Enhanced immune responses and protection by vaccination with respiratory syncytial virus fusion protein formulated with CpG oligodeoxynucleotide and innate defense regulator peptide in polyphosphazene microparticles

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ABSTRACT

Although respiratory syncytial virus (RSV) is the leading cause of serious respiratory tract disease in children, to date no RSV vaccine is available. To produce an effective subunit vaccine, a truncated secreted version of the F protein (ΔF) was expressed in mammalian cells, purified and shown to form trimers. The ΔF protein was then formulated with a CpG oligodeoxynucleotide (ODN) and an innate defense regulator (IDR) peptide in polyphosphazene microparticles (ΔF-MP). Mice immunized either intramuscularly (IM) or intranasally (IN) with ΔF-MP developed significantly higher levels of virus-neutralizing antibodies in the sera and lungs, as well as higher numbers of IFN-γ secreting cells than mice immunized with the ΔF protein alone. In contrast, the IM delivered ΔF induced high production of IL-5 while the IN delivered ΔF did not elicit a measurable immune response. After RSV challenge, essentially no virus and no evidence of immunopathology were detected in mice immunized with ΔF-MP regardless of the route of delivery. While the mice immunized IM with ΔF alone also showed reduced virus replication, they developed enhanced levels of pulmonary IgE, IL-4, IL-5, IL-13 and eotaxin, as well as eosinophilia after challenge. The level of protection induced by the ΔF-MP formulation was equivalent after IM and IN delivery. The efficacy and safety of the ΔF-MP formulation was confirmed in cotton rats, which also developed enhanced immune responses and were fully protected from RSV challenge after vaccination with ΔF-MP. In conclusion, formulation of recombinant ΔF with CpG ODN and IDR peptide in polyphosphazene microparticles should be considered for further evaluation as a safe and effective vaccine against RSV.

1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in infants and children. RSV causes serious illness especially in premature infants, infants with chronic lung disease or congenital heart disease [1,2], elderly and immuno-compromised hosts [3]. Despite the burden of disease there still is no RSV vaccine. A vaccine trial with formalin-inactivated RSV (FI-RSV) and aluminum hydroxide tested in the 1960s failed to induce neutralizing antibodies or protect children against RSV and, in younger infants, enhanced disease during subsequent natural infection [4].

RSV has three transmembrane glycoproteins, the heavily glycosylated G, fusion F, and small hydrophobic SH proteins. The F protein mediates viral penetration and fusion of infected cells with surrounding cells [5], and is highly conserved among RSV strains. This makes the F protein the major protective antigen of RSV, and thus an appropriate subunit vaccine candidate. To induce high-affinity neutralizing antibodies and facilitate cross-priming, the F protein needs to be formulated with an adjuvant which may consist of one or more compounds. In fact, several recently developed adjuvants contain multiple components [6]. The enhanced respiratory disease associated with FI-RSV immunization in children has been attributed to the development of Th2-biased

responses with low-affinity non-neutralizing antibodies due to lack of toll-like receptor (TLR) activation, which strongly supports the need for a TLR agonist in a RSV subunit vaccine formulation [7]. CpG ODN promotes Th1-biased or balanced immune responses, and thus is an appropriate choice [8]; however, co-formulation with other adjuvants is required for CpG ODN for optimal activity [9–14]. Natural host defense peptides (HDPs) are cationic amphipathic peptides with microbicidal and/or immunomodulatory properties [15]. HDPs can induce chemokine production leading to recruitment of neutrophils and monocytes [16]. Polyphosphazenes poly[di(sodium carboxylatophenoxy)]-polyphosphazene (PCPP) and poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) are synthetic water-soluble polymers containing an inorganic backbone of alternating phosphorus and nitrogen atoms. Polyphosphazenes have immunostimulatory properties [17] and can be formulated into microparticles [18,19] to facilitate mucosal delivery. CpG ODN, HDP and PCPP can act synergistically [9–11].

In this study we evaluated the immunogenicity of a truncated secreted form of RSV F protein (ΔF), formulated with CpG ODN and a HDP derivative defined as innate defense regulator (IDR) in PCEP microparticles (ΔF-MP). Vaccination of mice or cotton rats with ΔF-MP elicited robust immune responses and complete protection from RSV challenge without evidence of immunopathology after either intramuscular (IM) or intranasal (IN) delivery.

2. Materials and methods

2.1. Cells and virus

HEp-2 cells (American Type Culture Collection) were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 0.1 mM nonessential amino acids and 50 μg/ml gentamicin (Gibco-Invitrogen). The RSV A2 strain (American Type Culture Collection) was propagated in Hep-2 cells. Virus titers were determined by plaque assay as described previously [20], and screened for syncytia formation after crystal violet staining.

2.2. Preparation of truncated F protein

The ORF of the F protein was synthesized by Geneart in accordance with a reported codon-optimized sequence [21] (Genbank accession number EF566942) so as to encode for a truncated form of the native protein (amino acids 1–529) without the transmembrane domain (ΔF) but with a carboxyl terminal (Ser-Gly)$_{10}$ bridge followed by a his$_{10}$ tag. The ORF was proceeded by a Kozak sequence and cloned downstream of the human CMV promoter and intron A contained within an episomal vector containing the EBNA-1 antigen ORF and P origin; elements downstream of the ΔF protein ORF included a woodchuck hepatitis post-transcriptional regulatory element and bovine growth hormone poly-adenylation site. HEK293 cells were transfected with the ΔF-encoding episomal vector, and his-tagged ΔF protein was purified from the culture supernatants using TALON Superflow resin (Clontech) according to the manufacturer's instructions. The plasmid maps and sequences are available upon request.

