Induction of mucosal immunity and protection by intranasal immunization with a respiratory syncytial virus subunit vaccine formulation

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The majority of infections, including those caused by respiratory syncytial virus (RSV), occur at mucosal surfaces. As no RSV vaccine is available our goal is to produce an effective subunit vaccine with an adjuvant suitable for mucosal delivery and cross-presentation. A truncated secreted version of the RSV fusion (ΔF) protein formulated with polyI:C, an innate defence regulator peptide and polyphosphazene, induced local and systemic immunity, including affinity maturation of RSV F-specific IgG, IgA and virus-neutralizing antibodies, and F-specific CD8⁺ T-cells in the lung, when delivered intranasally. Furthermore, this ΔF protein formulation promoted the production of CD8⁺ central memory T-cells in the mediastinal lymph nodes and provided protection from RSV challenge. Formulation of ΔF protein with this adjuvant combination enhanced uptake by lung dendritic cells and trafficking to the draining lymph nodes. The ΔF protein formulation was confirmed to be highly efficacious and safe in cotton rats.

Respiratory syncytial virus (RSV) is a major cause of respiratory tract disease in infants, elderly and immunocompromised individuals (Falsey et al., 2005). There are no safe and effective RSV vaccines or specific treatments other than prophylaxis with passive antibody therapy (Impact–RSV Study Group, 1998). As RSV is a pneumotropic virus, our goal is to produce an effective subunit vaccine using an adjuvant platform that is suitable for mucosal delivery and cross-presentation. The fusion (F) protein is highly conserved and facilitates penetration of the virus into host cells and subsequent formation of syncytia, thus making it a suitable subunit vaccine candidate (Collins & Graham, 2008). Formulation with a toll-like receptor (TLR) agonist is critical for a killed or subunit vaccine to be effective and safe (Delgado et al., 2009). We showed that co-formulation with CpG ODN, an innate defence regulator (IDR) peptide and a polyphosphazene (Andrianov et al., 2009; Kovacs-Nolan et al., 2009a) promoted partially or fully protective immune responses to bovine or human, respectively, RSV F protein (Garlapati et al., 2012; Kovacs-Nolan et al., 2009b). As among the TLR agonists polyI:C has been shown to promote cross-presentation (Schulz et al., 2005), and induction of effector cytotoxic CD8⁺ T-cells has been suggested to be required for optimal protection (Graham, 2011) and recently shown to be protective (Lee et al., 2012), we replaced CpG ODN with polyI:C, examined affinity maturation of ΔF-specific IgG and induction of effector and memory CD8⁺ T-cells, and determined whether uptake of the ΔF protein by cells in the lungs and trafficking to the draining lymph nodes (LNs) is improved by formulation with the triple adjuvant (TriAdj).

Female BALB/c mice (6–8 weeks of age; n=10) (Charles River Laboratories) were immunized twice intranasally (i.n.) with a 3 week interval with 20 μl vaccine containing 1 μg ΔF formulated with PBS (ΔF/PBS), 10 μg polyol·C (ΔF/polyol·C) or 10 μg polyol·C (Invivogen), 20 μg IDR1002 (VQRWLIVWRIRK, Genscript) and 10 μg poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) (Idaho National Laboratory) in PBS (ΔF/Triadj). The ΔF protein with His tag was produced, purified and formulated as described previously (Garlapati et al., 2012). Two additional groups of mice received PBS i.n. (placebo). Three weeks after the second immunization, all except one of the placebo groups were challenged i.n. with RSV strain A2 (5 × 10⁵ p.f.u./50 μl). To evaluate the local mucosal immune responses, ΔF-specific IgG1, IgG2a, IgA and virus-neutralizing (VN) antibody levels were measured in the lungs as previously described (Garlapati et al., 2012), except that biotin-labelled goat anti-mouse IgG1 and IgG2a followed by streptavidin-alkaline phosphatase were used to detect Ig subtypes. Significantly higher IgG1 production was observed in mice immunized with ΔF/Triadj in comparison with...
animals immunized with ΔF/PBS or ΔF/polyI:C. However, mice immunized with either ΔF/polyI:C or ΔF/TriAdj had higher IgG2a titres than ΔF/PBS-immunized animals (Fig. 1a). Furthermore, ΔF/TriAdj generated significantly higher IgA and VN titres compared with ΔF/polyI:C or ΔF/PBS (Fig. 1b, c), while ΔF/polyI:C elicited stronger IgA and VN responses than ΔF/PBS. Based on these results the TriAdj formulation is superior for induction of mucosal immunity.

