that heat-killed spores can confer protection without co-delivery of antigen. Our dose-dependent studies show 100% protection for 14 days against a lower lethal dose (5 LD_{50}) of H5N2 and the most straightforward explanation is that of innate immunity. Such protection should be typically short-lived and indeed might account for why 4 weeks after challenge with spores (see Fig. 5) only 60% protection against H5N2 (20 LD_{50}). Interestingly though, against a lower 5 LD_{50} challenge dose 100% protection was achieved 27 days after the final immunization. Short-term innate protection however, is a concept that is now being questioned with recent studies showing a defined innate memory cell population that can protect against a lethal systemic infection of vaccinia virus [45]. There is evidence that in the GI-tract, specialized M (microfold) cells have been shown able to take up bacterial spores to the Peyer's patches located in the lamina propria [46]. This region together with the associated intraepithelial lymphocytes (IELs) contains resting memory T cells, B cells, NK cells, macrophages and dendritic cells. NK cells in mice have been shown to be activated following oral administration of both live and dead *B. subtilis* spores [47] while a number of cytokines (including IFN-γ) have been shown to be induced by macrophages infected with *B. subtilis* spores *in vitro* [48] and *in vivo* [23,35,47,49].

We have provided five strands of evidence showing that heat-killed spores induce innate immunity. First, immunization with spores alone can protect against H5N2 infection. Second, that spores efficiently stimulate maturation of DCs. Third, recruitment of NK cells into the lungs. Fourth, spores administered by a parenteral route enhance responses to H5N1 delivered nasally. Finally, that spores induce expression of the NK-κB pathway. We speculate then that interaction of a spore ligand with one or more TLRs could activate the innate immune system in the nasal-associated lymphoid tissue (NALT). Further work will of course be required to confirm which TLRs are targeted by spores and specifically, the identity of the ligand/s involved. However, it is notable that recent studies have shown clearly that pre-stimulation of TLR2 and TLR4 with appropriate ligands protects mice against lethal infection with highly pathogenic influenza viruses in animal models [50]. We predict then that spores may act similarly, by interacting with the TLRs and stimulating host defenses. In other studies we have shown that killed spores administered orally to Golden Syrian hamsters can significantly delay symptoms of disease and mortality when challenged with *Clostridium difficile* [14] suggesting that this adjuvant property is specific not only to H5N1. Interestingly, other microparticulate adjuvants appear able to induce TLR2 and TLR4 expression, for example, poly(anhydride) nanoparticles which promote T_h1 biased cellular responses [51] and adjuvants derived from Gram-positive bacteria including i.n. delivery of peptidoglycan-rich GEM particles (Gram-positive enhancer matrix; derived from food-grade

