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Intranasal immunization with a single dose of the fusion protein formulated with a combination adjuvant induces long-term protective immunity against respiratory syncytial virus

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ABSTRACT

Respiratory syncytial virus (RSV) is the most common cause of respiratory tract infections in both children and elderly people. In this study we evaluated the short- and long-term protective efficacy of a single intranasal (IN) immunization with a RSV vaccine formulation consisting of a codon-optimized fusion (F) protein formulated with poly(I:C), an innate defense regulator peptide and a polyphosphazene (ΔF/TriAdj). This vaccine induced strong systemic and local immune responses, including RSV F-specific IgG1 and IgG2a, SIgA and virus neutralizing antibodies in mice. Furthermore, ΔF/TriAdj promoted production of IFN-γ-secreting T cells and RSV F_{85-93}-specific $CD8^+$ effector T cells. After RSV challenge, no virus was recovered from the lungs of the vaccinated mice. To evaluate the duration of immunity induced by a single IN vaccination, mice were again immunized once with ΔF/TriAdj and challenged with RSV five months later. High levels of IgG1, IgG2a and virus neutralizing antibodies were detected in the ΔF/TriAdj-vaccinated animals. Moreover, this vaccine formulation induced robust local SIgA production and IgA-secreting memory B cell development, and conferred complete protection against subsequent RSV challenge. In conclusion, a single IN vaccination with RSV ΔF protein formulated with TriAdj induced robust, long-term protective immune responses against RSV infection.

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Introduction

Respiratory syncytial virus (RSV) is the leading cause of severe lower respiratory tract infection in infants, as well as older and immunocompromised individuals, worldwide.[1] While many RSV vaccine candidates, including live attenuated strains, vectored and viral protein subunit vaccines, are in development, there is no licensed vaccine or specific treatment, with exception of passive antibody therapy (Palivizumab), against RSV.[2] The first clinical trial with formalin-inactivated RSV (FI-RSV) failed to protect against infections and caused enhanced respiratory disease after natural RSV infection.[3]

RSV is an enveloped virus, encoding three transmembrane glycoproteins: the G (attachment), F (fusion) and SH (small hydrophobic) proteins. The F protein is essential for the fusion of the viral envelope to host cells and is highly conserved among RSV strains, making it a major target for subunit vaccine and antiviral drug development.[4] However, RSV F protein alone does not clear the infection after intranasal (IN) immunization;[5,6] therefore, it needs to be formulated with an adjuvant to establish long-lasting humoral and cell-mediated protective immune responses.

Previously, we developed a combination adjuvant platform (TriAdj) consisting of three components, namely poly(I:C), innate defense regulator (IDR) peptide and poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP). Synthetic double-stranded RNA, poly(I:C), is known to recognize toll-like receptor (TLR)3 and retinoic acid-inducible gene I(RIG-I) leading to induction of proinflammatory cytokines, which in turn activate various immune cells.[7,8] To stabilize and protect the poly(I:C) from degradation, two other immunmodulators, an IDR peptide and a polyphosphazene, were added. Innate defense regulator peptides are derivatives of natural host defense peptides with microbicidal and chemotactic properties,[9] whereas PCEP is a synthetic biodegradable water-soluble polymer with immunostimulatory properties. PCEP forms non-covalent complexes with antigens and/or other adjuvants to increase their stability and allow multimeric presentation.[10,11] Previously, we developed a novel vaccine candidate against RSV consisting of a truncated version of the RSV fusion protein (ΔF) formulated with TriAdj (ΔF/TriAdj). We have shown that two IN vaccinations with ΔF/TriAdj establishes long-lasting humoral and cell-mediated protective immune responses in mice, cotton rats and lambs.[12,13]

In the present study we demonstrated that a single IN administration of ΔF/TriAdj elicits mucosal and systemic immune responses and offers complete protection from RSV challenge in mice. Furthermore, ΔF/TriAdj induced effective local $CD8^+$ T cells, which is one of the hallmarks for successful vaccination against many viral infections. To assess the long-term immunity induced by this vaccine candidate after a single IN vaccination, we performed a long-term trial in mice. RSV

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ΔF formulated with TriAdj induced robust mucosal and systemic immune responses, which were still protective against RSV challenge five months after vaccination. Furthermore, protection was correlated with long-lived local IgA secreting memory B cell development and B cell IgA production, as well as memory T cell development

