Formulation of bovine respiratory syncytial virus fusion protein with CpG oligodeoxynucleotide, cationic host defence peptide and polyphosphazene enhances humoral and cellular responses and induces a protective type 1 immune response in mice

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Respiratory syncytial virus (RSV) is the leading cause of serious respiratory tract disease in children and calves; however, RSV vaccine development has been slow due to early observations that formalin-inactivated vaccines induced Th2-type immune responses and led to disease enhancement upon subsequent exposure. Hence, there is a need for novel adjuvants that will promote a protective Th1-type or balanced immune response against RSV. CpG oligodeoxynucleotides (ODNs), indolicidin, and polyphosphazene were examined for their ability to enhance antigen-specific immune responses and influence the Th-bias when co-formulated with a recombinant truncated bovine RSV (BRSV) fusion protein (ΔF). Mice immunized with ΔF co-formulated with CpG ODN, indolicidin, and polyphosphazene (ΔF/CpG/indol/PP) developed higher levels of ΔF-specific serum IgG, IgG1 and IgG2a antibodies when compared with ΔF alone, and displayed an increase in the frequency of gamma interferon-secreting cells and decreased interleukin (IL)-5 production by in vitro restimulated splenocytes, characteristic of a Th1 immune response. These results were observed in both C57BL/6 and BALB/c strains of mice. When evaluated in a BRSV challenge model, mice immunized with ΔF/CpG/indol/PP developed significantly higher levels of BRSV-neutralizing serum antibodies than mice immunized with the ΔF protein alone, and displayed significantly less pulmonary IL-4, IL-5, IL-13 and eotaxin and reduced eosinophilia after challenge. These results suggest that co-formulation of ΔF with CpG ODN, host defence peptide and polyphosphazene may result in a safe and effective vaccine for the prevention of BRSV and may have implications for the development of novel human RSV vaccines.

INTRODUCTION

Human respiratory syncytial virus (HRSV) is the leading cause of serious lower respiratory tract infections in infants, and almost all children will have been infected by the age of two (Glezen et al., 1986; Shay et al., 1999). Severe HRSV infection in infancy has been strongly associated with the development of asthma and allergic sensitization later in life (Sigurs et al., 1995, 2000), and it may also cause disease in adults, especially in elderly and immunosuppressed individuals (Meyer et al., 2008). Likewise, bovine respiratory syncytial virus (BRSV) causes considerable economic loss in the cattle industry (Gershwin, 2007). BRSV and HRSV are closely related, displaying similar epidemiology and pathogenesis (Van der Poel et al., 1994), making BRSV a good model for the study of RSV vaccines (Valarcher & Taylor, 2007).

Previous vaccination attempts in humans have not been successful, and most notably, infants immunized with a formalin-inactivated respiratory syncytial virus (RSV) preparation (FI-RSV) preparation induced considerable disease enhancement following subsequent natural infection, generally believed to be due to the generation of a type-2 immune response in naıve infants following vaccination (Graham et al., 2000;
Waris et al., 1996). More recent evidence has suggested that the failure of the FI-RSV vaccine may also have been due to the inability of the formalin-inactivated virus to prime for CD8+ T cell responses (Srikitkachorn & Braciale, 1997), as well as the resultant generation of low avidity, non-protective antibody responses (Delgado et al., 2009; Polack et al., 2002). To date, there is still no licensed vaccine against HRSV, and BRSV vaccines are only moderately effective. Therefore, the development of alternative vaccine strategies against HRSV and BRSV remains a priority.

RSV, an enveloped, non-segmented negative-strand RNA virus, is a member of the family Paramyxoviridae. The envelope contains three virally encoded surface glycoproteins, the fusion (F) protein, the attachment (G) protein, and the small hydrophobic (SH) protein, that are organized into spikes on the virion surface (Collins & Crowe, 2007). The F protein directs viral penetration of the host cell, and mediates fusion with neighbouring cells to form syncytia (Collins & Crowe, 2007). It is synthesized as an inactive 574 aa precursor F0, that is proteolytically cleaved to yield the disulfide-linked heterodimer F2 (residues 1–109)–F1 (residues 137–574), releasing the 27 aa peptide p27 (Collins et al., 1984; Zimmer et al., 2002). Although the protective correlates of RSV infection are not completely understood, the F glycoprotein appears to play an important role in protection against viral challenge, and several neutralization epitopes have been identified on the larger, carboxy-terminal F1 portion of the protein (Lopez et al., 1998).

Monoclonal antibodies against the F protein of RSV were found to passively protect rodents (Taylor et al., 1984; Walsh et al., 1984) against RSV challenge, and reduce RSV symptoms in children (Fenton et al., 1991) and adults (Hall et al., 1991) recovering from RSV infection. Furthermore, F protein-specific CD4+ and CD8+ T cell responses have been detected using human peripheral blood mononuclear cells (PBMCs), in contrast to the G protein which elicited only CD4+ T cell responses (de Waal et al., 2006).

The involvement of the F protein in both humoral and cellular immunity, as well as the high degree of conservation between antigenic groups (Johnson & Collins, 1988), makes it an attractive subunit vaccine candidate for protection against RSV. Subunit vaccines, however, are often poorly immunogenic and can result in weak and transient T-cell responses, thereby requiring adjuvants to boost the immune response. In the case of RSV vaccine formulation, the choice of adjuvant is critical in order to induce a protective Th1-type or balanced immune response that does not result in enhanced disease, and several compounds are promising as novel adjuvants for RSV vaccine development, including CpG oligodeoxynucleotides, host defence peptides and polyphosphazene polymers.

