Vaccination with the RSV fusion protein formulated with a combination adjuvant induces long-lasting protective immunity

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Respiratory syncytial virus (RSV) is one of the primary causative agents of upper and lower respiratory tract infections in young children, in particular infants. Recently, we reported the protective efficacy of a RSV vaccine formulation consisting of a truncated version of the fusion (F) protein formulated with a Toll-like receptor (TLR) agonist and an immunostimulatory peptide in a carrier system (ΔF/TriAdj). To evaluate the duration of immunity induced by this vaccine candidate, we carried out long-term trials. The ΔF was formulated with triple adjuvant (TriAdj) containing either polyinosinic : polycytidylic acid (polyI : C) or cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs) and administered intranasally to mice. One year after the second vaccination all mice were challenged with RSV. Both ΔF/TriAdj formulations mediated the induction of high levels of IgG1, IgG2a and virus-neutralizing antibodies, and IgA in the lungs. Based on the numbers of IFN-γ- and IL-5-secreting cells in the spleen, the immune response was slightly T-helper cell type 1 (Th1)-biased. This was confirmed by the presence of F 85–93 -specific CD8+ effectors in both ΔF/TriAdj(polyI : C) and ΔF/TriAdj(CpG)-immunized mice. Both ΔF/TriAdj formulations induced ΔF/TriAdj(CpG)-immunized mice. However, ΔF/TriAdj(polyI : C) generated significantly higher IgG affinity maturation and higher numbers of RSV-specific CD8+ effectors in lungs and CD8+ central memory T cells in spleen and lymph nodes than ΔF/TriAdj(CpG). After RSV challenge, no virus replication and no evidence of vaccine-induced pathology were detected in mice immunized with either of the ΔF/TriAdj formulations, demonstrating that the duration of immunity induced with these vaccines is at least one year.

INTRODUCTION

Respiratory syncytial virus (RSV) is a major cause of serious lower respiratory tract (LRT) illness in infants, elderly and immunocompromised individuals (Falsey et al., 2005). The development of a RSV vaccine has been hampered by the outcome obtained with the formalin-inactivated (FI) virus vaccine in the 1960s, which failed to induce RSV-neutralizing antibodies and caused vaccine-enhanced disease after natural RSV infection (Kim et al., 1969). Although a number of RSV vaccine candidates have been evaluated with varying success, there are still no licensed RSV vaccines or specific treatments other than prophylaxis with passive antibody (The 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.

RSV encodes 11 proteins, and the envelope contains three virus-encoded transmembrane surface glycoproteins: the major attachment protein G, fusion protein F and small hydrophobic SH protein. The highly conserved F protein facilitates penetration of the virus into host cells and subsequent formation of syncytia, thus making it a suitable subunit vaccine candidate (Collins & Graham, 2008). However, mucosal immunization with F protein alone does not result in protective immunity (Garlapati et al., 2012; Vaux-Peretz et al., 1992). Thus, the F protein needs to be formulated with an adjuvant to induce robust immune responses.

Vaccination with FI-RSV resulted in low-avidity anti-RSV antibodies due to poor Toll-like receptor (TLR) activation, which strongly supports the need for a TLR agonist in a RSV subunit vaccine (Delgado et al., 2009). It is also well established that a RSV vaccine needs to induce a balanced...
T-helper cell types 1/2 (Th1/Th2) adaptive immune response, which promotes the generation of high-affinity neutralizing antibodies and stimulation of effector cytotoxic CD8+ T cells (Graham, 2011). Polynosinic:polycytidylic acid (polyI:C), a synthetic double-stranded RNA, has efficient immunostimulatory properties and has been tested as adjuvant in viral vaccines (Matsumoto & Seya, 2008; Salazar et al., 1996; Trumpfheller et al., 2012). Similarly, cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODNs) that mimic bacterial CpG-containing unmethylated DNA enhance immune responses when co-administered with antigens (Garlapati et al., 2012; Ioannou et al., 2002; Krieg, 2002). For intranasal (i.n.) immunization it is crucial to stabilize and protect the TLR ligand from degradation. We found a combination of two components to be suitable for this purpose, namely an innate defence regulator (IDR) peptide and a polyphosphazene. IDR peptides are derivatives of natural host defence peptides, which are cationic amphipathic peptides with microbicidal, chemotactic and/or immunomodulatory properties (Yeung et al., 2011). Poly[d(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) is a synthetic water-soluble polymer with immunostimulatory properties and forms non-covalent complexes with antigens and/or other adjuvants to enhance their stability and allow multimeric presentation (Andrianov et al., 2009; Kovacs-Nolan et al., 2009).