Results

A single mucosal vaccination with ΔF/TriAdj induces strong systemic immune responses

Antibodies play an important role in antiviral immunity. To characterize the quality of the humoral immune responses induced by a single vaccination with ΔF/TriAdj, serum IgG1 and IgG2a levels were measured. As shown in Fig. 1A and B, the mice immunized with ΔF/TriAdj developed significantly higher IgG1 and IgG2a titers than PBS-immunized animals, both before and after challenge with RSV. These results indicate that formulation of ΔF protein with TriAdj promotes a balanced humoral immune response. Neutralizing antibody provides essential protection against most viral infections. To evaluate the biological function of the ΔF-specific serum antibodies, virus neutralization (VN) titers were determined. Mice immunized with ΔF/TriAdj developed significantly higher neutralizing antibody titers than the ones immunized with PBS, both before and after challenge with RSV (Fig. 1C).

To investigate the phenotype of the cellular immune response, ΔF-induced secretion of IFN-γ and IL-5 by splenocytes was measured four days after challenge. Significant numbers of IFN-γ secreting cells were generated, while the numbers of IL-5 secreting cells were low in the mice vaccinated with the ΔF/TriAdj formulation (Fig. 1D). This suggests that ΔF/TriAdj elicits a cell-mediated immune response.

Intranasal vaccination with a single dose of ΔF protein formulated with TriAdj induces a robust protective mucosal immune response

Mucosal immunity provides a first line of defense to the host by blocking the establishment and spread of RSV infection in the lung. Thus, an effective RSV vaccine should induce local IgA antibodies in the respiratory tract. To evaluate the mucosal immune responses induced by ΔF/TriAdj, we measured ΔF-specific IgA levels in the lungs. Significantly higher IgA production was observed in mice immunized with ΔF/TriAdj in comparison with mice immunized with PBS (Fig. 2A). To determine whether a single IN vaccination with the ΔF/Triadj formulation would affect viral clearance, all mice except those in one of the two Placebo groups were challenged IN with RSV. No infectious virus was recovered from lungs of mice immunized with ΔF/TriAdj, showing full protection from RSV infection (Fig. 2B).

To investigate whether protection is correlated with higher cross-presentation and cell-mediated immune responses, we examined infiltration of RSV F-specific $CD8^+$ T cells into the lung after RSV challenge with a F_{85-93} pentamer. Previously it has been shown that the F_{85-93} epitope is conserved between RSV strains.[14] The ΔF/TriAdj induced a significantly higher number of F_{85-93} specific $CD8^+$ T cells in the lungs than PBS (Fig. 2C), which suggests that vaccination with ΔF/TriAdj promotes a cytolytic $CD8^+$ T cell response to RSV infection. The virus-specific effector $CD8^+$ T-cell response has been shown to play a critical role in RSV clearance.[15] Therefore, we analyzed the effector function of the $CD8^+$ T cells according to their expression of IFN-γ by flow cytometry. After RSV challenge, the ΔF/TriAdj-immunized animals showed higher frequencies of IFN-γ-expressing $CD8^+$ T cells than PBS-immunized, RSV-challenged mice in the lungs (Fig. 2D). This suggests that