DNA sequences containing an unmethylated CpG dinucleotide, flanked by two 5′ purines and two 3′ pyrimidines, stimulate innate immune responses and trigger the production of Th-1 type cytokines, including gamma interferon (IFN-γ), interleukin (IL)-6, IL-12, and tumour necrosis factor (TNF)-α, via interaction of the CpG motif with toll-like receptor (TLR)-9 on dendritic cells, macrophages and B lymphocytes (Klinman et al., 1996, 1999; Krieg, 2006; Krieg et al., 1995). Co-immunization of protein antigens with synthetic CpG ODNs has been found to increase the production of antigen-specific IgG and direct T-cell responses toward a Th1 phenotype (Ioanou et al., 2002).

Likewise, cathelicidins, a class of endogenous mammalian host defence peptides, have been found to exert a number of immune-modulating functions. Besides their well-documented antimicrobial activity, cathelicidins act as chemotactic factors, induce cytokine and chemokine expression, alter gene expression in host cells, and modulate dendritic cell function (Bowdish et al., 2005; Brown & Hancock, 2006; Hancock, 2001). Recent evidence has also shown that the human cathelicidin LL-37 (An et al., 2005) and mouse cathelin-related antimicrobial peptide (CRAMP) (Kurosaka et al., 2005) were able to enhance adaptive immune responses. Indolicidin, one of the smallest known host defence peptides, is a linear 13 aa peptide found in the cytoplasmic granules of bovine neutrophils (Selsted et al., 1992). In vitro it was found to inhibit lipopolysaccharide-induced TNF-α secretion by human macrophage-like cells and to induce production of the chemokine IL-8 in human bronchial epithelial cells (Bowdish et al., 2005); however, its activity as an adjuvant in vivo has not been explored.

Polyphosphazenes are high molecular mass, water-soluble polymers, containing a backbone of alternating phosphorus and nitrogen atoms (Payne & Andrianov, 1998). They exert adjuvant activity when incorporated into a number of vaccine formulations, including influenza (Mutwiri et al., 2007; Payne et al., 1998), human rotavirus (McNeal et al., 1999), and cholera vaccines (Wu et al., 2001). Polyphosphazene activity is not due to the formation of an injection-site depot, but rather may be linked to the ability of the polymer to form water-soluble, non-covalent complexes with antigens, stabilizing them and allowing efficient presentation to immune cells (Andrianov et al., 2005; Payne & Andrianov, 1998).

Central to the development of RSV candidate vaccines is the need to identify novel adjuvant compounds that will induce a protective balanced or Th1 immune response and will not lead to disease enhancement. Here, we report on the co-formulation of a recombinant BRSV fusion (F) glycoprotein with the adjuvant compounds CpG ODN, indolicidin and polyphosphazene, and their ability to elicit Th1 immune responses and mediate protection against BRSV infection in mice.

**METHODS**

**Cells and virus.** HEK 293 cells were grown in minimum essential medium (MEM) (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 0.1 mM non-essential amino acids
The 375 strain of BRSV (ATCC) was propagated in bovine turbinate (BT) cells (ATCC) maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 1 % (v/v) heat-inactivated FBS, 10 mM HEPES and 50 μg ml−1 gentamicin. Four to seven days following infection, cells were collected by scraping and were stored at −70 °C until used for preparation of challenge virus. Virus titres were determined by plaque assay and visualized by immunostaining as described previously (Mapletoft et al., 2008).

To prepare challenge virus, infected cell-lyse was thawed and centrifuged for 30 min at 1940 g, at 4 °C. The pellet was then resuspended in 1/100 of the original volume in serum-free DMEM and disrupted by sonication.

**Constitution of expression plasmid.** The construction of a plasmid expressing a truncated version of the BRSV F protein (aa 1-522), lacking the transmembrane anchor domain to facilitate secretion from the cells, was carried out as follows. Briefly, the truncated F gene (ΔF) was PCR-amplified from the plasmid pMASIA-ΔF, using the following primers: 5’-TCGGATCCATATGACGT-CGACGCGTCTG-3’ and 5’-GCCGATATCTATGACGT-CGACGCGTCTG-3’. The resulting PCR product was digested with HindIII and BamHI (New England BioLabs) and ligated into the expression vector pcDNA6/V5-His (Invitrogen), digested with the same restriction enzymes. The pcDNA-ΔF expression plasmid was then digested with NotI (New England BioLabs) and dephosphorylated using Antarctic Phosphatase (New England BioLabs). Synthetic oligonucleotides (Sigma-Genosys Canada) encoding ten repeating serine-glycine (Ser/Gly) residues followed by ten histidine (His) residues were annealed, and ligated into pcDNA-ΔF, to produce the expression vector pcDNA-ΔF-10His.

**Expression and purification of truncated F protein.** HEK 293 cells were transiently transfected with pcDNA-ΔF-10His using Lipofectamine Plus Reagent (Invitrogen) in Opti-MEM medium (Invitrogen), according to the manufacturer’s instructions. After 48 h, the medium was collected and replaced with fresh medium. Medium was collected again at 96 and 144 h post-transfection. Medium collected from transfected cells was clarified by centrifugation and concentrated by ultrafiltration using a YM 10 membrane (MWCO 10 kDa) (Millipore).