An essential requirement for any vaccine is the induction of long-term protective immunity. However, many RSV vaccine candidates have failed to stimulate long-term protective responses in humans (Hall, 2001; Power, 2008), illustrating our lack of understanding of the immune mechanisms necessary to generate long-term, protective anti-RSV immune responses. Previously, we reported immunity for up to one month after i.n. vaccination with a RSV vaccine formulation consisting of a truncated version of the F protein formulated with a TLR agonist and an IDR peptide in PCEP (ΔF/TriAdj) (Garg et al., 2014; Garlapati et al., 2012). In order to evaluate the duration of immunity induced by this vaccine candidate, we carried out long-term trials. After i.n. delivery, ΔF formulated with triple adjuvant (TriAdj) containing either polyI:C or CpG ODN elicited long-term mucosal and systemic immune responses, including memory CD8+ T cells, and complete protection from RSV challenge in mice, one year after the second vaccination.

**RESULTS**

**Long-term systemic immune responses induced by ΔF with TriAdj containing polyI:C or CpG ODNs**

Mice were immunized intranasally with ΔF and TriAdj containing either polyI:C [ΔF/TriAdj(polyI:C)] or CpG ODN [ΔF/TriAdj(CpG)] to assess the induction of persistent immune responses. Both groups of mice developed robust IgG and virus-neutralization (VN) titres compared with mice that received PBS (placebo) (Fig. 1a, b). After a slight decrease, these titres remained relatively constant for approximately 55 weeks. No differences were observed between the formulations with polyI:C and CpG ODN. As a lack of antibody affinity maturation may have been responsible for the enhanced respiratory disease in several children vaccinated with the Fl-RSV vaccine (Delgado et al., 2009), we investigated whether ΔF/TriAdj containing polyI:C or CpG ODN promoted affinity maturation of the ΔF-specific IgG. Interestingly, while both ΔF/TriAdj-vaccinated groups developed high-avidity F-binding antibodies, levels were higher in the group vaccinated with ΔF/TriAdj(polyI:C) than in the group that received ΔF/TriAdj(CpG) (Fig. 1c, d).

To determine the type of immune response induced, we examined whether i.n. vaccination promoted the induction of ΔF-specific serum IgG1 and IgG2a. Both groups immunized with ΔF/TriAdj had significantly higher IgG1 and IgG2a levels than placebo groups both before (day 388) and after (day 393) challenge with RSV (Fig. 2a, b). The production of both Ig subtypes with a slight predominance of IgG2a supports the induction of a balanced to Th1-biased humoral immune response. To define further the bias of the immune response, the ΔF-induced secretion of IFN-γ and IL-5 by in vitro restimulated splenocytes was measured. Both ΔF/TriAdj formulations generated a significantly higher frequency of IFN-γ-secreting cells compared with the placebo (Fig. 2c), while the number of IL-5-secreting cells was low in all vaccinated mice (Fig. 2d), suggesting a Th1 bias. No differences were observed between the polyI:C- and CpG ODN-containing adjuvant formulations in either isotype or cytokine profiles.