Also, as mucosal immunization is expected to induce systemic immunity, the antibody levels in the serum were measured. Mice immunized with ΔF/polyI:C or ΔF/TriAdj produced significantly more IgG1 and IgG2a than the animals vaccinated with ΔF/PBS, while ΔF/TriAdj induced higher IgG1 and IgG2a titres than ΔF/polyI:C (Fig. 1d). Furthermore, the group of mice immunized with ΔF/TriAdj developed higher serum VN antibody levels than the groups

Fig. 1. Immune responses to RSV ΔF protein in mice immunized with ΔF protein formulations, and challenged with RSV A2. Lung IgG1 and IgG2a (a), IgA (b) and VN (c) antibody titres. Serum IgG1 and IgG2a (d), and VN (e) antibody titres. Serum IgG antibody affinity (f). Numbers of IFN-γ and IL-5 secreting splenocytes (g). Proportion of ΔF-specific CD8+ T-cells in the lung determined by pentamer staining (h). Presence of TCM+ cells expressing CD62L and CD127 in the lung draining LN (i). ELISA titres are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Virus neutralization titres are expressed as the highest dilution of serum that resulted in ≤50% of cells displaying cytopathic effects. Cytokine-secreting cell numbers are expressed as the difference in the number of spots between ΔF-stimulated wells and medium-control wells. Bars represent median values with interquartile ranges. Data were analysed using GraphPad PRISM version 5 for Windows. Differences among groups were examined using one-way ANOVA, followed by a Newman–Keuls post-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, *P<0.05, **P<0.01, ***P<0.001.
immunized with either ΔF/polyI:C or ΔF/PBS (Fig. 1e). The IgG2a to IgG1 ratios also show that both TriAdj and polyI:C promoted a Th1-biased humoral immune response, while ΔF/PBS induced a very low, balanced response. As a further measure of the quality of the antibody response, we investigated whether TriAdj promotes affinity maturation of the ΔF-specific IgG by incubating ΔF protein-coated ELISA plates with serum from different vaccine groups, followed by washing with increasing concentrations of urea and calculating the percentage of antibody bound in comparison with a PBS wash. Indeed, the ΔF-specific antibodies elicited by ΔF/TriAdj were bound with significantly higher avidity than those induced by ΔF/polyI:C or ΔF/PBS (Fig. 1f). Thus, in terms of ΔF-specific serum antibody production, the ΔF/TriAdj formulation was superior in comparison to ΔF/polyI:C and ΔF/PBS.

To further investigate the bias of the immune response, the ΔF-induced secretion of IFN-γ and IL-5 by in vitro restimulated splenocytes was measured in an ELISPOT assay as described previously (Garlapati et al., 2012). The ΔF/TriAdj formulation generated a higher frequency of IFN-γ secreting cells when compared with all other vaccine formulations, but no IL-5 secreting cells (Fig. 1g). In contrast, the responses elicited by ΔF/PBS or ΔF/polyI:C were similar to those in the placebo-immunized RSV-challenged mice. These data show that the addition of TriAdj to the ΔF protein resulted in a stronger, Th1-biased immune response, which is in agreement with the enhanced IgG2a production. The ability of the polyI:C or TriAdj to promote cross-presentation and cell-mediated immunity was also examined. For analysis of RSV F85–93-specific CD8+ T-cells, lung mononuclear cells were surface-stained with H-2Kd-F85–93 MHC class I pentamer (Proimmune) together with labelled antibodies specific for CD8+ T-cells (BD Pharmingen). Both ΔF/polyI:C and ΔF/TriAdj induced a considerable increase in RSV F85–93-specific CD8+ T-cells in the lungs (Fig. 1h) when compared with ΔF/PBS and placebo, which suggests that vaccination with ΔF/polyI:C or ΔF/TriAdj promotes a cytolytic CD8+ T-cell response to RSV.

Furthermore, we analysed the memory phenotype of the CD8+ T-cells in the ΔF/TriAdj-immunized mice. Single-cell suspensions from the mediastinal LNs were stained with anti-CD8, anti-CD127 and anti-CD62L (IL-7Rα) (Biolegend) antibodies and analysed by flow cytometry. This showed that the ΔF/TriAdj-immunized group displayed higher frequencies of CD8+ T-cells with a central memory phenotype (CD8+CD127+CD62L+) when compared with the placebo group in the lung draining LNs (Fig. 1i). In contrast to the mice immunized with placebo or ΔF/PBS, which showed peak virus titres of 4.4×10^5 or 2.8×10^5 p.f.u. per gram of lung tissue, respectively, on day 4 post-challenge, no infectious virus was detected in mice immunized with ΔF/TriAdj or ΔF/polyI:C.