Recombinant ΔF was purified from the concentrated transfection supernatant by nickel ion affinity chromatography, under native conditions, using ProBond nickel-chelating resin (Invitrogen). Impurities were removed from the resin by subsequent washes with 50 mM monobasic sodium phosphate, 500 mM sodium chloride, pH 8.0, containing 20 mM and 30 mM imidazole (Sigma-Aldrich), respectively. Bound protein was eluted with 50 mM monobasic sodium phosphate, 500 mM sodium chloride, pH 8.0, containing 300 mM imidazole. Purified protein was dialysed against PBS, pH 7.4. Purity and molecular mass were confirmed by SDS-PAGE, according to the method of Laemmli (1970), and densitometry analysis and mass approximation were carried out using a Molecular Imager Gel Doc XR System with Quantity One 1-D Analyst Software (Bio-Rad Laboratories). Protein concentration was determined by the bicinchoninic acid (BCA) assay (Pierce). Western immunoblotting was carried out to confirm the presence of both the F1 and F2 regions of the protein. Purified ΔF protein was resolved by SDS-PAGE under both reducing and non-reducing sample conditions, and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked and probed using anti-ΔF rabbit serum, followed by IRDye 680 labelled goat anti-rabbit antibody, diluted 1:500 and 1:15,000, respectively, in 1 % (w/v) skim milk powder in TBS, and ΔF protein was visualized using an Odyssey Infrared Imaging System (LI-COR Biosciences).

**Immunization and challenge.** To evaluate the efficacy of the various adjuvant compounds, six- to eight-week-old female C57BL/6 mice (Charles River Laboratories) were immunized twice subcutaneously at 3 week intervals with 100 μl of vaccine containing 0.5 μg ΔF, either alone, or co-formulated with CpG ODN 1826 (CpG), indolcinid (indol), and/or polyphosphazene (PP) in 10 mM Tris/HCl, pH 7.6, containing 0.27 M sorbitol (Sigma-Aldrich). CpG ODN, indolcinid and polyphosphazene were given at doses of 10, 20 and 50 μg, respectively. Negative control animals were immunized with Tris-sorbitol buffer (placebo). CpG ODN 1826 (TCCATGAGCTTCCGAGTCTG) was provided by Merial, and contained a phosphorothioate-modified backbone. Indolcinid (ILPWKWPWPPWRR-NH2) was chemically synthesized on a Pioneer solid-phase peptide synthesizer (PerSeptive Biosystems) using 9-fluorenlymethoxy carbonyl (Fmoc) chemistry. The polyphosphazene polymer, a 90 % substituted poly(di-p-dicarboxylatophenoxo)phosphazene (PCP/P)10% hydroxylate (90 : 10 PCP/OH), was synthesized as described previously (Andrianov et al., 2004) and was provided by Dr Klæhn, Idaho National Laboratory (Idaho Falls, ID, USA).

The vaccine composed of ΔF formulated with CpG ODN, indolcinid and polyphosphazene (ΔF/CpG/indol/PP) was further evaluated for its ability to enhance protection against BRSV. Six- to eight-week-old female BALB/c mice (Charles River Laboratories) were immunized twice subcutaneously at 3 week intervals with 0.5 μg ΔF alone or combined with CpG ODN 1826, indolcinid and polyphosphazene (ΔF/CpG/indol/ PP). Negative control animals were immunized with buffer alone (placebo). Two weeks following the second immunization, mice were challenged intranasally with 107 p.f.u. of BRSV strain 375, in a final volume of 50 μl. Mock challenged animals were given 50 μl of saline intranasally. Four days following challenge, the mice were humanely euthanized and lungs were collected from half of the mice for detection of viral RNA and IL-5, IL-4, IL-13, IFN-γ and eotaxin production. Six days following challenge, the remaining mice were humanely euthanized and spleens were collected for analysis by enzyme-linked immunosopot (ELISPOT) assay, and bronchoalveolar lavage (BAL) fluids were collected from each group and pooled. Cytospin slides were prepared using 1 × 105 and 5 × 104 cells and stained with Wright-Giemsa stain (Bayer HealthCare). The numbers of macrophages, neutrophils, lymphocytes and eosinophils for each group were determined by examination of at least 200 cells. All procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care.

**ELISA.** Ninety-six-well microtitre plates (Immulon 2; Thermoflone) were coated with concentrated ΔF transfection supernatant diluted 1:100 in sodium carbonate buffer, pH 9.6. Plates were incubated overnight at 4 °C with serially diluted mouse sera. Total ΔF-specific serum IgG titres were measured using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories), diluted 1:5000, and bound antibodies were visualized with p-nitrophenyl phosphate (PNPP) (Sigma-Aldrich). ΔF-specific IgG1 and IgG2a titres were measured using biotinylated goat anti-mouse IgG1 and IgG2a antibodies (Caltag Laboratories), diluted 1:5000, followed by streptavidin-AP (Jackson ImmuoResearch Laboratories) at a dilution of 1:20,000. ELISA titres were expressed as the reciprocal of the highest dilution resulting in a value of 2 SD above the negative control serum.