2.3. Entrapment of vaccine components into microparticles

Microparticles were prepared by coacervation according to Garlapati et al. [18,19], but PCEP was used instead of PCPP. Poly[di(sodium carboxylatoethylphenoxy)]-phosphazene was synthesized at Idaho National Laboratory. Phosphorothioate-stabilized single-stranded CpG ODN10101 (TCGTCGTTTTCGCGCGCGCGCCG) was provided by Pfizer. IDR1002 (VQRWLIVWRIRK) [16] was synthesized at Genscript. PCEP, IDR1002, and CpG ODN10101 were
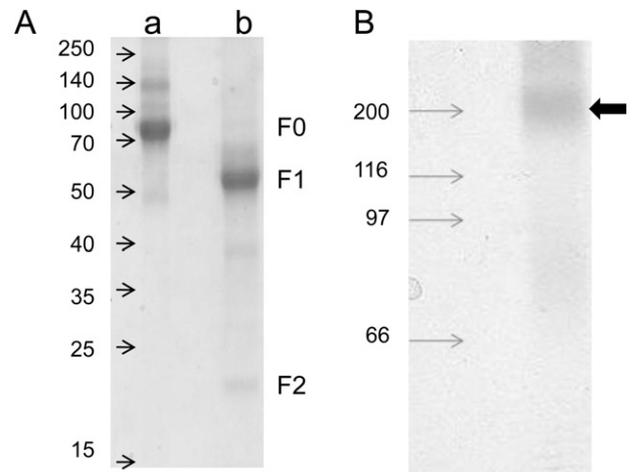
Fig. 1. Analysis of affinity-purified ΔF protein. (A) The pure ΔF protein was resolved in a 10% polyacrylamide gel under non-reducing (lane a) and reducing (lane b) conditions, or in a 7% native gel (B) and visualized by staining with Coomassie Brilliant Blue. Molecular mass markers are shown in the left margins.

formulated in a ratio of 1:2:1, with each animal receiving 10 μg, 20 μg and 10 μg, respectively. CpG ODN10101 and IDR1002 were mixed and incubated at 37 °C, and after 30 min, PCEP with ΔF protein was added. To this mixture 6.6% NaCl was added dropwise. After 20 min at room temperature 8% CaCl$_2$ was added and then the mixture was rocked for 10 min. The particles were collected by centrifugation at 1390 $\times$ g for 10 min, washed with ddH$_2$O, and resuspended in phosphate-buffered saline (PBS). The efficiency of encapsulation was estimated to be 89 $\pm$ 2% using Alexa-Fluor 488-labeled ΔF protein.

2.4. Vaccination and challenge

Four groups of 10 naive female BALB/c mice (8–10 weeks of age) (Charles River Laboratories) were vaccinated with 1 μg of ΔF protein in PBS or formulated with CpG ODN and IDR peptide in PCEP microparticles in a total volume of 20 μl IN or 50 μl IM. The ΔF dose was based on a previous titration in mice. Two additional groups of mice received PBS (Placebo). Three weeks later a second immunization was administered and three weeks after the second dose all, except one of the Placebo groups, were challenged IN with RSV strain A2 (5 $\times$ 10^5 PFU in 50 μl). The mice were lightly anesthetized with isoflurane for IN immunization and RSV challenge. Four days post-challenge half of the mice from each group was euthanized for detection of virus in the lungs. The other half was sacrificed six days post-challenge to collect bronchoalveolar lavage (BAL) fluids, set up lung fragment cultures, generate lung homogenates, and isolate splenocytes. Serum samples were collected immediately before and 21, 42, 46 and 48 days after primary immunization.