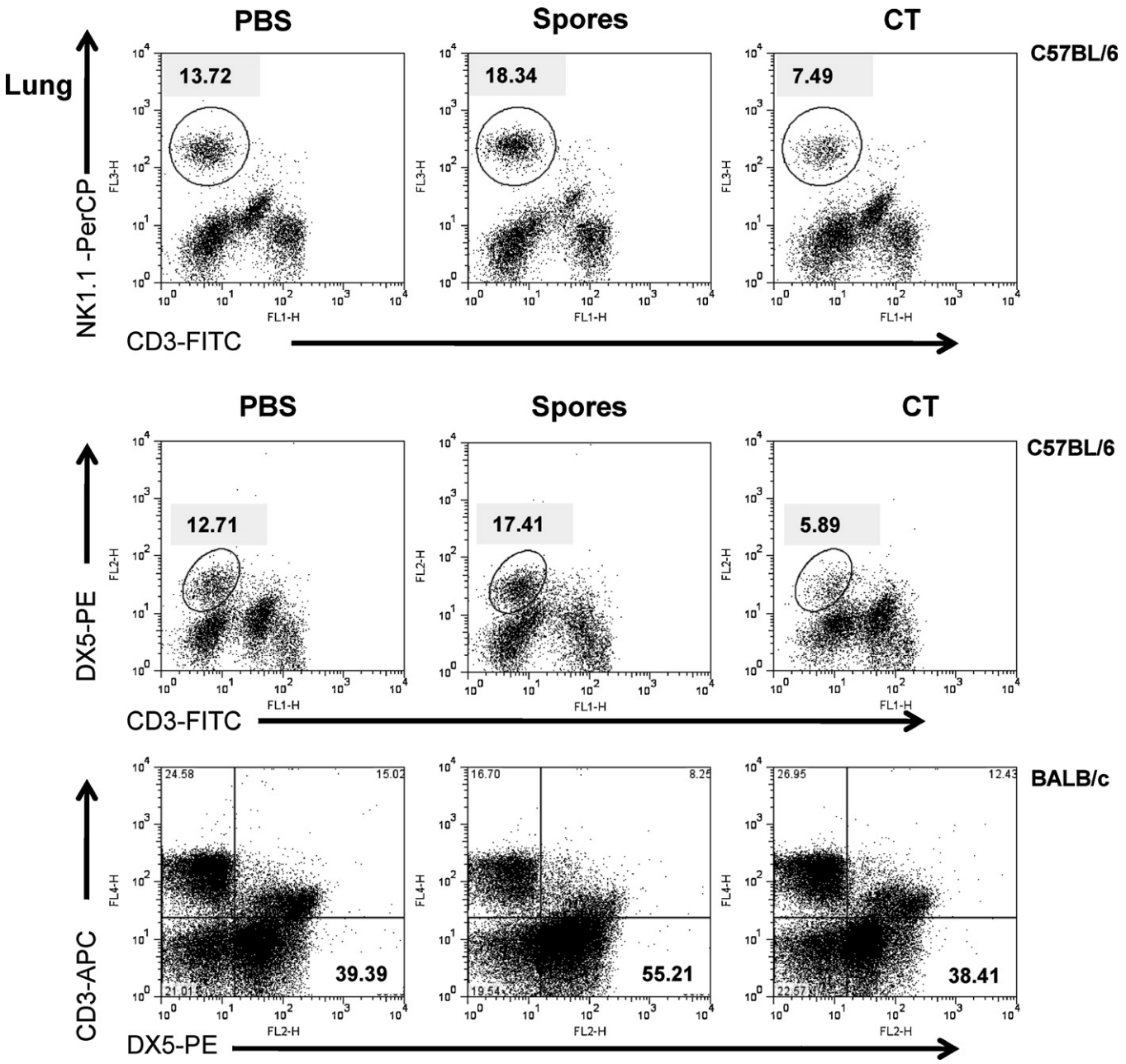

Fig. 10. Killed spores induce NK cell recruitment in the lungs. Flow cytometric analysis of NK cell populations in lungs from BALB/c and C57BL/6 mice was performed 24 h after treatment with PBS, 2×10^9 CFU of spores or 2 μg of CT intranasal route. The two different types of NK cells were defined as $NK1.1^+(CD3^-\ NK1.1^+)$ and $DX5^+$ ($CD3^-\ DX5^+$). The frequency of NK cells was presented as a percentage of total lymphocytes.

Lactococcus lactis) [52]. Studies using *B. firmus* DFPs have also shown upregulation of a number of type 1 interferons, cytokines and TLR genes [40] as well as short-term (7 days) protection to H1N1 [41,53] and live *Bacillus cereus* spores administered in animal feed have been shown to enhance specific PBMC proliferative responses to Influenza H1N1 and H3N2 antigens in piglets [54].

Regarding how the virion binds, in previous work we have shown that proteins can readily bind to negatively charged spores when the pH of the aqueous phase falls below the pI of the respective protein [20]. In addition to electrostatic binding hydrophobic bonding also contributes and this may be a factor in virion adsorption and, in addition to the two viral proteins (neuraminidase and hemagglutinin) it is possible that the lipid component of the viral envelope contributes to binding. As shown here though, binding to the spore is not essential for generating systemic and mucosal responses but these are significantly improved if combined. Therefore, spores serve as both a delivery system and adjuvant. As a delivery system they resemble other microparticulate adjuvants under development that mimic the size range of natural pathogens (20 nm to 2 μm), such as liposomes [55], ISCOMs [56] and emulsions [5] but offer the additional advantage of simplified production and stability. As a microparticulate adjuvant it is predicted that spores would introduce adsorbed antigens directly into the MHC class I and class II presentation pathways [23] and this would occur either in parallel or independently to the interaction of spores with the TLR.

Killed spores may be a viable vaccine-adjuvant for influenza with regard to safety. We show here that the number of spores that are required for generating serum antibody responses can be reduced by 2 logs without any significant effect on antibody titer.

This area will need further empirical investigation but it is possible that the number of spores used to generate protection can be significantly reduced. In summary we demonstrate that inactive spores then can augment mucosal and systemic responses to H5N1 as well as provide an explanation for how this might occur. As such, spores have potential as an influenza adjuvant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2012.03.016.

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