**Virus-neutralization assay.** BT cells were cultured overnight in 96-well flat bottom tissue culture plates (Corning) to 70–80 % confluency. Sera were diluted in 96-well plates (Corning) in DMEM supplemented with 1 % (v/v) FBS and 10 mM HEPES, starting at a dilution of 1:20 and continuing in serial twofold dilutions. BRSV strain 375 (500 p.f.u. per well) was added to the wells and incubated for 1 h at 37 °C. The serum–virus mixtures were transferred to duplicate BT cell plates and incubated for 7 days at 37 °C. BRSV plaques were visualized by immunostaining as described previously (Mapletoft et al., 2008), and virus-neutralization titres were expressed as the highest dilution of serum that resulted in <50 % of cells displaying cytopathic effects.
**IFN-γ and IL-5 ELISPOT assays.** Ninety-six-well nitrocellulose plates (MultiScreen-HA; Millipore) were coated overnight, at 4 °C, with 0.2 μg per well of mouse IFN-γ or IL-5-specific monoclonal antibodies (BD Biosciences). Plates were washed with PBS (Invitrogen) and blocked with 1 % (w/v) BSA (Sigma-Aldrich) in PBS. Splenocytes were isolated as described previously (Baca-Estrada et al., 1996) and resuspended to a final concentration of 10^6 cells ml^-1 in AIM V medium (Invitrogen) containing 0.1 mM non-essential amino acids, 1 mM sodium pyruvate (Invitrogen), 10 mM HEPES and 50 μM 2-mercaptoethanol (Sigma-Aldrich). Splenocytes were added to the plates at a concentration of 10^6 cells per well, and cultured in the presence of 1 μg ml^-1 AF or medium for 18–20 h. Plates were washed and incubated with biotinylated rat anti-mouse IFN-γ or IL-5 (BD Biosciences) at 2 μg ml^-1. Bound antibodies were detected using streptavidin-AP (Jackson Laboratories) diluted 1:1000 and visualized using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitro blue tetrazolium chloride (NBT) substrate tablets (Sigma-Aldrich). Plates were washed with water and air-dried. The spots were counted, and the numbers of IFN-γ- and IL-5-secreting cells were expressed as the difference between the number of spots in AF-stimulated wells and control (medium) wells per 10^6 cells.

**Cytokine ELISAs.** Lungs were homogenized in DMEM supplemented with 10 mM HEPES, 50 μg gentamicin ml^-1, 10 μg aprotinin ml^-1 (Sigma-Aldrich), 10 μg leupeptin ml^-1 (Sigma-Aldrich), 0.1 mM EDTA (Sigma) and 1× antibiotic/antimycotic (Invitrogen), using a Mini-BeadBater (BioSpec Products) with 2.4 mm zirconia microbeads (BioSpec Products). Homogenates were clarified by centrifugation at 10 000 g for 1 min, and IL-4, IL-5, IL-13, IFN-γ and eotaxin were quantified using Quantikine mouse ELISA kits (R&D Systems), according to the manufacturer’s instructions.

**Detection of viral RNA.** Detection of viral RNA was carried out as described previously (Mapleton et al., 2008). Briefly, lungs were collected into TRIzol reagent (Invitrogen) 4 days following infection, and homogenized. Total RNA was isolated according to the manufacturer’s instructions, and genomic DNA removal and reverse transcription were carried out using a QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR (qPCR) was performed with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) using primers designed to amplify a 168 bp fragment of the BRSV F gene (5’-AACCGGCCCTCCCTTGAGA-3’; 5’-TGGACACGTCACTACACCATT-3’). A standard curve was constructed using a plasmid containing the AF gene (pMASIA-AF) at known concentrations, allowing the quantification of BRSV F protein cDNA in each of the samples. Results were expressed as the number of viral RNA copies per ml of lung homogenate.

**Statistical analysis.** Statistical analysis was carried out using GraphPad Prism version 5.00 for Windows (GraphPad Software). As outcome variables were not normally distributed, differences among all groups were examined using the Kruskal–Wallis test. If a significant difference was found among the groups, median ranks between pairs of groups were compared using the Mann–Whitney U test. Differences were considered significant if P<0.05.

**RESULTS**

**Expression and purification of recombinant ΔF protein**

A vector expressing a truncated version of the BRSV F protein (ΔF) with a C-terminal serine/glycine linker and 10× histidine (His) tag was constructed and used to transiently transfect HEK 293 cells. Soluble ΔF protein was purified from the transfection supernatant using nickel ion affinity chromatography. SDS-PAGE analysis indicated that the affinity-purified protein was 92 % pure, with a molecular mass of 52 kDa, corresponding to the mass of the F1 region of the F protein (Fig. 1a). When the ΔF protein was analysed under non-reducing conditions (Fig. 1b) the protein had an apparent molecular mass of approximately 75 kDa, corresponding to AF0, indicating that both the F1 and F2 regions of the ΔF protein were expressed and remained associated. The identity of the ΔF protein was confirmed by mass spectrometry.

**Humoral and cell-mediated immune responses after immunization with ΔF formulated with CpG ODN, indolicidin and polyphosphazene**

CpG ODN 1826, indolicidin and polyphosphazene were examined for their ability to enhance antigen-specific immune responses when administered with the AF protein, either alone, or co-formulated with one another. In order to examine the efficacy of each of the adjuvant compounds, vaccines were formulated with a suboptimal amount of ΔF protein (0.5 μg per dose), a dose that had been established.