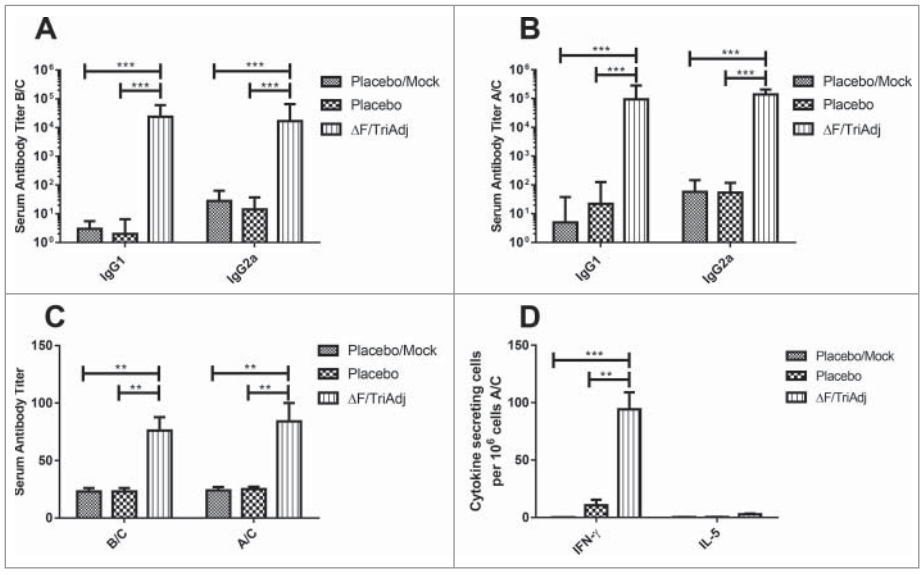

Figure 1. RSV ΔF-specific systemic humoral immune responses in mice. (A) Serum IgG1 and IgG2a titers before challenge (B/C), (B) Serum IgG1 and IgG2a titers after challenge (A/C) with RSV, (C) Serum VN antibody titers determined before (B/C) and after (A/C) RSV challenge, and (D) Numbers of IFN-γ and IL-5 secreting splenocytes determined in response to *in vitro* restimulation with ΔF protein. BALB/c mice were immunized once IN with ΔF formulated with TriAdj and challenged 3 weeks later with RSV. Control groups were immunized with PBS and challenged with RSV (Placebo) or mock-challenged (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 cell numbers are expressed as the difference in the number of spots between ΔF-stimulated wells and medium-control wells. Bars indicate median values with interquartile ranges. $^{**}P < 0.01$; $^{***}P < 0.001$.

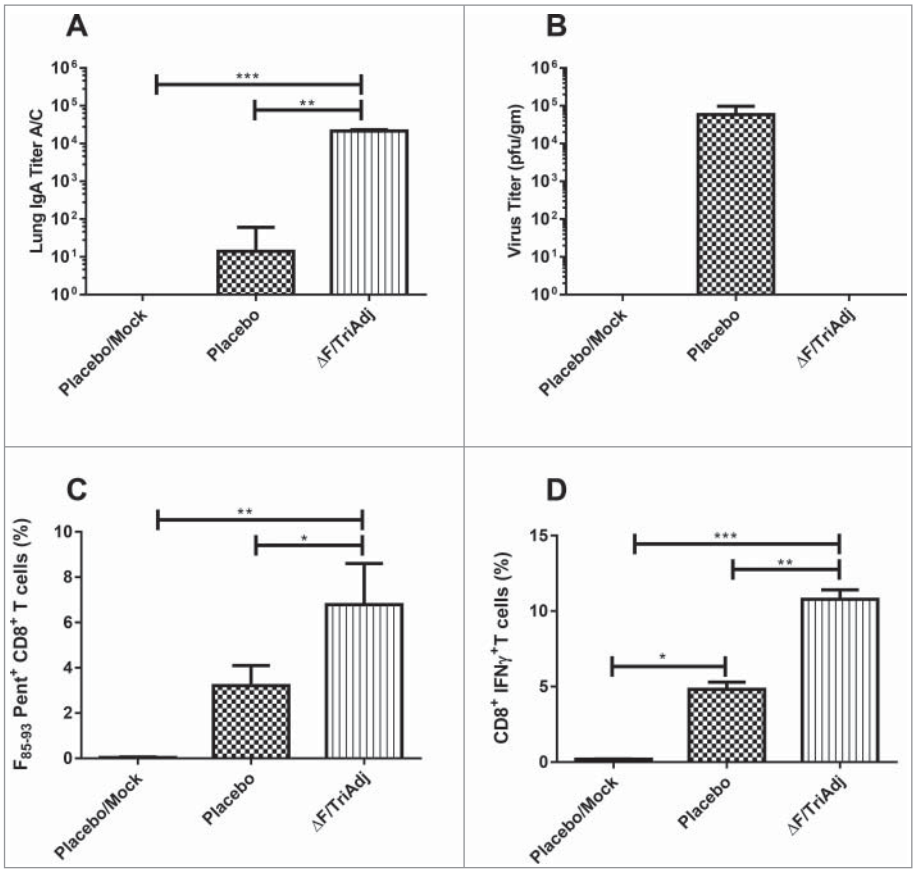

Figure 2. Mucosal immune responses to RSV ΔF protein in mice after challenge with RSV. The IgA titers (A), virus titers (B), percentages of ΔF-specific $CD8^+$ T cells (C), and ΔF-specific IFN-γ secreting $CD8^+$ T cells (D) were measured in the lung after RSV challenge. Mice were immunized and challenged as described in the legend for Fig. 1. 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 replication in the lungs is expressed as pfu per gram of lung tissue. The proportion of RSV F-specific $CD8^+$ T cells was determined in the lung by KYKNAVTEL-MHC I pentamer staining. IFN-γ expression by $CD8^+$ T cells was determined by intracellular cytokine staining. Bars represent median values with interquartile ranges. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ΔF/TriAdj promotes a strong effector $CD8^+$ T cell response to RSV infection.