**Long-term mucosal immune responses induced by ΔF with TriAdj containing polyI:C or CpG ODN**

We next examined whether i.n. vaccination promotes the induction of long-term ΔF-specific IgG1, IgG2a and IgA in the lungs. Mice immunized with ΔF/TriAdj developed significantly higher IgA levels when compared with placebo groups (Fig. 3a). The ΔF-induced secretion of IgA by lung mononuclear cells in vitro restimulated with ΔF protein was measured after RSV challenge. While both ΔF/TriAdj formulations generated a significantly higher frequency of IgA-secreting cells in comparison with PBS, this was higher in the ΔF/TriAdj(polyI:C) group (Fig. 3b). As observed for the systemic response, determination of lung IgG subclass titres revealed significantly higher IgG1 and IgG2a production in mice immunized with ΔF/TriAdj compared with mice immunized with PBS, both before and after challenge (Fig. 3c, d), but there were no differences between the polyI:C- and CpG ODN-containing formulations. In order to investigate whether protection is correlated with increased cell-mediated immune responses, we measured the proportion of F-specific CD8+ T cells infiltrating the lungs with F85–93 pentamers. RSV F85–93-specific CD8+ T cells were increased in the lungs of mice immunized with ΔF/TriAdj compared with PBS-treated mice (Fig. 3e). Furthermore, ΔF/TriAdj containing polyI:C generated significantly more...
F85-93-specific CD8$^+$ T cells than ΔF/TriAdj with CpG ODN. As there is evidence that RSV-specific effector CD8$^+$ T-cell responses play a major role in virus clearance (Graham, 2011), we further investigated whether ΔF/TriAdj generated IFN-γ-producing CD8$^+$ T cells after RSV infection in the lungs. Mice vaccinated with ΔF/TriAdj and then challenged with RSV appeared to have a higher frequency of IFN-γ-expressing CD8$^+$ T cells in the lungs after peptide restimulation than PBS-immunized, RSV-challenged mice; however, this difference was only significant for ΔF/TriAdj(polyI : C) (Fig. 3f), suggesting that ΔF/TriAdj containing polyI : C promotes a stronger effector CD8$^+$ T-cell response to RSV than ΔF/TriAdj containing CpG ODN.

**Formulation of ΔF with TriAdj induces effective long-term protection from RSV challenge**

To determine viral clearance after vaccination with ΔF/TriAdj containing either polyI : C or CpG ODN, all mice except those in one of the two placebo groups were challenged intranasally with RSV on day 389. No infectious virus was recovered from mice immunized with ΔF/TriAdj containing either polyI : C or CpG ODN, showing that these mice still had sufficient immunity to be completely protected from challenge virus replication in the lungs (Fig. 4a). This result corroborates the observation that sera from mice vaccinated with ΔF/TriAdj had significant neutralization titres on day 388. Furthermore, we evaluated the cytokine profile in lungs following challenge with RSV. ΔF/TriAdj induced higher secretion of IFN-γ and tended to increase IL-12 production (Fig. 4b, c), while reducing the secretion of IL-13 (Fig. 4d). Similar to IL-13, very low levels of IL-4 and IL-5 secretions were observed (data not shown). This suggests that ΔF/TriAdj promoted a Th1 cytokine profile without Th2 cytokine responses; however, even the IFN-γ level was very low. Differential counts of the cells in the lung lavages after challenge were performed to assess eosinophilia. After i.n. vaccination and RSV challenge, mice immunized with ΔF/TriAdj had no eosinophils in the bronchoalveolar lavage (data not shown). This result agrees well with the fact that the generation of eosinophils is highly dependent on Th2 cytokines.

**Fig. 1.** Long-term systemic immune responses to RSV ΔF protein. IgG (a) and VN (b) titres were measured at different times after vaccination. Serum IgG antibody affinity was measured one day before (day 388) (c) and four days after challenge (day 393) (d) with RSV. BALB/c mice were immunized twice intranasally with ΔF formulated with TriAdj containing either polyI : C (●) or CpG ODN (○) and challenged with RSV on day 389. Control groups were immunized with PBS (●, placebo). ELISA titres are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. VN titres are expressed as the highest dilution of serum that resulted in <50% of cells displaying cytopathic effects. The antibody affinity is calculated as a percentage of the titres obtained after urea washes compared with those after PBS washes. Symbols represent median values with interquartile ranges. *P<0.05.
Immunization with ΔF/TriAdj induces RSV ΔF-specific effector memory CD8+ T cells in lungs and a central memory phenotype in lymph nodes and spleen

On the basis of their homing characteristics, memory cells are classified into two functionally distinct subsets: effector memory (TEM) and central memory (TCM) T cells. CD127 (IL-7Rα) is expressed on long-lived antigen-independent memory CD8+ T cells, while CD62L expression on T cells is required for homing. TEMs are predominant in secondary lymphoid organs [lymph nodes (LNs) and spleen], whereas T cells in peripheral compartments (lungs) show a TEM phenotype. TEMS express CD127 but downregulate CD62L and TCMs are CD127/CD62L double-positive, representing the pool of circulating long-lived memory CD8+ T cells. We analysed the memory phenotype of the ΔF-specific CD8+ T cells according to their expression of the surface markers CD62L and CD127 by flow cytometry. After challenge, the CD8+ T cells in the lungs of all ΔF/TriAdj-immunized mice mainly displayed an effector memory phenotype (CD8+CD127−CD62L+); however, ΔF/TriAdj(polyI:C) generated a significantly stronger effector memory phenotype than ΔF/TriAdj(CpG) (Fig. 5a–c). In addition, the group immunized with ΔF/TriAdj(polyI:C) displayed higher frequencies of central memory phenotype (CD8+CD127+CD62L+) compared with that immunized with ΔF/TriAdj(CpG) in the lung-draining LNs and spleen of mice (Fig. 5d, e).