Four groups of five cotton rats (Sigmodon hispidus) (Sigmovir Biosystems Inc.) were anesthetized by isoflurane inhalation. Two groups were given 1 μg of ΔF protein alone or ΔF-MP in a total volume of 50 μl IM, one group received 5 $\times$ 10^5 PFU of live RSV IN in 50 μl and the fourth group received saline. All cotton rats, except the live RSV-immunized group, were re-vaccinated three weeks later. After another three weeks the cotton rats were challenged IN with RSV strain A2 (1 $\times$ 10^6 PFU in 100 μl). Blood samples were obtained by cardiac puncture under anesthesia before inoculation and at regular intervals afterwards. All procedures were approved and performed in accordance to the standards of the Canadian Council on Animal Care.
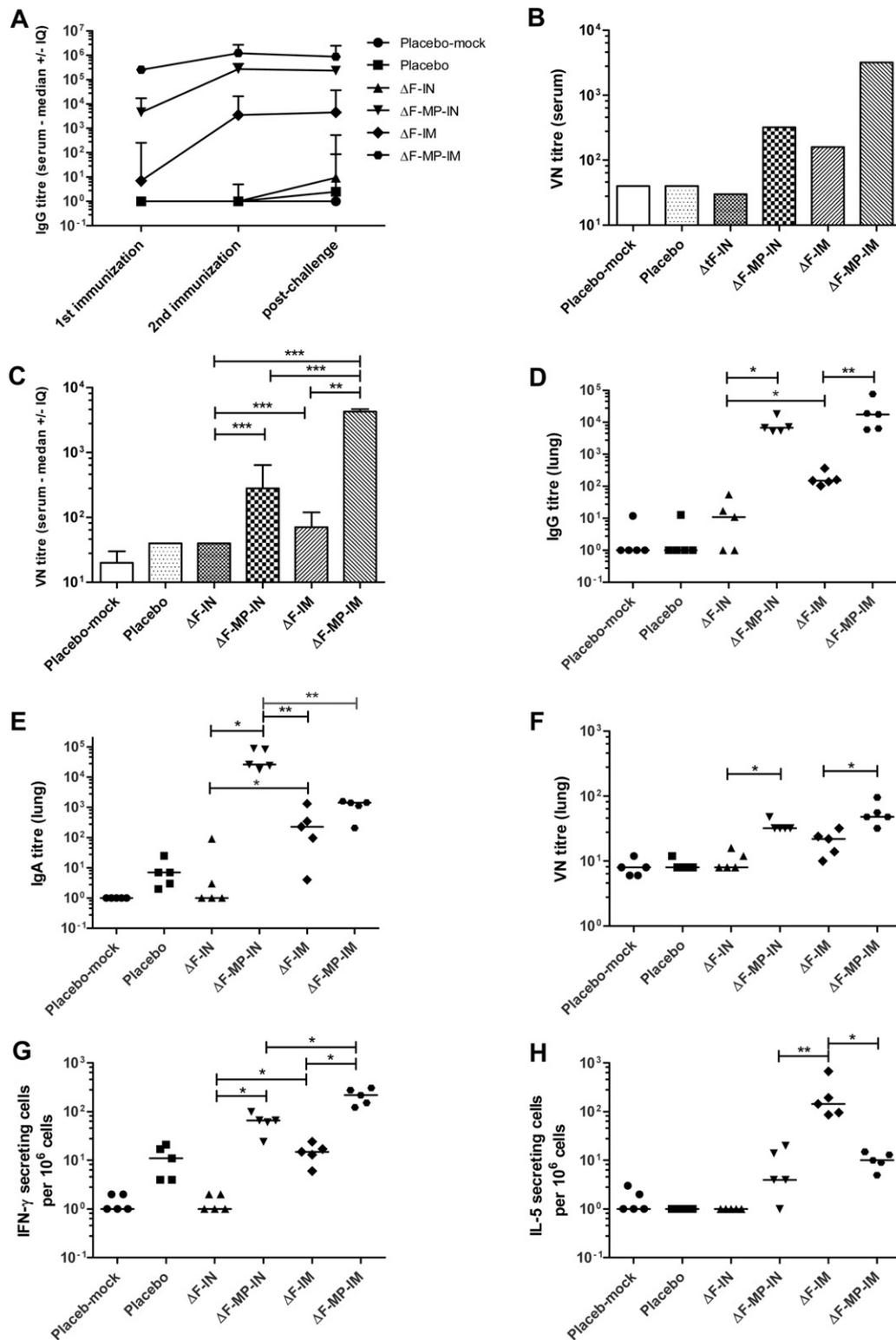
Fig. 2. Immune responses to RSV ΔF protein in mice. (A) Serum IgG titers specific for ΔF after one and two immunizations, and after challenge. (B) Serum VN antibody titers prior to challenge. (C) Serum VN antibody titers after RSV challenge. (D) Lung IgG titers specific for ΔF after RSV challenge. (E) Lung IgA titers specific for ΔF after RSV challenge. (F) Lung VN titers after RSV challenge. Numbers of IFN-γ (G) and IL-5 (H) secreting splenocytes in response to *in vitro* re-stimulation with ΔF protein. Cytokine production was measured on day 6 after challenge. BALB/c mice were immunized twice IM or IN with ΔF alone or with ΔF-MP and challenged three weeks later with RSV. Control groups were immunized with buffer and then challenged with RSV (Placebo) or mock-challenged with saline (Placebo–mock). ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Virus-neutralization titers are expressed as the highest dilution of serum that resulted in <50% of cells displaying cytopathic effects. Cytokine secreting cells are expressed as the difference between the number of spots per 10^6 cells in ΔF-stimulated wells and the number of spots per 10^6 cells in medium-treated wells. In (A) median values and interquartile ranges are shown. In (B) bars indicate values obtained from pooled sera. In (C) bars indicate median values and interquartile range. In (D–H) each data point represents an individual animal, and median values are indicated by horizontal bars. $^*P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$.

2.5. Lung fragment cultures

Lung fragment cultures were prepared as previously described [20]. Briefly, six days after challenge, lavaged lungs of euthanized mice were cut into pieces and cultured in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamicin, and antibiotic/antimycotic (Gibco-Invitrogen) for 5 days at 37 °C. Cell-free supernatants were collected and stored at −80 °C.

2.6. ELISA and virus neutralization assay

Serum and lung fragment culture supernatants were analyzed for RSV ΔF-specific IgG, IgE and IgA by ELISA. Briefly, 96-well microtiter plates (Thermo Scientific) were coated overnight at 37 °C with ΔF protein at 50 ng/ml. Samples were serially diluted, added to the ΔF-coated plates, and incubated overnight at 4 °C. Bound ΔF-specific antibody was quantified using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories), or biotin labeled goat anti-mouse IgA (Invitrogen) or anti-mouse IgE (AbD serotec) followed by streptavidin-AP (Jackson ImmunoResearch Laboratories Inc.) and p-nitrophenyl phosphate (Sigma–Aldrich Inc.).