**Fig. 1.** Analysis of affinity-purified ΔF protein by SDS-PAGE and Western blotting. Proteins were resolved using 10 % acrylamide, and visualized by staining with Coomassie brilliant blue (a) or transferred to nitrocellulose and probed with anti-ΔF rabbit serum and IRDye 680-labelled goat anti-rabbit antibody (b). Protein was prepared in reducing, R (presence of 2-mercaptoethanol), or non-reducing, NR (no 2-mercaptoethanol), sample buffer. Molecular mass markers are shown in the left lane.
in a previous dose-titration study (data not shown). C57BL/6 mice were immunized twice subcutaneously, and serum total anti-ΔF IgG titres were measured after each immunization by ELISA.

While the total IgG titres elicited by all adjuvants after one immunization were significantly higher than those of groups immunized with ΔF alone (Fig. 2a), groups immunized with vaccines containing CpG ODN tended to develop higher titres than those immunized without it. Likewise, following two immunizations the IgG titres of all groups, with the exception of those immunized with ΔF/indol and ΔF/indol/PP, were significantly higher than that of groups immunized with ΔF alone (Fig. 2b). Titres induced by immunization with ΔF/indol or ΔF/indol/PP were not significantly higher than those induced by ΔF alone and were significantly lower than those elicited by all other adjuvant groups.

Determination of serum IgG1 and IgG2a subclass titres revealed that all of the vaccine formulations were capable of eliciting high IgG1 antibody titres, with the highest titres observed in the groups immunized with ΔF/CpG/PP and ΔF/CpG/indol/PP (Fig. 2c). However, CpG-containing formulations also resulted in IgG2a titres that were significantly higher than those observed in the groups not immunized with CpG ODN (Fig. 2d), suggesting the induction of a Th1-biased or balanced immune response. This shift towards a more balanced humoral immune response was also evident in the serum subclass IgG1/IgG2a ratios (Fig. 2e).

ΔF-induced secretion of IFN-γ and IL-5 by in vitro restimulated splenocytes was measured after the second immunization, in order to further evaluate the immune response induced by the ΔF protein when co-formulated with the different adjuvant compounds. A marked increase in the frequency of IFN-γ-secreting cells was observed in mice immunized with ΔF/CpG/indol/PP when compared with IFN-γ secretion in all other vaccine groups (Fig. 3a). Significantly fewer IL-5-secreting cells were observed in all groups immunized with CpG-containing formulations (Fig. 3b).

These results suggest that the ΔF protein induced a Th2-biased immune response. However, the inclusion of CpG ODN in the vaccine formulations resulted in a shift towards a Th1 or balanced immune response. On the other hand, indolicidin and polyphosphazene, in the absence of CpG ODN, appeared to sustain the type-2 immune response induced by ΔF. Only the combination of all three adjuvants, ΔF/CpG/indol/PP, demonstrated a significant enhancement of both the ΔF-specific humoral and cellular immune responses, and was therefore selected for further characterization as a potential BRSV vaccine candidate.

**Comparison of immune responses in C57BL/6 and BALB/c mice**

BALB/c mice are most commonly used for challenge studies, as they can be infected with RSV, even though they are not fully permissive; however, they are more likely to develop a Th2-skewed immune response than C57BL/6 mice. Therefore, the immune response to ΔF formulated with the combination of CpG ODN, indolicidin and polyphosphazene was further evaluated in both strains of mice.

Serum subclass titres were measured in order to determine whether immunization with ΔF/CpG/indol/PP could similarly increase antigen-specific IgG1 and IgG2a levels in both strains of mice. Mice immunized with ΔF/CpG/indol/PP developed significantly higher IgG1 (Fig. 4a) and IgG2a (Fig. 4b) titres than those immunized with ΔF alone. There was no significant difference in the number of IFN-γ-secreting cells produced in either strain compared with those immunized with ΔF alone (Fig. 4c), and both strains demonstrated a decrease in the number of IL-5 secreting cells when immunized with ΔF/CpG/indol/PP (Fig. 4d).

**ΔF-specific humoral and cell-mediated immune responses in mice immunized with ΔF protein formulated with CpG ODN, indolicidin and polyphosphazene, and challenged with BRSV**

In order to evaluate the protective efficacy of the ΔF protein when formulated with CpG ODN, indolicidin and polyphosphazene against BRSV, BALB/c mice were immunized twice with either ΔF alone or ΔF/CpG/indol/PP, and challenged 2 weeks after the second immunization with strain 375 of BRSV. Control mice were immunized with buffer and were challenged with BRSV or mock-challenged with saline.

As observed in the previous trials, serum total IgG responses were significantly higher in mice immunized with ΔF/CpG/indol/PP than in mice immunized with ΔF alone (Fig. 5a). This difference was evident in both the primary and secondary immune responses, as well as after challenge. In both groups, titres increased following the second immunization as well as after challenge, suggesting the induction of an anamnestic response. Likewise, determination of serum subclass titres revealed significantly higher IgG1 and IgG2a production in mice immunized with ΔF/CpG/indol/PP, both after two immunizations and after challenge (Supplementary Table S1, available in JGV Online). The ability of the resulting serum antibodies to neutralize the virus in vitro was assessed using a virus neutralization assay. Immunization with ΔF/CpG/indol/PP induced neutralizing antibody titres that were significantly higher than those elicited by ΔF alone (Fig. 5b) and increased approximately fourfold following challenge with BRSV.