A single intranasal vaccination with ΔF/TriAdj induces long-term systemic immune responses

To assess whether a single dose of ΔF/TriAdj can induce long-term memory, mice were again immunized once IN, and serum IgG and VN antibody levels were measured at different time points during a period of five months. When compared with the PBS-immunized animals, the mice immunized with ΔF/TriAdj developed higher IgG and VN titers, which remained elevated for at least 25 weeks (Fig. 3A and B). Next, we examined whether IN vaccination promoted the induction of long-term ΔF-specific serum IgG1 and IgG2a. The ΔF/TriAdj-vaccinated group had significantly higher ΔF-specific serum IgG1 and IgG2a titers than the placebo group, both before and after challenge with RSV (Fig. 4A and B). Virus neutralization titers were also determined after RSV challenge to evaluate the functional quality of the ΔF-specific serum antibodies. Mice immunized with ΔF/TriAdj developed significantly higher neutralizing antibody titers when compared with the PBS-immunized animals (Fig. 4C). To further define the phenotype of the immune response, ΔF-induced secretion of IFN-γ and IL-5 by splenocytes was measured four days after challenge with RSV. The ΔF/TriAdj formulation generated a significantly higher frequency of IFN-γ secreting cells in comparison to PBS, while the numbers of IL-5 secreting cells were low (Fig. 4D).

Intranasal vaccination with a single dose of ΔF/TriAdj induces a long-term protective mucosal immune response

To access the long-term local immune responses after immunization with ΔF/TriAdj, induction of ΔF-specific IgA in the lungs was examined by ELISA. Mice immunized with ΔF/TriAdj developed significantly higher IgA levels when compared with the PBS-immunized, mock- and RSV-challenged groups (Fig. 5A). Furthermore, we checked the development of IgA-secreting memory B cells in draining lymph nodes (LNs) after RSV challenge. The ΔF/TriAdj formulation generated a significantly higher frequency of RSV ΔF-specific IgA-secreting memory B cells in LNs in comparison to PBS (Fig. 5B).

To evaluate viral clearance after a single vaccination with ΔF/TriAdj, all mice except those in one of the two placebo groups were challenged IN with RSV on day 150. The mice were killed after four days and virus titers in the lungs were

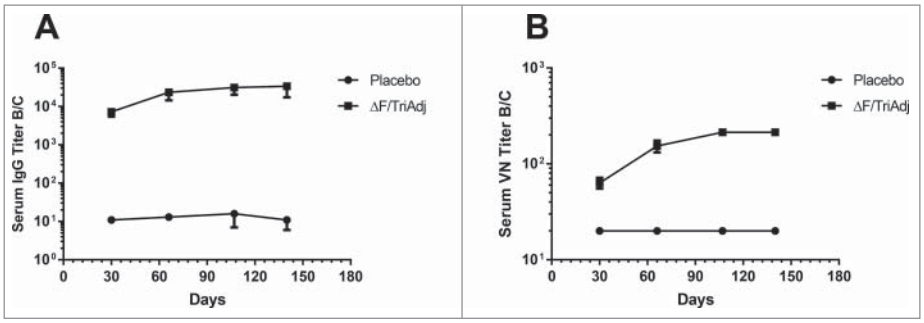

Figure 3. Long-term systemic immune responses to RSV ΔF protein. IgG (A) and VN (B) titers were measured at different times after vaccination. BALB/c mice were immunized once IN with ΔF formulated with TriAdj and a control group was immunized with PBS (Placebo). ELISA titers are expressed as the reciprocal 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. Symbols represent median values with interquartile ranges.

determined. No infectious virus particles were recovered from mice vaccinated with ΔF/TriAdj, showing that these mice still had a high level of immunity that protected them from RSV (Fig. 5C).