RSV-specific neutralization tests were performed as plaque reduction assays. Sera and lung fragment culture supernatants were heat-inactivated at 56 °C for 30 min and serially diluted in 96-well plates (Corning Incorporated) in DMEM supplemented with 1% FBS and 10 mM HEPES. The diluted samples were incubated with 500 PFU/well of RSV strain A2 for 1 h at 37 °C. Subsequently, the sample-virus mixtures were transferred to duplicate HEp-2 cell monolayers. After incubation for 4 days at 37 °C, the cells were fixed and stained with 0.5% crystal violet.

2.7. Enzyme-linked immunospot (ELISPOT) assays

Isolation of splenocytes and ELISPOT assays were performed as previously described [22]. Briefly, 96-well Multiscreen-HA ELISPOT plates (Millipore) were coated overnight at 4 °C with murine IFN-γ- or IL-5-specific monoclonal antibodies (BD PharMingen). Splenocytes (10^6 cells/well) were stimulated with 2 μg/ml ΔF protein per well or medium only. After overnight incubation at 37 °C, the plates were washed and sequentially incubated with biotinylated IFN-γ- or IL-5-specific polyclonal antibody (BD Biosciences) and AP-conjugated streptavidin (Jackson Immuno-Research Laboratories Inc.). Spots representing IFN-γ- and IL-5-secreting cells were visualized with BCIP/NBT substrate.

2.8. Cytokine ELISA

Lungs from mice were homogenized in DMEM supplemented with 10 mM HEPES, 50 μg/ml gentamycin, 10 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma–Aldrich), 0.1 mM EDTA, and antibiotic/antimycotic (Invitrogen Corp.) with 2.4 mm zirconia microbeads in a Mini-Beadbeater™ (BioSpec Products Inc.). The homogenates were centrifuged for 1 min at 10,000 × g, and the supernatants collected and stored at −80 °C. IFN-γ, IL-5, IL-13 and eotaxin were quantified in the cell-free supernatants of lung homogenates using Quantikine Mouse Immunoassay Kits (R&D Systems).

2.9. Analysis of bronchoalveolar lavage fluids

Pulmonary eosinophilia was assessed in the BAL fluids six days after challenge. The relative percentage of eosinophils, neutrophils, lymphocytes and macrophages was determined by examination
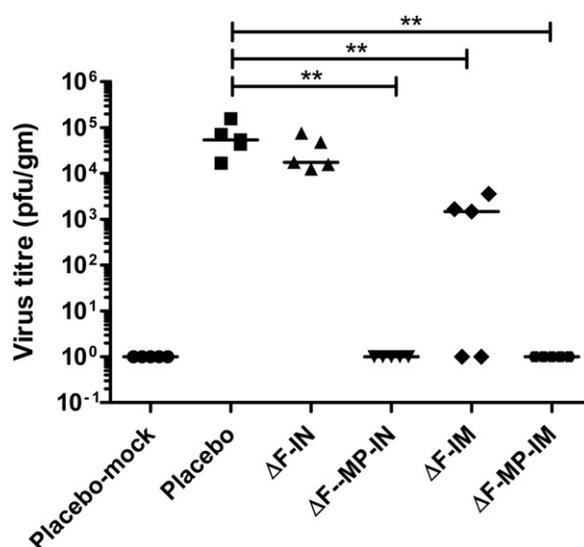
Fig. 3. Detection of RSV in lung tissue following immunization and RSV challenge. BALB/c mice were treated as described in the legend for Fig. 2. The virus titers were measured on day 4 after RSV challenge and expressed as pfu per gram of lung tissue. Due to the log scale 10^{-1} was used as value instead of 0 when no virus was detected. Each data point represents an individual animal, and median values are indicated by horizontal bars. **$P < 0.01$.

of at least 200 leukocytes in cytospin-preparations stained with Wright-Giemsa stain (Bayer Corp.).

2.10. Histopathology of cotton rat lungs

The left lung lobe was instilled with and fixed in 10% neutral buffered formalin, embedded in dorsoventral position in paraffin, and sectioned serially until ∼75% of the bronchial tree was exposed. Subsequently, 5 μm-thick sections were obtained and stained with hematoxylin and eosin (H&E). Using a semiquantitative scale (0 to 4+) (0 = absent and 4 = maximum/severe), a board-certified pathologist evaluated all slides for peribronchial and/or peribronchiolar inflammatory infiltration, perivascular inflammatory infiltration, intra-alveolar inflammatory infiltration and alveolar septal thickening as previously described [23]. The last two parameters are integral components of the alveolar inflammatory process (alveolitis), and markers for formalin-inactivated RSV vaccine-enhanced pulmonary pathology in cotton rats [23]. The pathologist was not aware of the treatment groups.

2.11. Statistical analysis

All data were analyzed using GraphPad PRISM version 5 for Windows (GraphPad Software). 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 $P < 0.05$.