To investigate the mechanism by which the TriAdj enhanced the ΔF-specific immune responses, we hypothesized that formulation of ΔF with TriAdj may influence its uptake by cells in the lungs and trafficking to the draining LN. To explore this, Alexa 647-labelled ΔF (ΔF*) protein was formulated in PBS or with TriAdj, and delivered i.n. at the same dose used in the vaccine trial, and antigen uptake by lung and mediastinal LN cells was analysed by flow cytometry. At both 4 h and 24 h after delivery, higher frequencies of total Alexa 647+ cells were detected in the lungs of ΔF*/TriAdj-immunized mice, when compared with ΔF*/PBS-immunized animals, indicating that TriAdj promotes crossing of the ΔF antigen through the initial airway barriers, possibly through increased uptake by DCs (Fig. 2a). In addition, the proportion of Alexa 647+ LN cells was increased in mice immunized with ΔF*/TriAdj when compared with ΔF*/PBS, both at 4 h and 24 h after delivery (Fig. 2b). Uptake by dendritic cells (DCs) was confirmed based on the frequencies of antigen-loaded DCs (CD11c+ MHC-II+), which were increased in the mediastinal LNs in mice that received ΔF*/TriAdj compared with animals immunized with ΔF*/PBS (Fig. 2c). These results suggest that formulation of ΔF with TriAdj promotes its uptake by DCs and trafficking to the draining LN.

![Fig. 2](http://vir.sgmjournals.org)  
**Fig. 2.** Antigen uptake in lungs and trafficking to draining LN. Alexa647-labelled ΔF was formulated in PBS or with TriAdj, and administered i.n. to mice. Four hours and 24 h after delivery, the proportion of Alexa 647+ cells was determined in lung (a) and draining LN (b) by flow cytometry. (c) Proportion of CD11c+ MHC-II+ DCs positive for ΔF* in the LN determined 24 h after delivery. Data were analysed as described in Fig. 1. *P<0.05.
We further confirmed the efficacy and safety of the ΔF protein formulated with TriAdj in cotton rats. Cotton rats (*Sigmodon hispidus*) (Sigmovir Biosystems) (*n* = 5) were vaccinated under anaesthesia by i.n. inoculation with 50 μl of ΔF (3 μg), either in PBS or formulated with polyI:C or TriAdj. Control groups were immunized with FI-RSV or 1 × 10⁵ p.f.u. live RSV i.n. in 100 μl, and the last group received PBS i.n. (placebo). Formalin-inactivated RSV was prepared according to a previously published protocol (Kim *et al.*, 1969; Kovacs-Nolan *et al.*, 2009a, b). All cotton rats were revaccinated after 3 weeks, with exception of the live RSV-vaccinated group. Three weeks after the second immunization, the cotton rats were challenged i.n. with live RSV strain A2 (1 × 10⁶ p.f.u./100 μl). The ΔF/TriAdj- and live RSV-vaccinated cotton rats developed significantly higher ΔF-specific serum IgG titres than ΔF/polyI:C-, ΔF/PBS- or FI-RSV-vaccinated animals (Fig. 3a). Furthermore, groups immunized with ΔF/TriAdj or live RSV developed significantly increased VN titres compared with those immunized with FI-RSV, ΔF/polyI:C or ΔF/PBS (Fig. 3b). Significantly higher IgA production was observed in the group of cotton rats immunized with ΔF/TriAdj than in all other groups (Fig. 3c). To assess potential pulmonary immunopathology after RSV challenge, the lungs of all