DISCUSSION

Natural RSV infection in humans frequently fails to protect against subsequent infection because RSV is a poor inducer of innate immune defences, and consequently fails to stimulate long-lived immunological memory against repeated infections with the same strain of virus. An effective RSV vaccine must thus stimulate more protective and longer-lasting immune responses than those developed during natural infection (Pulendran & Ahmed, 2011). In the present study, we demonstrated that vaccination with ΔF protein formulated with TriAdj containing polyI:C or CpG ODN resulted in stable systemic IgG and local IgA production and, more importantly, robust neutralizing antibody titres for a period of over one year. Furthermore, ΔF/TriAdj provided a balanced to Th1-type response including RSV F-specific effector CD8+ T cells in the lungs. Finally, vaccination with ΔF/TriAdj provided full protection against RSV infection in mice. Previously, it was shown that single RSV infection in mice, as in humans, stimulates short-lived neutralizing antibodies without detectable antibody-secreting cells and memory B cells (Schmidt...
et al., 2012). The similar longevity of the responses to RSV infection in mice and humans suggests that mucosal immunization with RSV ΔF protein formulated with TriAdj could also induce long-lasting protective RSV-specific immune responses in humans—a critical criterion for an effective RSV vaccine.

In other studies, effective mucosal immunity has been achieved by antigen delivery to the total respiratory tract (TRT) (Minne et al., 2007; Southam et al., 2002; Vujanic et al., 2012). According to one study, i.n. delivery of a small volume (5 μl) completely retained the antigen in the upper respiratory tract (URT), while 25 μl partially reached the LRT (5–30%) depending on the level of anaesthesia, and with 50 μl a larger proportion (~55%) was found in the LRT (Southam et al., 2002). As we used 20 μl with light anaesthesia, most of the vaccine probably remained in the URT, with some reaching the LRT. This would suggest that in humans an i.n. spray should be used that distributes the

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**Fig. 3.** Mucosal immune responses to RSV ΔF protein. IgA titres (a), numbers of IgA-secreting lung cells (b), IgG1 titres (c) and IgG2a titres (d) were determined one day before (day 388, B/C) and four days after (day 393, A/C) RSV challenge. Percentages of ΔF-specific CD8+ T cells (e) and ΔF-specific IFN-γ-secreting CD8+ T cells (f) were measured in the lung after RSV challenge. White bars, placebo/mock; light grey bars, placebo; dark grey bars, ΔF/TriAdj(polyI : C); black bars, ΔF/TriAdj(CpG). Mice were immunized and challenged with RSV as described in the legend to Fig. 1. ELISA titres 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. Bars represent median values with interquartile ranges. *P<0.05. ***P<0.001.
vaccine through the TRT; however, as natural RSV infection in humans starts in the URT, i.n. delivery to the URT tract might be adequate.

When used as adjuvant, polyI:C induces inflammation and long-lasting T-cell immunity (Stahl-Hennig et al., 2009; Trumpfheller et al., 2008). The adjuvant effect of polyI:C is probably caused by direct interaction with TLR3 and retinoic acid-inducible gene 1 (RIG-I), leading to activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and production of type 1 IFN, which is critical for cross-presentations to generate cytotoxic CD8⁺ T cells (Le Bon et al., 2006). Accordingly, we demonstrated that mice immunized with the ΔF/TriAdj containing polyI:C generated significantly more F-specific effector CD8⁺ T cells than ΔF/TriAdj with CpG ODN in the lungs. Similarly, i.n. vaccination of polyI:C with inactivated influenza virus haemagglutinin (HA) vaccine induced both nasal IgA and serum IgG and cross-protective immune responses against homologous and heterologous variant influenza virus infection in mice and non-human primates (Ichinohe et al., 2010, 2007, 2005). However, it has been shown that i.n. administration of higher doses of polyI:C to the lungs of mice caused marked inflammation accompanied by impaired lung function (Aeffner et al., 2011; Boukhvalova et al., 2010; Stowell et al., 2009), suggesting that sustained TLR3 activation may also contribute to exacerbation of chronic pulmonary disease. By contrast, this was not observed in the mice vaccinated with ΔF/TriAdj containing polyI:C, which were fully protected without any evidence of inflammatory or immunopathological responses; this can be attributed to the low dose of polyI:C together with the ability of IDR to mitigate TLR ligand-induced inflammatory responses (Mookherjee et al., 2006).