To examine the cellular immune responses generated by each of the vaccines, ΔF-induced secretion of IFN-γ and IL-5 by splenocytes was measured 6 days after challenge. Although not significantly different, a trend towards increased frequencies of ΔF-specific IFN-γ-producing splenocytes was observed in mice immunized with ΔF/CpG/indol/PP (Fig. 6a). In contrast, the number of
**Fig. 2.** ΔF-specific antibody responses in sera of immunized mice. Total serum anti-ΔF IgG titres were measured after one (a) and two (b) immunizations. IgG1 (c) and IgG2a (d) titres and IgG1/IgG2a ratios (e) were measured after two immunizations. C57BL/6 mice were immunized twice subcutaneously with 0.5 μg ΔF alone, or co-formulated with CpG ODN 1826 (CpG), indolicidin (indol), and/or polyphosphazene (PP), given at 10, 20 and 50 μg per dose, respectively. Control mice were immunized with buffer (Placebo). One mouse in the ΔF/PP group died and was excluded from analysis. ELISA titres are expressed as the reciprocal of the highest dilution resulting in a value of 2 SD above the negative control serum. Each data point represents an individual animal, and median values are indicated by horizontal lines. *P<0.05; **P<0.01; ***P<0.001.
IL-5-producing cells was significantly reduced in mice immunized with ΔF/CpG/indol/PP when compared with immunization with ΔF alone (Fig. 6b). These results further substantiate the ability of the formulation of CpG ODN, indolicidin and polyphosphazene to shift the immune response generated by ΔF from a Th2 response to a Th1-biased response.

Cytokine/chemokine induction and cell populations in the lungs

Lung homogenates were examined for the presence of IL-4, IL-5, IL-13, IFN-γ and eotaxin 4 days after challenge. Significantly higher levels of the Th2 cytokines IL-4, IL-5, IL-13 and eotaxin, a potent eosinophil chemoattractant, were detected in mice immunized with ΔF alone, when compared with mice immunized with ΔF/CpG/indol/PP (Fig. 7a–d). Similar results were observed in lungs from animals 6 days after challenge (data not shown). Accordingly, IFN-γ levels were elevated in mice immunized with ΔF/CpG/indol/PP when compared with mice immunized with ΔF alone (Fig. 7e).

The effect of formulation of ΔF with CpG ODN, indolicidin and polyphosphazene on the pulmonary immune response following BRSV infection was also examined. The percentage of eosinophils present in BAL fluids of mice immunized with ΔF was 63%, in contrast to 2% in mice immunized with ΔF/CpG/indol/PP (Fig. 7f). No eosinophils were observed in either of the control groups. The percentage of both neutrophils and alveolar macrophages was higher in the control mice and mice immunized ΔF/CpG/indol/PP when compared with ΔF alone, whereas the percentage of lymphocytes was low in all the groups.

Detection of viral RNA in the lungs of infected mice

To determine whether immunization with ΔF/CpG/indol/PP could reduce viral replication, and thereby prevent BRSV infection, viral RNA in the lungs of mice was measured 4 days after infection using qRT-PCR. The amount of viral RNA detected in mice immunized with ΔF/CpG/indol/PP was significantly lower than that detected in the placebo group, as was that in mice immunized with ΔF alone (Fig. 8).

DISCUSSION

Vaccination remains the single most effective method for the prevention of infectious disease (Klinman, 2004). Despite this, there still exists no licensed HRSV vaccine, and BRSV vaccines are only moderately effective. Efforts to develop RSV vaccines have been slowed by the inability of candidate vaccines to induce protection against natural infection and the induction of exaggerated disease upon subsequent exposure to the virus, widely accepted to be due to vaccine-induced type-2 polarized T cell responses (Meyer et al., 2008). In addition, evidence for a dual role of cytotoxic T-lymphocytes (CTL) in RSV infection has been reported. CD8+ T cells may play an important role in the regulation of differentiation and activation of Th2 CD4+ T cells and eosinophil recruitment during RSV infection (Srikiatkhachorn & Braciale, 1997). Transfer of RSV-specific CD8+ T-cells immediately after RSV infection

Fig. 3. Numbers of IFN-γ-secreting (a) and IL-5-secreting (b) splenocytes in response to in vitro restimulation with ΔF. C57BL/6 mice were immunized twice subcutaneously with 0.5 μg ΔF alone, or co-formulated with CpG ODN 1826 (CpG), indolicidin (indol) and/or polyphosphazene (PP), given at 10, 20 and 50 μg per dose, respectively. Control mice were immunized with buffer (Placebo). Results are expressed as the difference between the number of cytokine-secreting cells in ΔF-stimulated wells and medium-control wells per 10⁶ cells. Each data point represents an individual animal, and median values are indicated by horizontal lines. *P<0.05; **P<0.01; ***P<0.001.
was shown to lead to viral clearance, but also augmented lung pathology (Alwan et al., 1992; Cannon et al., 1988), although the treatment time and numbers of transferred CTLs might not correspond to the in vivo response to RSV infection. However, in humans decreased clinical symptoms were associated with increased CTL numbers in the peripheral blood of previously infected adults (Bangham et al., 1986; Isaacs, 1991). Recent evidence also suggests that the inability of the formalin-inactivated virus to prime for CD8$^+$ T-cell responses (Srikiatkhachorn & Braciale, 1997), as well as the generation of low avidity, non-protective antibody responses (Delgado et al., 2009; Polack et al., 2002) may have led to the lack of protection and disease enhancement observed in children immunized with FI-RSV. It is therefore essential to identify novel adjuvant candidates that will overcome these obstacles and induce protective balanced or Th1-biased immune responses when co-administered with RSV vaccines. We have already demonstrated that the adjuvant combination of CpG ODN, indolicidin and polyphosphazene could induce