![Fig. 3. Immune responses and protection in cotton rats immunized with RSV ΔF protein formulations, live RSV or FI-RSV, and challenged with RSV A2. Serum IgG (a), serum VN (b) and lung IgA (c) titres. Semiquantitative histological evaluation (score 0–4) of the extent and severity of peribronchitis/peribronchiolitis (d), intra-alveolar inflammatory cellular infiltration (e), and alveolar septal thickening (f). Representative photomicrographs for the FI-RSV (g) and ΔF/TriAdj (h) immunized groups demonstrate peribronchiolitis (arrows) and alveolitis characterized by alveolar septal thickening and intra-alveolar inflammatory cellular infiltration (high magnification inserts). Virus replication in the lungs determined on day 4 after challenge (i). Bars represent median values with interquartile ranges. Data were analysed as described in Fig. 1. *P* < 0.05, **P** < 0.01, ***P** < 0.01.](image-url)
cotton rats were subjected to a histopathological evaluation, as described previously (Garlapati et al., 2012). Animals immunized with FI-RSV and challenged with RSV revealed the characteristic hallmarks of enhanced disease: a significant increase in severity and prevalence of peribronchiolitis and alveolitis characterized by alveolar septal thickening and intra-alveolar inflammatory cellular infiltration (Fig. 3d–f, g). In contrast, cotton rats immunized with live RSV or ΔF/TriAdj and challenged with RSV had markedly reduced peribronchiolitis and showed no alveolitis (Fig. 3d–f, h). Thus, ΔF/TriAdj elicited protection from pulmonary inflammation in terms of all histopathological parameters studied here, demonstrating the safety of ΔF/TriAdj in cotton rats. Immunization with either ΔF/TriAdj or live RSV induced sufficient immunity to protect the cotton rats completely from challenge virus replication in the lungs (Fig. 3i). In contrast, neither ΔF/polyl:C nor FI-RSV provided any protection from RSV challenge.

RSV infects the upper respiratory tract, which suggests that secretory antibodies play a critical role in protection from infection. In fact, in humans, protection against RSV is more related to the levels of RSV-specific nasal IgA than to serum antibody (Watt et al., 1990). Consequently, the i.n. route is an attractive route of immunization. Intranasal vaccination against influenza virus promoted the induction of secretory IgA at the mucosal epithelium leading to more efficient production of cross-protective immunity when compared to serum IgG (Tamura et al., 1992). However, while RSV F protein with cholera toxin induced mucosal IgA responses in mice, virus replication in the lung was reduced by one log, and potential vaccine-induced lung pathology was not investigated (Singh et al., 2007). In contrast, our results clearly demonstrate that i.n. immunization with the ΔF/TriAdj vaccine induces strong serum and mucosal antibody responses, as well as complete protection from experimental RSV infection. The antibody levels, numbers of IFN-γ secreting splenocytes and protection were equivalent to those induced with TriAdj containing CpG ODN reported previously (Garlapati et al., 2012). Importantly, in the current study we also showed that TriAdj mediated affinity maturation, which correlated with effective virus neutralization. Furthermore, vaccination with ΔF/TriAdj successfully generated RSV F-specific CD8+ central memory T cells (T CM-cells) in the lungs, which is important as immunological memory is a crucial feature of adaptive immune responses after vaccination (Salk & Salk, 1977), and there is evidence that CD8+ T CM is correlated with protection from disease (Castiglioni et al., 2004; Wherry et al., 2003). The TriAdj markedly enhanced the uptake of ΔF protein by lung cells and trafficking to the draining LNs in DCs, which is a crucial step for induction of effective pulmonary immune responses (Vermaelen et al., 2001), and in agreement with other reports in which i.n. administered particulate antigens were shown to be taken up and transported to the draining LN DCs, which present the antigen to CD8+ T-cells (GeurtsvanKessel et al., 2008; Jakubzick et al., 2008).

While ΔF with polyl:C alone induced protective immunity to RSV in mice, this was not observed for cotton rats. Notably, this is the first time i.n. vaccination with polyl:C as adjuvant in a RSV vaccine in cotton rats has been reported. The polyl:C dose might have been too low, but increasing the dose is probably not advisable from a viewpoint of safety. In contrast, i.n. immunization with ΔF/TriAdj induced sufficient immunity to protect cotton rats completely from RSV replication in the lungs, without inducing immunopathology. This demonstrates that a combination of polyl:C, IDR and PCEP mediates optimal enhancement of the RSV-specific immune response in the more susceptible cotton rats, which is promising for future studies in large animal species, including humans. Polypseudazenes exert their adjuvant effects through the formation of non-covalent complexes with protein (Andrianov et al., 2005) and enhance antigen-specific humoral immunity, while IDRs can enhance cell-mediated immune responses and modulate excessive consequences of TLR signalling (Bodish et al., 2005; Hancock & Sahl, 2006), suggesting important contributions from both of these compounds. In summary, our data show that the ΔF/TriAdj formulation is safe and induces local and systemic, humoral and cell-mediated immune responses, as well as protective immunity upon i.n. vaccination in mice and cotton rats, and that the TriAdj at least partially acts through enhanced uptake of the vaccine antigen by DCs.

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