CpG ODNs have been found to increase the immunogenicity of protein antigens. However, while the addition of CpG ODN to F protein in alum enhanced Th1-biased responses, accelerated clearance of RSV from the lungs and reduced eosinophilia, this did not lead to complete protection (Hancock et al., 2001). Similarly, mucosal immunization of cotton rats with RSV F protein co-adjuvanted with only CpG ODN resulted in only modest

Fig. 4. Detection of RSV and cytokines in lung tissue. Virus titre (a) and concentrations of IFN-γ (b), IL-12 (c) and IL-13 (d) were determined in the lungs. Mice were immunized as described in the legend to Fig. 1 and challenged with RSV on day 389. Virus replication in the lungs is expressed as p.f.u. per gram of lung tissue. Bars represent median values with interquartile ranges. *P<0.05.
**Fig. 5.** Detection of $\Delta F$-specific memory CD$^+$ T cells. The memory phenotype of pentamer positive CD$^+$ T cells was determined in the lungs of $\Delta F$/TriAdj(polyI:C)- (a) and $\Delta F$/TriAdj(CpG)- (b) vaccinated mice six days after RSV challenge. The presence of antigen-specific memory CD$^+$ T cells expressing CD62L and CD127 was analysed in the lungs (c), lung-draining LNs (d), and spleen (e). Grey bars, $\Delta F$/TriAdj(polyI:C); black bars, $\Delta F$/TriAdj(CpG). Bars represent median values with interquartile ranges. *$P<0.05$. 

Induction of long-term immunity by RSV subunit vaccine
protection from viral challenge and did not prevent the development of enhanced pulmonary pathology (Prince et al., 2003). However, the mice vaccinated with ΔF/TriAdj containing CpG ODN were fully protected from RSV without development of any enhanced disease.

Preclinical studies in animal models play a crucial role in evaluating the safety of vaccines for humans. However, the translation of preclinical observations to human disease is intricate due to species-specific variations between rodents and humans. In mice TLR9 is expressed in B cells, macrophages, dendritic cells (DCs) and activated T cells, whereas in humans it is expressed in B cells and plasmacytoid DCs (Hornung et al., 2002; Rothenfusser et al., 2002). This may influence the translation of preclinical trials with CpG ODNs to clinical studies. However, CpG ODNs combined with vaccines enhanced immune responses in human clinical trials designed to prevent influenza (Cooper et al., 2004), malaria (Sagara et al., 2009) and hepatitis B (Cooper et al., 2008). By contrast, the efficacy of CpG ODNs in cancer vaccines was not confirmed, as robust cytotoxic T-lymphocyte (CTL) responses are required to clear an established tumour. TLR3 is expressed on myeloid DCs (mDCs) and macrophages in mice and on mDCs in humans. This has an advantage because the mDCs are most proficient as antigen-presenting cells including the ability to cross-present. TLR3 agonists have been most widely examined in humans as a stand-alone treatment against infectious diseases and cancer (Ewel et al., 1992; Lampkin et al., 1985). Due to degradation by serum nucleases, polyI:C had limited success in humans and non-human primates (McFarlin et al., 1985; Nordlund et al., 1970), while in phase I–II clinical trials with higher doses of polyI:C in cancer patients, safety issues such as kidney failure and shock were observed (Robinson et al., 1976). However, these problems may be overcome by using a more stable compound such as poly-ICLC (Butowski et al., 2009) or poly-I:C;G2U (Thompson et al., 1996), or by combining polyI:C with IDR and PCEP, which allows us to limit the dose of polyI:C and yet retain optimal enhancement of the activity, stability and safety. Thus, with improvements in formulation, polyI:C and CpG ODNs are likely to find use as adjuvants in vaccines and/or stand-alone applications.