Discussion

RSV is one of the major pathogens causing a broad spectrum of respiratory illnesses in children and elderly worldwide. There still is no licensed RSV vaccine, in part due to the disastrous outcomes observed following vaccination of naïve children with FI-RSV.[3] Natural infection with RSV fails to protect against subsequent infections, because it stimulates modest immunity and short-lived immunological memory. Similar to natural RSV infection, many promising RSV vaccines have failed to generate long-lasting protective immune responses.[16,17] For broad clinical applications, a RSV vaccine needs to be safe and easy to administer, and must stimulate stronger and longer protective immunity than natural RSV infection.[18] Protein subunit vaccines have a high safety profile, but generally are poorly immunogenic and induce short-lived humoral and cellular immunity.[15] Therefore, adjuvants are added to subunit vaccines to stimulate faster, stronger, and long-lasting immune responses.

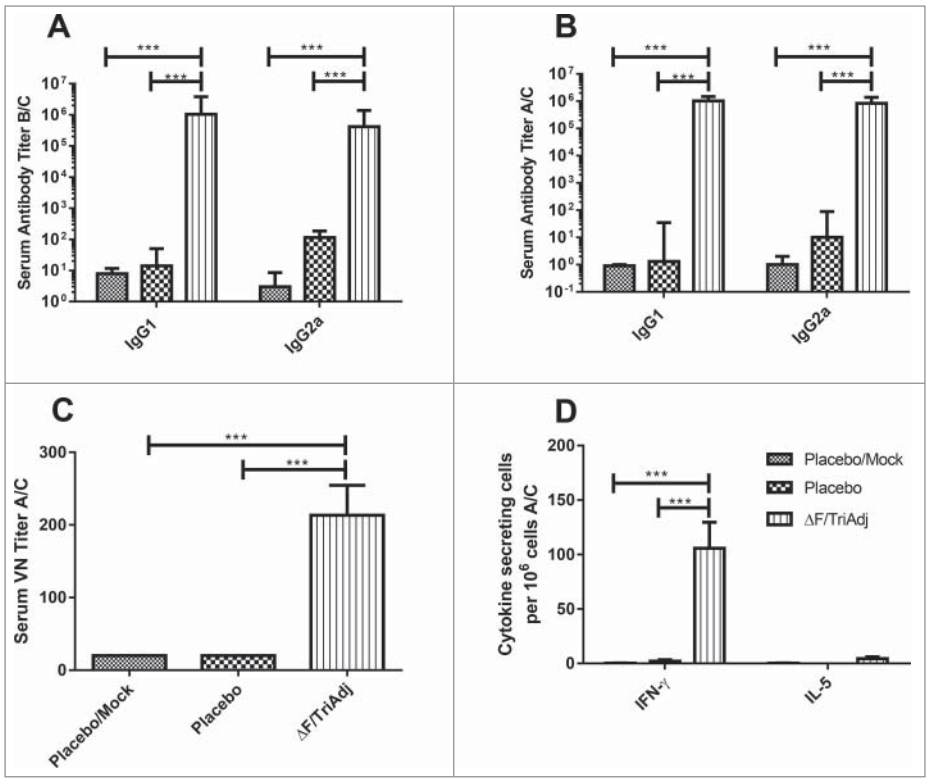

Figure 4. Systemic immune responses to RSV ΔF protein. (A) Serum IgG1 and IgG2a titers before challenge (B/C), (B) Serum IgG1 and IgG2a titers after RSV challenge (A/C), (C) Serum VN antibody titers after RSV challenge (A/C), and (D) Numbers of IFN-γ and IL-5 secreting splenocytes determined in response to *in vitro* restimulation with ΔF protein. BALB/c mice were immunized once IN with ΔF formulated with TriAdj and challenged with RSV on day 150. Control groups were immunized with PBS and challenged with RSV (Placebo) or mock-challenged (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 cell numbers are expressed as the difference in the number of spots between ΔF-stimulated wells and medium-control wells. Bars indicate median values with interquartile ranges. $^{***}P < 0.001$.