3. Results

3.1. Production and purification of RSV ΔF protein

RSV ΔF protein with a C-terminal his tag was generated, produced in HEK293 cells and purified on TALON Superflow resin. Analysis by SDS-PAGE indicated that the affinity-purified ΔF protein was ≥90% pure (Fig. 1). Under reducing conditions two bands with apparent molecular weights of ∼50 kD and ∼20 kD, corresponding to F1 and F2, respectively, were identified, while
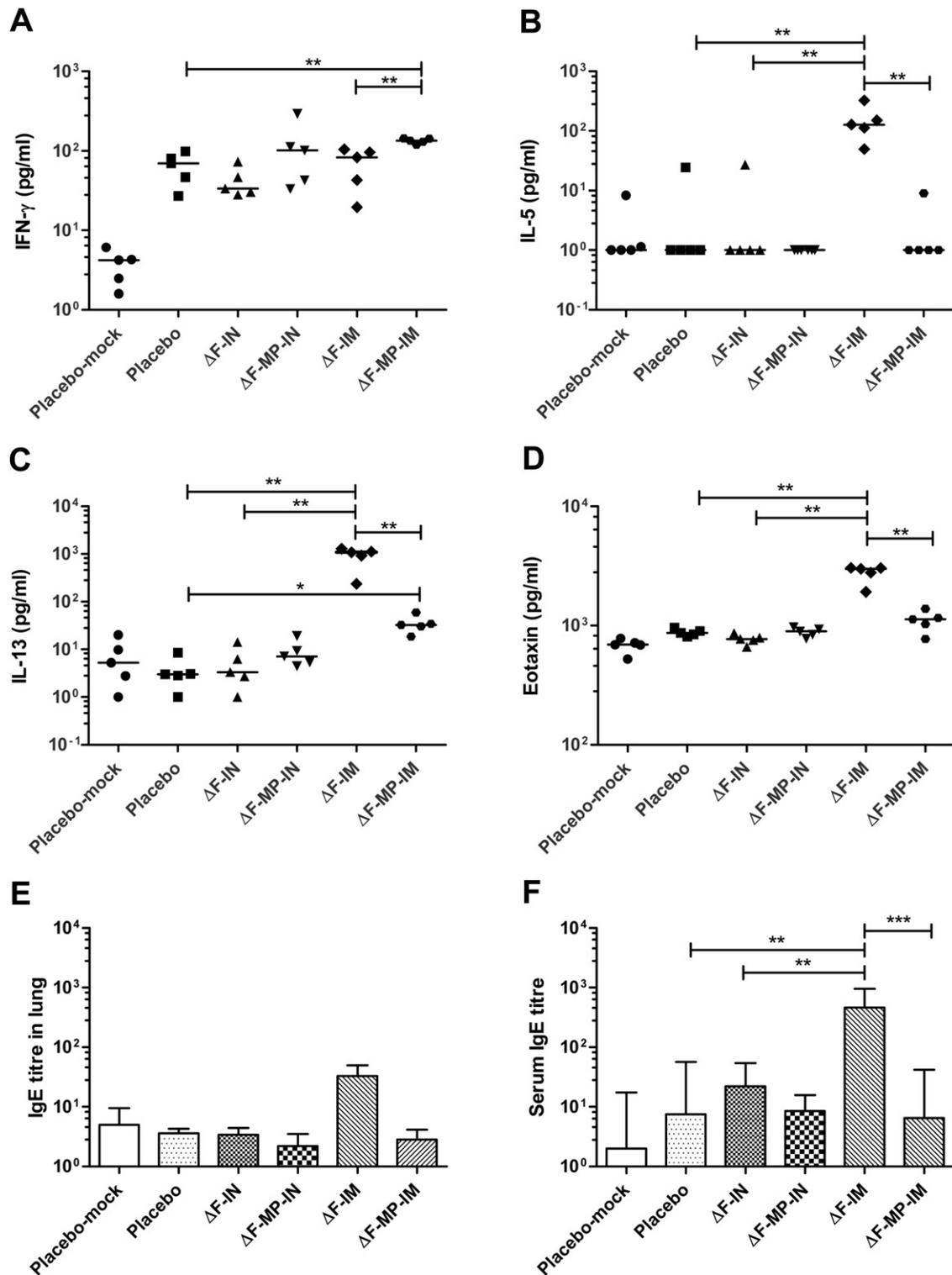
Fig. 4. Concentrations of lung cytokines and IgE. BALB/c mice were treated as described in the legend for Fig. 2. Concentrations of (A) IFN-γ, (B) IL-5, (C) IL-13 and (D) eotaxin in lung homogenate supernatants were measured 6 days after challenge. Each data point represents an individual animal, and median values are indicated by horizontal bars. IgE titers specific for ΔF in lung fragment culture supernatants (E) and serum (F) were measured 6 days after RSV challenge. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Bars indicate median and interquartile range values. **$P < 0.01$ and ***$P < 0.001$.

non-reducing conditions demonstrated a ∼70 kD protein band corresponding to F0. When analyzed under native conditions a band of ∼210 kD was observed indicating that the ΔF protein forms a trimer. The identity of the ΔF protein was further confirmed by Western blotting and N-terminal sequencing (data not shown).

3.2. The ΔF protein formulated with CpG ODN and IDR in PCEP microparticles induces protective immunity in mice

Significantly higher IgG titers were observed in mice immunized with ΔF formulated with microparticles compared to ΔF protein

alone regardless of the route of immunization (Fig. 2A). Furthermore, the antibody titers induced by ∆F-MP were higher when administered IM than after IN delivery. The difference between the antibody levels elicited by ∆F alone when delivered IN or IM was also highly significant. The VN titers elicited by ∆F-MP were significantly higher than those induced by ∆F whether administered by IN or IM route (Fig. 2B and C). Furthermore, ∆F-MP generated more than 10-fold higher VN titers when delivered IM compared to IN. These results showed that parenteral immunization with ∆F-MP induced stronger systemic humoral responses than mucosal administration.