A key factor in virus control is the functional capacity for cytokine expression by virus-specific CD8+ T cells. To define the generation of functional high-quality CD8+ T-cell responses induced by TriAdj, we measured levels of IFN-γ positive RSV-specific CD8+ T cells in lungs; the results suggested that ΔF/TriAdj(polyI:C) promoted a stronger effector CD8+ T-cell response to RSV than ΔF/TriAdj(CpG). While this was not reflected in the level of protection from RSV infection in this study, induction of antigen-specific, activated CD8+ T cells would be expected to be beneficial, in particular in the long term beyond one year, when the antibody levels are expected ultimately to decrease such that they may not prevent initiation of infection. Previously, it has been shown that exogenous IFN-γ expression in the lungs of BALB/c mice protects against RSV infection (Kumar et al., 1999). Similarly, infants with moderate or severe RSV disease had less RSV-specific IFN-γ recall responses than infants with mild RSV infection (Lee et al., 2007).

The most effective approach to provide long-term protection against subsequent infection and disease may be the induction of a combination of robust, long-lived antibody and T-cell immunity (Bevan, 2011). In the respiratory tracts of mice, influenza-specific CD8+ T cells have a long life (Wiley et al., 2001) when compared with RSV-specific CD8+ T cells, suggesting that the protective CD8+ T-cell-mediated immunity declines relatively quickly (Connors et al., 1991). Immunological memory is a crucial feature of adaptive immune responses after vaccination (Salk & Salk, 1977). We demonstrated that vaccination with ΔF/TriAdj containing polyI:C generated significantly higher frequencies of RSV F-specific CD8+ TCM cells in the LNs and spleen when compared with ΔF/TriAdj(CpG). While the role of memory antibody responses in protection against infection is well understood, not much is known about the quality of memory T cells required for protection against disease. However, there is evidence that in a murine model of influenza, CD8+ TCM is correlated with protection from disease (Castiglioni et al., 2004; Wherry et al., 2003).

The challenge of vaccinating infants lies in the limitations of the neonatal immune response. Neonates and young infants are less capable of mounting an adequate protective cell-mediated and humoral immune response to RSV infection. Indeed it was shown that infants infected with RSV recruited low numbers of CD4+ and CD8+ T cells to the lung (Johnson et al., 2007; Welliver et al., 2007). Successful induction of early protection must also overcome the inhibitory influence of maternal antibodies. Since ΔF/TriAdj contains a Th1-promoting adjuvant and induces strong T-cell immunity, it might overcome the difficulty of neonates in developing a cell-mediated immune response. Furthermore, mucosal immunization is more likely to avoid vaccine inactivation by circulating maternal antibodies. Alternatively, as there is a rapid decrease in passive protection such that by the age of 6 months only ~30% of infants still have significant maternal antibody concentrations, maternal immunization with a RSV vaccine appears to be a reasonable approach to protect young infants against severe RSV infection.

In summary, our data show that RSV ΔF protein formulated with TriAdj induces long-term protective immunity by stimulating long-lived neutralizing antibodies, RSV-specific antibody-secreting cells, and RSV-specific CD8+ T cells upon i.n. vaccination in mice, thus warranting further evaluation as a vaccine against RSV in clinical trials.

METHODS

Virus and vaccine formulation. The RSV A2 strain (American Type Culture Collection, Manassas, VA, USA) was generated in Hep-2 cells

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(American Type Culture Collection). The ΔF protein with his-tag was produced and purified as described previously (Garlapati et al., 2012). Briefly, HEK-293T cells were transfected with an episcopal vector expressing the ΔF protein using Turbofect (Fermentas). Subsequently, the cells were collected and spun down, and supernatants were centrifuged at 20,000 g for 20 min. The ΔF protein with his-tag was purified using TALON Superflow resin (Clontech).

The ΔF protein was formulated in 20 μg IDR-1002 and 10 μg PCEP with 10 μg poly: C [ΔF/TriAdj(poly:C)], or 10 μg CpG ODN [ΔF/TriAdj(CpG)]. Poly: C (Invivogen) or CpG ODN 10101 (Pfizer) and IDR-1002 (Genscript) were mixed at a 1:2 ratio at 37 °C. PCEP (Idaho National Laboratory) along with ΔF protein was added after 30 min to make a final 1:2:1 ratio of poly:C or CpG ODN: IDR: PCEP.