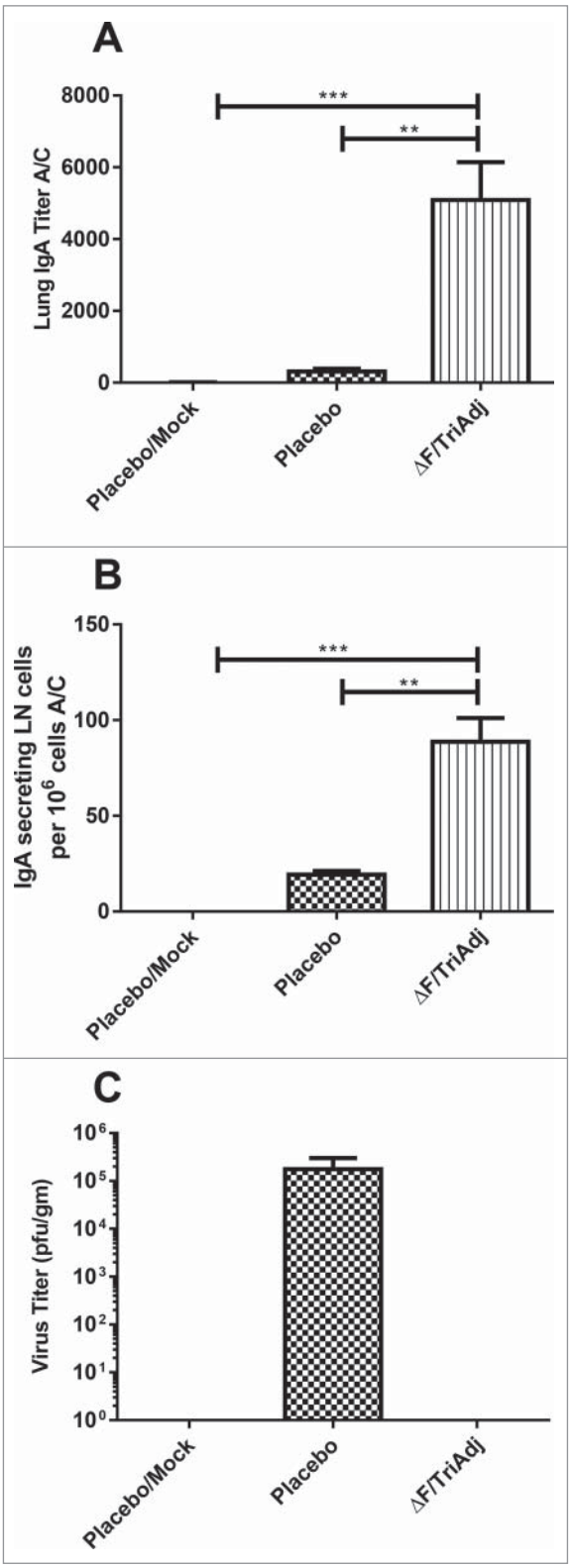

Figure 5. Long-term mucosal immune responses to RSV ΔF protein. IgA titers (A), numbers of IgA secreting LN cells (B) and virus titers (C) were determined after RSV challenge (A/C). Mice were immunized and challenged with RSV as described in the legend for Fig. 4. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. IgA secreting cell numbers are expressed as the difference in the number of spots between ΔF-stimulated wells and medium-control wells. Virus replication in the lungs is expressed as pfu per gram of lung tissue. Bars represent median values with interquartile ranges. $^{**}P < 0.01$; $^{***}P < 0.001$.

Previously, we and others have reported that strong, protective immune responses are induced after two immunizations with RSV F protein when formulated with an adjuvant.[19-21] At the clinical level, a multi-dose vaccine is expensive and has potential compliance problems, whereas a single vaccination is more cost-effective, and more convenient for health-care workers and patients.

In the present study, we demonstrated induction of short- and long-term protective immunity, including stable systemic IgG, local IgA and neutralizing antibodies, by a RSV ΔF protein formulated with TriAdj in mice after a single IN immunization. Furthermore, ΔF/TriAdj promoted effector $CD8^+$ T cells and complete protection against RSV infection; previously we also demonstrated that this vaccine does not induce vaccine-enhanced pathology in the lungs.[12] While a single mucosal vaccination with an adenovirus-based RSV vaccine has also been proved effective in the short term,[22] this is the first report showing long-term protective efficacy of a single mucosal vaccination with an adjuvanted RSV F protein subunit vaccine in mice.

Mucosal immunization via the IN route is non-invasive and suitable to generate both local and systemic immunity. RSV targets the respiratory tract, so mucosal antibodies, specifically IgA, play an important role in prevention and clearance of virus. Previously, it has been shown that protection against RSV infection is correlated with the levels of mucosal IgA rather than to systemic antibody in humans.[23] In mice, RSV infection induces short-lived neutralizing antibodies with absence of antibody-secreting memory B cells.[24] Interestingly, we have recently demonstrated that RSV-infected mice develop significantly lower levels of local IgA production and IgA-secreting memory B cells indicating an impaired local antibody response that allows RSV re-infection and explains the short-term protective immunity generated by natural infection with RSV.[25] In contrast, IN vaccination with a single dose of ΔF/TriAdj promoted long-lasting IgA-secreting memory B cell development, which in turn stimulates local B cell IgA production, a major criterion for an effective RSV vaccine.