![Fig. 4. Comparison of ΔF-specific immune responses in C57BL/6 (black symbols) and BALB/c (open symbols) mice. Mice were immunized subcutaneously with ΔF alone or ΔF/CpG/indol/PP. Control mice were immunized with buffer (Placebo). After two immunizations, IgG1 (a) and IgG2a (b), titres were measured, and numbers of IFN-γ-secreting (c) and IL-5-secreting (d) splenocytes in response to in vitro restimulation with ΔF were determined. In vitro restimulation results are expressed as the difference between the number of cytokine-secreting cells in ΔF-stimulated wells and medium-control wells per 10$^6$ cells. ELISA titres are expressed as the reciprocal of the highest dilution resulting in a value of 2 SD above the negative control serum. Each data point represents an individual animal, and median values are indicated by horizontal lines. One BALB/c mouse in the ΔF/CpG/indol/PP group died and was excluded from analysis. **P<0.01; ***P<0.001.](http://vir.sgmjournals.org)
balanced immune responses when co-administered with ovalbumin, as well as enhance antigen processing via both major histocompatibility complex (MHC) I and II pathways (Kovacs-Nolan et al., 2009). Furthermore, the inclusion of the TLR ligand CpG ODN should help to overcome the limitations in affinity maturation observed with inactivated RSV vaccines (Delgado et al., 2009).

Subunit protein vaccines offer a safer alternative to conventional vaccines such as inactivated or live attenuated pathogens; however, they are typically less immunogenic and thus require co-formulation with adjuvants (Perrie et al., 2008). Evaluation of the adjuvant effects of CpG ODN, indolicidin and polyphosphazene, when co-formulated with the DF protein, revealed that they were most efficacious when used in combination. Adjuvant doses were selected based on previous reports of adjuvanticity of the individual components. Complete immunostimulatory effects of CpG ODN 1826 have been observed with doses as low as 10 μg (Davis et al., 1998), and polyphosphazene when administered at 50 μg per dose has demonstrated enhancement of immune responses even in the presence of low amounts of antigen (Mutwiri et al., 2007). Examination of the effect of indolicidin dose and adjuvant activity when co-administered with CpG ODN 1826 indicated that 20 μg of peptide enhanced antigen-specific

Fig. 6. Numbers of IFN-γ-secreting (a) and IL-5-secreting (b) splenocytes in response to in vitro restimulation with ΔF in mice challenged with BRSV. BALB/c mice were immunized twice subcutaneously with ΔF alone or with ΔF/CpG/indol/PP and challenged 2 weeks later with BRSV. Control groups were immunized with buffer and then challenged with BRSV (Placebo/BRBV or mock-challenged with saline (Placebo/saline). Results are expressed as the difference between the number of cytokine-secreting cells in ΔF-stimulated wells and medium-control wells per 10⁶ cells. Numbers of IFN-γ and IL-5-secreting-cells observed in medium-control wells were 2 for IFN-γ and 20 for IL-5. Each data point represents an individual animal, and median values are indicated by horizontal lines. **P<0.01.
immune responses and effected class switching to IgG2a better than when the peptide was administered at a tenfold higher dose (unpublished results). Similar to findings described previously (Hancock et al., 1995; 2001), the ΔF protein, when administered alone, resulted in a predominantly Th2 immune response. In our study, immunization of mice with ΔF co-formulated with a combination of all three adjuvants at the indicated doses led to a significant increase in serum total IgG, IgG1 and IgG2a titres, and increased IFN-γ production, which is important in establishing a protective Th1 antigen-specific immune response to RSV infection, and preventing vaccine-induced disease enhancement (Durbin et al., 2002).

The effects of CpG ODNs as adjuvants and immune modulators have been well established. CpG ODNs have been found to increase the immunogenicity of protein antigens, induce IgG isotype class switching to favour IgG2a production, and increase the production of Th1...
cytokines, such as IFN-\(\gamma\) (reviewed by Klinman, 2004); and CpG ODN-mediated immunomodulatory effects have been found to be enhanced when the ODNs were retained in close proximity to the antigen (Klinman, 2004; Klinman et al., 1999). Hancock et al. (2001) found that the RSV F protein, when administered with CpG ODN in the absence of another adjuvant, did not induce significant levels of anti-F total or subclass IgG, or neutralizing antibody. Likewise, mucosal immunization of cotton rats with RSV F protein co-adjuvanted with only CpG ODN resulted in only modest protection from viral challenge and did not prevent the development of enhanced pulmonary pathology (Prince et al., 2003). Polyphosphazenes have been suggested to exert their adjuvant effects through the formation of water-soluble antigen–polyphosphazene complexes (Andrianov et al., 2005), and this complex formation would satisfy the requirement for antigen–ODN association. In fact, Mapleton et al. (2008) observed an increase in antigen-specific IgG titres and frequency of INF-\(\gamma\)-secreting cells following mucosal immunization of mice with a Fl-BRSV vaccine co-formulated with CpG ODN and polyphosphazene. Indeed, the combination of AF with CpG ODN and polyphosphazene did enhance antigen-specific humoral immunity; however, inclusion of the cationic cathelicidin peptide indolicidin further enhanced cell-mediated immune responses, suggesting it also plays an important role in vaccine formulation.