The ∆F-MP formulation generated higher IgG titers in the lungs compared to mice immunized with ∆F (Fig. 2D). Furthermore, mice immunized IN with ∆F-MP developed higher IgA levels compared to mice immunized IM with ∆F or ∆F-MP, or IN with ∆F (Fig. 2E). In contrast, ∆F alone elicited higher IgG and IgA levels when delivered IM than IN, which agreed with the data presented above and suggested that ∆F likely was degraded or washed out during IN delivery, or possibly less effectively taken up by antigen presenting cells. Interestingly, the VN titers induced in the lungs by ∆F-MP were equivalent in IN and IM immunized mice (Fig. 2F). This suggests that the local VN titers in the lung were due to both IgA and IgG in the IN vaccinated mice and to primarily IgG in the IM immunized animals, which probably resulted from a serum transudate.

The ∆F-MP adjuvant formulation generated higher numbers of IFN-γ secreting cells compared to ∆F delivered by IM or IN route (Fig. 2G). Furthermore, IM delivery resulted in higher numbers of IFN-γ secreting cells than IN administration. In contrast, the number of IL-5 secreting cells was significantly higher in mice immunized IM with ∆F compared to the ∆F-MP formulation (Fig. 2H). Again, no immune response was observed in mice immunized IN with ∆F protein alone. Thus, formulation of ∆F protein with CpG ODN and IDR in microparticles shifted the immune response from Th2-type to Th1-biased after IM administration.

With exception of a few particles in one mouse from the IM group, no virus was detected in mice immunized with ∆F-MP regardless of the route of delivery; thus these mice were fully protected from infection (Fig. 3). Immunization with ∆F alone resulted in reduced replication after IM delivery, while there was no difference between mice immunized IN with ∆F alone and Placebo-treated RSV-challenged mice, which correlates to the lack of detectable immune responses in this group of mice.

3.3. Lung cytokine/chemokine profiles and IgE levels are dependent on formulation of ∆F with CpG ODN and IDR in PCEP microparticles in mice

No differences were observed in cytokine profiles between unvaccinated mice and mice vaccinated with IN delivered ∆F-MP or ∆F after RSV challenge. However, IFN-γ production was increased in mice immunized IM with the ∆F-MP formulation compared to mice immunized with ∆F alone and unvaccinated mice (Fig. 4A). In contrast, ∆F alone induced significantly enhanced production of IL-5, IL-13 and eotaxin, and ∆F-MP more IL-13, when delivered IM (Fig. 4B–D). The group of mice immunized IM with ∆F alone was the only one showing enhanced serum IgE when compared to the unvaccinated RSV-challenged group, as well as all other vaccine groups. While not significant, a similar trend was observed for lung IgE (Fig. 4E and F), which is consistent with the enhanced IL-13 levels observed in the group immunized IM with ∆F alone.

To evaluate potential eosinophilia post-vaccination and challenge, differential counts of the cells in lung lavages after challenge were performed. In mice immunized IM with ∆F 41% of the BAL cells constituted of eosinophils, while mice immunized IM with ∆F-MP had 1% eosinophils. Intranasally immunized mice, whether with ∆F or with ∆F-MP, had no eosinophils in the BAL. These results indicate that the mice immunized with ∆F-MP did not show any evidence of immunopathology in contrast to mice immunized with IM delivered ∆F protein.

3.4. The ∆F protein formulated with CpG ODN and IDR in PCEP microparticles elicits protection from RSV challenge in cotton rats