A clinical trial with FI-RSV failed to induce protection against RSV due to generation of poorly neutralizing antibodies and weak TLR activation, which led to vaccine-associated enhanced disease.[26] The inclusion of a TLR ligand such as poly (I:C) in a vaccine formulation is expected to overcome the limitations of non-replicating RSV vaccines. The adjuvant effect of poly(I:C), which promotes long-lasting T cell immunity, is likely caused by the direct interaction with pattern recognition receptors (PRR) such as TLR3, melanoma differentiation-associated gene 5 (MDA5) and RIG-I, leading to production of pro-inflammatory cytokines and chemokines. Interestingly, it has been shown that alveolar macrophages and lung DCs induced strong IgA and IgG antibodies by administration of TLR3 ligands.[27] However, IN administration of higher doses of poly (I:C) has been shown to cause marked production of inflammatory cytokines/chemokines associated with impaired lung function,[28-30] suggesting that excessive stimulation of local immune responses can result in detrimental effects. In contrast, the TriAdj has an advantage over poly(I:C) alone as the combination of poly(I:C), IDR peptide and PCEP promotes balanced RSV-specific immune responses, without causing adverse

effects. We believe that the IDR peptide is one of the major anti-inflammatory components in our vaccine formulation as it has been previously shown to increase cell-mediated immune responses and to regulate severe outcomes of TLR signaling,[31,32] whereas polyphosphazenes enhance antigen-specific humoral immunity through the formation of non-covalent complexes with protein,[33] suggesting major roles for both of these compounds in this vaccine formulation.

For an RSV vaccine to be effective, the generation of effector $CD8^+$ T cells in the lungs is required. Previously, it has been shown that mild to severe infection with RSV failed to induce virus-specific IFN-γ recall responses in infants.[34] In our study RSV F-specific pentamer staining demonstrated marked induction of IFN-γ secreting RSV-specific $CD8^+$ T cells in the lungs, which supports stimulation of local effector $CD8^+$ T cell responses by ΔF/TriAdj. Interestingly, exogenous IFN-γ expression was shown to protect against RSV infection in the lungs of BALB/c mice.[35] This evidence suggests that complete protection against RSV is associated with the presence of IFN-γ^+ RSV-specific $CD8^+$ T cells.

There are some limitations in our current study. We have not evaluated the safety of ΔF/TriAdj in this study; however, previously we have shown that IN vaccination with RSV ΔF protein formulated in TriAdj promotes strong protective mucosal immunity without causing any lung immunopathology upon RSV challenge in a standard cotton rat model.[19] It is known that RSV does not replicate effectively in mouse lung, hence it causes little or no clinical disease. Inclusion of other RSV strains such as mucus producing isolates as well as other animal models might be important for further studies of this vaccine candidate. This would also provide additional information about cross-protection of this vaccine from other strains. The fact that we previously showed mucosal delivery (prime-boost) of ΔF/TriAdj to induce both systemic and mucosal immune responses in lambs provides support for mucosal vaccination with the ΔF/TriAdj vaccine.[13]

In summary, our data show that RSV ΔF protein formulated with TriAdj represents a safe, effective and promising RSV vaccine candidate. A major advantage associated with this mucosal RSV vaccine is the induction of long-term protective immunity after a single IN immunization, by stimulating long-lived RSV-specific neutralizing antibodies, memory B cells and $CD8^+$ T cells. This supports further evaluation of this RSV vaccine in clinical trials.