It has been demonstrated that cathelicidin peptides have the potential to not only act as innate immune stimulators, but also promote the induction of adaptive immune responses (Kurosaka et al., 2005). There is evidence suggesting that cationic peptides may form a transient depot at the injection site and enhance the association of antigen with antigen-presenting cells (Fritz et al., 2004; Lingnau et al., 2002). In the present study, indolicidin on its own did not appear to be an effective adjuvant, and promoted a Th2-biased response. The combination of indolicidin, however, along with CpG ODN and polyphosphazene enhanced AF-specific humoral and cellular immune responses and generated a Th1 response. Furthermore, we have recently demonstrated that indolicidin acts synergistically with CpG ODN to increase the production of Th1 cytokines, such as IL-12 and IFN-\(\gamma\), as well as the chemotactic factor MCP-1 (Kovacs-Nolan et al., 2009).

The combination of all three adjuvants was required for optimal enhancement of the immune response, as well as to shift the resulting anti-AF immune response from Th2 to Th1. However, the mechanisms of this immune enhancement are unclear. Synergism between CpG ODN and other adjuvants has been reported, suggested to arise due to the protection of CpG and/or antigen from degradation, depot formation or an enhancement of uptake by antigen-presenting cells (reviewed by McCluskie & Krieg, 2006). Likewise, short cationic peptides, such as indolicidin, have been shown to enhance the association of antigen to antigen-presenting cells and form a depot at the site of injection (Lingnau et al., 2002). Formulation with polyphosphazene, then, which forms non-covalent complexes with protein, may ensure that the vaccine components remain closely associated. The enhanced immunogenicity and Th1-biasing effect of the combination of all three adjuvants with AF could therefore occur as a result of the stabilization and prolonged effect of CpG ODN and/or AF and an increased uptake of CpG ODN and AF into antigen-presenting cells.

The vaccine consisting of AF/CpG/indol/PP was further evaluated using a BRSV challenge model in mice to examine if this vaccine could also enhance protection. Serum antibody titres, both before and after challenge, as well as the enhancement of IFN-\(\gamma\) secretion and concomitant decrease in IL-5 production confirmed the results of the previous trials, that AF/CpG/indol/PP could enhance the immune response when compared to AF alone, and could effect a shift from a Th2 to Th1 response. Moreover, mice immunized with AF/CpG/indol/PP developed serum neutralizing antibody titres that were significantly higher than titres induced by AF alone, both before and after challenge.

The presence of Th2 cytokines, including IL-4, IL-5 and IL-13, has been found to correlate with eosinophilic, vaccine-enhanced lung pathology (Castilow et al., 2008; Johnson et al., 2003). Here, the production of IL-4, IL-5, IL-13 and eotaxin was significantly reduced in the lungs of mice immunized with AF/CpG/indol/PP. Th2 cytokines exhibit multiple effects on eosinophils. IL-5 can act systemically to stimulate eosinophil mobilization from the bone marrow, thus increasing the number of circulating eosinophils, as
well as locally in the lung tissue to increase their responsiveness to mediators that stimulate chemotaxis and degranulation (Palframan et al., 1998; Rankin et al., 2000), and IL-13 can act as a chemoattractant and prolong the survival of eosinophils (Castilow et al., 2008). Eotaxin, a CC chemokine, is a potent and selective chemoattractant for eosinophils. The potency and rapid action of eotaxin in inducing selective eosinophil accumulation suggests an integral role for this chemokine in eosinophil trafficking (Rankin et al., 2000). Indeed, levels of IL-4, IL-5, IL-13 and eotaxin in the lungs of mice immunized with ΔF alone correlated with the increased incidence of eosinophils. This influx of eosinophils, which is associated with RSV disease enhancement, is similar to that observed both in mice (Waris et al., 1996) and calves (Antonis et al., 2003) following immunization with formalin-inactivated vaccines. At the same time, the percentage of neutrophils and alveolar macrophages, which have been shown to be important in the clearance of BRSV during infection (Viuff et al., 2002), were lower in mice receiving ΔF alone. Moreover, the production of IFN-γ, which plays an important role in establishing a protective Th1 response in RSV infection (Durbin et al., 2002), was increased in the lungs of mice immunized with ΔF/CpG/indol/PP when compared with those immunized with ΔF alone.

Detection of virus in the lungs of infected mice by qRT-PCR revealed a significant reduction in the number of viral RNA copies in the lungs of mice vaccinated with ΔF/CpG/indol/PP compared with the unvaccinated mice, suggesting more effective clearance of the virus following vaccination. Viral RNA also appeared to be reduced in the mice immunized with ΔF alone. The observation that vaccination with ΔF/CpG/indol/PP did not eliminate viral replication in the lungs may be the result of the low dose of ΔF used in the vaccine formulations. Protection following immunization with F subunit vaccines is typically observed with antigen doses greater than 1 μg (Hancock et al., 1994, 1995; Walsh, 1994). Therefore, higher doses of the recombinant ΔF may be required for complete protection when co-formulated with CpG ODN, indolicidin and polyphosphazene, and warrants further study. Likewise, further study will be required to determine the roles of CpG ODN, indolicidin and polyphosphazene in enhancing the antigen-specific immune response and promoting viral clearance and BRSV prevention.

Taken together, these results suggest that the co-formulation of recombinant ΔF with CpG ODN, indolicidin and polyphosphazene may result in a safe and effective vaccine for the prevention of BRSV infection, and may have significant implications for the development of novel human RSV vaccines.

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REFERENCES