Significantly higher VN titers were observed in cotton rats immunized with ∆F, ∆F-MP or RSV in comparison with placebo animals (Fig. 5A). In addition, the ∆F-MP- and RSV-vaccinated cotton rats had 10-fold higher median VN titers than the animals immunized with ∆F alone, although due to one cotton rat having a high VN titer in the ∆F group there was no significant difference. After RSV challenge no virus was detected in cotton rats immunized with ∆F-MP (Fig. 5B); in contrast, although there was a 10-fold reduction in virus replication in the lungs from cotton rats
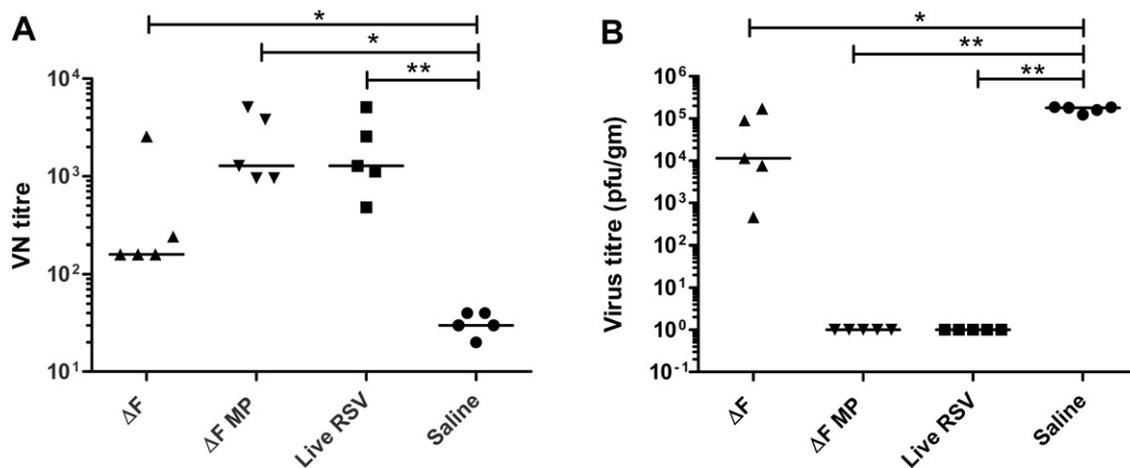
Fig. 5. Immune responses and virus replication in cotton rats vaccinated twice IM with a 3-week interval with ∆F protein alone or ∆F-MP. One group of cotton rats received live RSV once and another group saline (Placebo). All cotton rats were challenged with RSV virus strain A2 after another three weeks. (A) Virus neutralizing antibodies in serum; (B) Virus replication in the lungs, expressed as pfu per gram of lung tissue. Due to the log scale 10^{-1} was used as value instead of 0 when no virus was detected. *$P < 0.05$ and **$P < 0.01$.
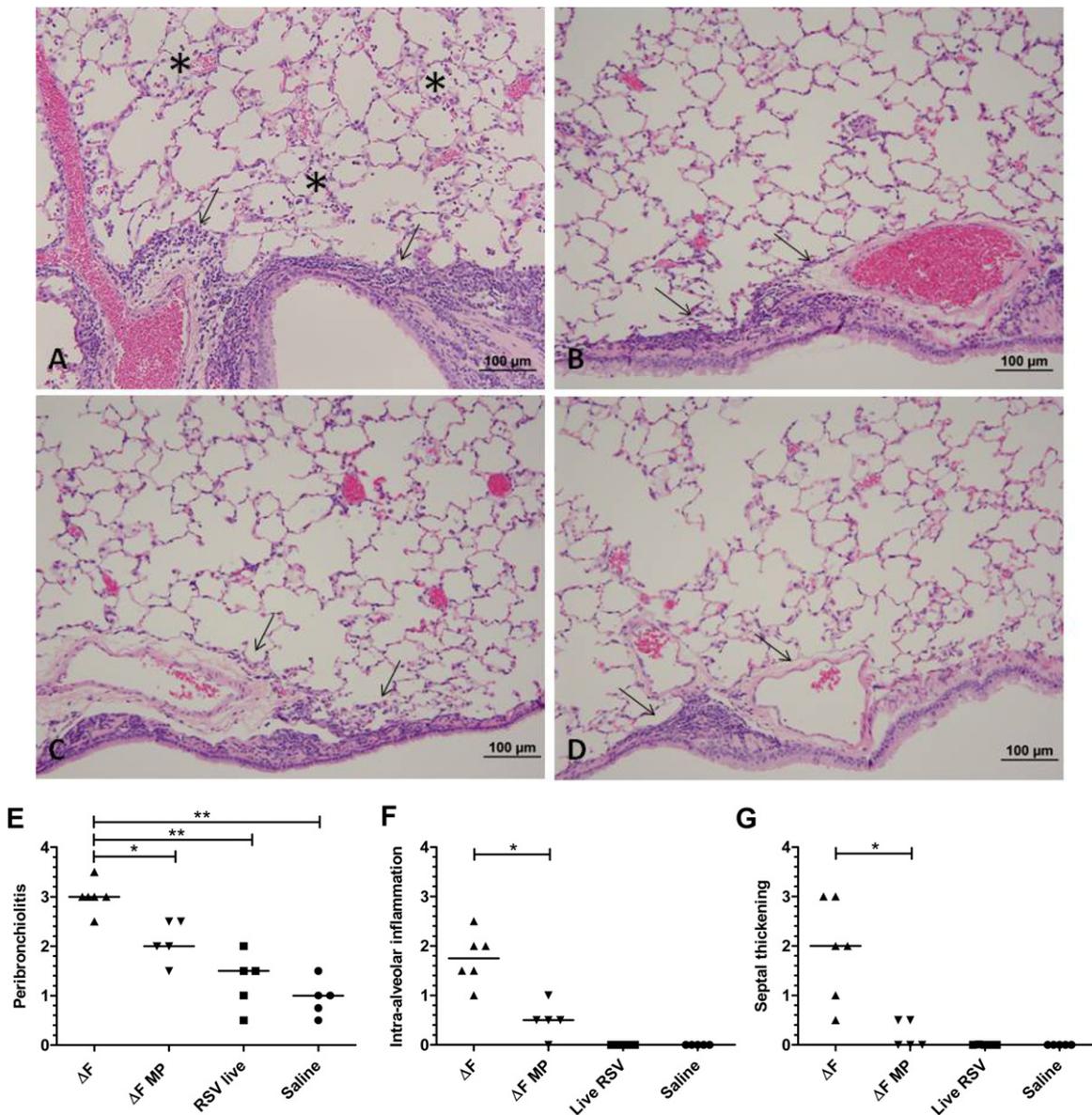
Fig. 6. Histology of H&E stained lung sections of cotton rats vaccinated twice IM with a 3-week interval with ΔF protein alone or ΔF-MP. One group of cotton rats received live RSV once and another group saline (Placebo). All cotton rats were challenged with RSV virus strain A2 after another three weeks. Representative images of a cotton rat from each group are shown (A–D). A semiquantitative scale (0 to 4+) was used to evaluate the lungs based on peribronchial and/or peribronchiolar inflammatory (predominantly lymphocytic) infiltration, perivascular inflammatory (predominantly lymphocytic) infiltration, intra-alveolar inflammatory (predominantly neutrophils) infiltration and alveolar septal thickening. Peribronchial and perivascular inflammatory infiltration (arrows) is severe in a cotton rat immunized with ΔF (A), mild to moderate in a cotton rat immunized with ΔF-MP (B), and mild to absent in cotton rats exposed to RSV (C) or saline (D). Mild alveolar septal thickening and intra-alveolar inflammatory infiltration (*) is present only in cotton rat A. Semiquantified (score 0–4) peribronchitis/peribronchiolitis (E), intra-alveolar inflammation (F), and alveolar septal thickening (G) observed in lungs of cotton rats of the above stated groups (A–D). Each data point represents an individual animal, and median values are indicated by horizontal bars. *$P < 0.05$ and **$P < 0.01$.

vaccinated with ΔF in comparison with the unvaccinated animals, this difference was not significant.