Materials and methods

Virus and vaccine formulation

The RSV A2 strain was propagated in Hep-2 cells (American Type Culture Collection, VR-1540). The RSV ΔF protein was synthesized without the transmembrane domain but with a carboxyl terminal (ser-gly)10 bridge followed by a his10 tag to facilitate purification as described previously.[6] Briefly, an episomal vector containing the ΔF protein was transfected into HEK-293T cells and the ΔF protein was purified by TALON Superflow resin (Clontech,) according to the manufacturer's instructions. Ten μg poly(I:C) (Invivogen, tlrl-picw), and 20 μg IDR1002 (Genscript, 818360) were mixed and incubated at 37°C, and after 30 min, 10 μg PCEP (Idaho National Laboratory) containing 1 μg ΔF protein was added as described previously.[12]

Animals, immunizations and challenge

Six to eight week-old female BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were housed under pathogen-free conditions. Mice were vaccinated once IN with ΔF/TriAdj and as control, two additional groups of mice received PBS (Placebo). To detect protective immune responses in the lungs, 3 weeks (short-term trial) or 25 weeks (long-term trial) post-vaccination, all mice, except those in one of the Placebo groups, were challenged IN with RSV strain A2 (5×10^5 PFU/50μl) and killed four days later. Blood samples were collected at regular intervals after vaccination and after RSV challenge. The University of Saskatchewan Animal Research Ethics Board approved all procedures, in accordance with the standards stipulated by the Canadian Council on Animal Care.

Lung fragment cultures and enzyme-linked immunosorbent assay (ELISA)

Lung fragment cultures were performed as described previously.[36] Briefly, four days after challenge, lavaged lungs of killed mice were cut into small pieces and cultured in medium at 37°C. After a 5-day incubation, cell-free supernatants were clarified and stored at −80°C. ELISAs were performed with sera and lung fragment culture supernatants as described previously.[12] Samples were serially diluted 4-fold and added to the RSV ΔF-coated plates to be incubated overnight at 4°C. Bound ΔF-specific IgG1, IgG2a and IgA were detected by addition of biotin-labeled goat anti-mouse IgG1, IgG2a (Southern Biotech, 1070–08, 1080–08) or IgA (Gibco, M3115) followed by streptavidin-alkaline phosphatase (AP) (Jackson ImmunoResearch Laboratories Inc., 016–050–084). Lastly, the reaction was developed with *p* -nitrophenyl phosphate (Sigma-Aldrich, N3254) substrate and read in a SPECTRAmax 340 PC Microplate Reader (Molecular Devices). ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum.

Enzyme-linked immunospot (ELISPOT) assays

ELISPOT assays were performed as described previously.[6,25] Briefly, splenocytes and LN cells were stimulated with ΔF protein (2 μg/ml) and spots were developed with biotinylated IFN-γ- or IL-5-specific goat anti-mouse IgG (BD Biosciences, 554410, 554397) or biotinylated goat anti-mouse IgA-specific antibody, followed by AP-conjugated streptavidin and BCIP/NBT (Sigma-Aldrich, B5655) as the substrate.

Analysis of $CD8^+$ T cells from lungs by flow cytometry

Lung single-cell suspensions were generated as described previously.[12] To identify the RSV F_{85-93}-specific $CD8^+$ T cells, lung mononuclear cells were stained with H-2Kd-F_{85-93} MHC class I pentamer (ProImmune, F149) along with anti-mouse CD8a

antibody (BD PharMingen, 553031). To detect IFN-γ producing $CD8^+$ T cells, lung mononuclear cells were treated with 1 μM of F_{85-93} peptide (KYKNAVTEL) and then GolgiStop (BD PharMingen, 554715) was added 6 h before harvesting the cells. Cell surface staining was performed with FITC-conjugated anti-mouse CD8a antibody followed by intracellular cytokine staining using Cytofix/Cytoperm (BD PharMingen, 554715), as per manufacturer's instructions, and addition of APC-conjugated anti- mouse IFN-γ antibody (BD Biosciences, 554413). After staining, cells were acquired by flow cytometry using a FACS Calibur (BD Biosciences), and data were analyzed using Kaluza Software (Version 1.2). Cells were gated for live cells, singlets and lymphocytes, and then analyzed for indicated markers.

Virus titration and virus neutralization assay

Virus titrations were performed with individual lungs on day 4 post- challenge as described previously [12] and data are expressed as PFU/g of lung tissue with a detection limit of 10 PFU/ml. RSV-specific neutralization titers were determined by plaque reduction assays. Two-fold serial dilutions of serum samples were mixed 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 and incubated at 37 °C. After a 4-day incubation, cells were fixed in 75% ethanol/ 25% acetic acid and stained with 0.5% crystal violet.

Statistical analysis

GraphPad PRISM version 6 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze data. A student t-test or one-way ANOVA was used to examine the difference among all groups followed by a Newman-Keuls post-test. Differences were considered statistically significant if $P < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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