After RSV challenge, a lower degree of peribronchitis/peribronchiolitis was observed in cotton rats exposed to RSV or immunized with ΔF-MP than in ΔF-immunized animals (Fig. 6A–E). Similarly, the degree of alveolitis consisting of both intra-alveolar inflammatory infiltration (Fig. 6F) and alveolar septal thickening (Fig. 6G), was significantly higher in the ΔF-immunized cotton rats (Fig. 6A) than in the ΔF-MP-immunized (Fig. 6B) or RSV-exposed animals (Fig. 6C and D). However, although there appeared to be slightly higher alveolitis and spatial thickening in ΔF-MP-immunized than in RSV-exposed animals, this difference was not significant. Since alveolitis is a specific marker for vaccine-enhanced pathology in cotton rats, these data demonstrate that

the ΔF-MP formulation is significantly safer than ΔF alone in cotton rats.

4. Discussion

In this study we demonstrated that RSV ΔF protein formulated with CpG ODN and IDR in PCEP microparticles elicited robust and balanced immune responses, and complete protection from RSV infection in mice, without any indication of immunopathology after either IM or IN immunization. In cotton rats the ΔF protein also induced protective immunity when formulated in the combination adjuvant microparticles, without inducing vaccine-enhanced pathology. This is in contrast to ΔF alone, which did not induce

significant protection and caused significantly more alveolitis in comparison with RSV infection.

Various strategies have been pursued to develop an RSV vaccine including live attenuated/genetically engineered viruses, purified proteins, and viral vector- and DNA-based vaccines [24]. Modern vaccinology paradigms indicate the need for highly purified antigenic components, as well as more powerful adjuvant/delivery systems [6]. Various modified forms of the F protein have been tested. An F protein fragment (aa412-524) associated with cholera toxin B pentamer induced mucosal and systemic VN antibody responses, as well as a Th1/Th2 mixed cytokine response, when administered IN. However, virus titers in the lungs were only reduced 10-fold [25]. Recently, a very stable form of the F protein was produced by removing the fusion peptide, transmembrane region and cytoplasmic tail. This F version when formulated with alum induced VN antibodies and reduced virus replication in cotton rats upon RSV challenge, which is promising although the lung tissues were not examined for immunopathology [26].

The beneficial effect of TLR ligand in a RSV vaccine formulation has been shown in various RSV vaccine formulations. Whereas cotton rats immunized with FG subunit vaccine developed mild alveolitis and interstitial pneumonia upon RSV challenge, this was eliminated by formulation with Monophosphoryl lipid A [27]. The addition of CpG ODN to F protein in alum enhanced Th1-biased responses, accelerated clearance of RSV from the lungs and reduced eosinophilia [28]. In these trials animals were immunized IM. Recently, β-propiolactone-inactivated RSV was formulated with a combination of CpG ODN and L18-MDP (NOD2 agonist). This promoted a shift to a Th1-biased response and affinity maturation of RSV-specific antibodies, and improved protection from RSV challenge without inducing immunopathology. This formulation was suitable for mucosal delivery which resulted in local IgA production [29].

Particulate delivery systems are promising for both parenteral and IN vaccine delivery due to their inherent adjuvant property and ability to increase the residence time of the delivered content in the nasal cavity. Micro- or nanoparticles are preferentially taken up by microfold-cells, which present their content to the underlying antigen presenting cells for immune induction [30,31]. The success of virus-like particles (VLP)/virosomes for immunization against hepatitis B and human papilloma virus has led to increased interest in this technology as an approach to develop a RSV vaccine [32]. Recently, recombinant F protein in virosomes was shown to induce VN antibodies in IM immunized mice [33]. An oil-in-water emulsion with a cationic detergent (nanoemulsion) inactivated infectivity when mixed with RSV. After IN immunization with this RSV-nanoemulsion long-lived RSV-specific systemic and local humoral immunity, as well as protection from RSV challenge, without immunopathology, was induced [34].

In our study formulation of the ΔF protein in the combination adjuvant microparticles significantly improved immune responses elicited after IM injection, and was critical for induction of immunity after IN delivery, making this formulation suitable for parenteral and mucosal administration, or possibly a combination of both. The presence of a TLR agonist appears to be critical for the efficacy and safety of a RSV vaccine. Reports on TLR expression in newborn infants, one of the primary target populations, are controversial. While cord blood cells of full-term infants expressed normal TLR levels, the responses to lipopeptide, lipopolysaccharide and imiquimod were found to be significantly lower than those of adult blood monocytes, and monocyte-derived and plasmacytoid dendritic cells [35]. However, according to other reports TLR8 and 9 agonists induce robust immune responses in human neonatal antigen presenting cells, as well as in newborn mice *in vivo* [36–38], which suggests that CpG ODN is an appropriate choice for neonates. Host defense peptides may act through induction of

chemokines leading to recruitment of neutrophils and monocytes [16], while polyphosphazenes can promote Th1-biased immune responses [9,39], and both polyphosphazene and HDP mediate a depot effect [40]. Further studies will be required to determine the actions of these components in neonates.

In conclusion, the results of the vaccination-challenge studies demonstrated that the ΔF-MP formulation is safe and effective whether delivered IM or IN, and thus warrants further evaluation as a vaccine against RSV.

Acknowledgements

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