**Animals, immunizations and challenge.** Female BALB/c mice (6–8 weeks of age; 15 mice per group) (Charles River Laboratories) were immunized twice intranasally under light anaesthesia at a three-week interval, with 20 μl vaccine containing 1 μg ΔF formulated with TriAdj containing either poly:C or CpG ODN. Two additional groups of mice received PBS intranasally (placebo). Fifty-two weeks after the second immunization, all groups except one placebo group were challenged intranasally with RSV strain A2 (5 × 10^6 p.f.u. in 50 μl). Serum samples were collected before immunization and at regular intervals afterwards until the end of the trial. Five mice from each group were euthanized one day before and five mice four days after RSV challenge to generate lung fragment cultures, obtain bronchoalveolar lavage fluids, produce lung homogenate supernatants, isolate splenocytes and assess virus replication in the lungs. The remaining mice were sacrificed six days after challenge to collect lungs for detection of F-pentamer (ProImmune)-specific CD8 T-cells, 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). To identify the memory phenotype, single-cell suspensions were stained with FITC anti-CD8 (BD Biosciences), allophycocyanin(ABC) anti-CD127 and PerCP-Cy5.5 anti-CD62L (BioLegend). For intracellular detection of IFN-γ in CD8^+^ T cells, lung mononuclear cells were stimulated with 1 μM of F85-93 peptide (KYKNAVTEL) for 6 h, and then Golgi-plug (BD Pharmingen) was added for another 6 h at 37 °C in 5% CO2. Cell surface staining was performed, followed by intracellular cytokine staining using Cytofix/Cytoperm (BD Pharmingen) and APC-conjugated anti-IFN-γ antibody (BD Biosciences). Cells were acquired by flow cytometry (BD Biosciences), and data were analysed using Kaluza Software (v. 1.2). Cells were gated for live cells, singlets and lymphocytes and then analysed for indicated markers.

**Lung fragment cultures, ELISA and virus-neutralization assay.** Lung fragment cultures were prepared as described previously (Mapletoft et al., 2008). RSV AF-specific IgG, IgG1, IgG2a and IgA were evaluated in serum and lung fragment culture supernatants by standard ELISA. Briefly, 96-well microtitre plates (Thermo Scientific) were coated with RSV protein (50 ng ml⁻¹) overnight at 4 °C. After washing, fourfold serially diluted samples were applied to the AF-coated plates and incubated overnight at 4 °C. Bound AF-specific antibodies were detected using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories), or biotin-labelled goat anti-mouse IgG1, IgG2a (Southern Biotech) or IgA (Gibco-Invitrogen) followed by streptavidin-AP (Jackson Immuno-Research Laboratories), and developed with 5-nitrophenyl phosphate (Sigma-Aldrich) substrate.

RSV AF-specific IgG avidity was determined by incubating AF protein-coated ELISA plates with serum from different vaccine groups, followed by washing with PBS or different concentrations of urea (6 M, 3 M and 1 M) at 37 °C for 15 min. The subsequent steps were performed in accordance with the standard ELISA protocol described above. The antibody affinity was calculated as a percentage of the titres obtained after urea washes compared to the titres after PBS washes.

**ELISPOT assays.** Isolation of splenocytes and ELISPOT assays were performed as previously described (Garlapati et al., 2012). Briefly, ELISPOT plates (Millipore) were coated overnight with murine IFN-γ- or IL-5-specific monoclonal antibodies (BD Pharmingen). Splenocytes (10^6 cells per well) were stimulated with ΔF protein (2 μg ml⁻¹) or medium only and incubated overnight at 37 °C. Spots were developed using biotinylated IFN-γ- or IL-5-specific polyclonal antibody (BD Biosciences), AP-conjugated streptavidin (Jackson Immuno-Research Laboratories) and BCIP/NBT (Sigma-Aldrich) as the substrate.

**Cytokine multiplex assay.** Lungs from mice were homogenized with 2.4 mm zirconia microbeads in a Mini-Beadbeater (BioSpec Products) in minimum essential medium (MEM) supplemented with 10 mM HEPES, Sigmafast protease inhibitor (Sigma-Aldrich) and antibiotic/antimycotic (Gibco-Invitrogen). The homogenates were centrifuged for 1 min at 10,000 g, and the supernatants were collected and stored at −80 °C. Lung cytokines and chemokines were quantified in the cell-free supernatants of lung homogenates using the electrochemiluminescence (ECL) detection-based Meso-scale discovery (MSD) multiplex platform and Sector Imager 2400 (MSD) according to the manufacturer’s instructions. The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations based on several log calibration curves.

**Virus titration.** On day 4 post-challenge, lungs of euthanized mice were excised, weighed and homogenized in a Mini-Beadbeater™. Homogenates were clarified by centrifugation at 10,000 g for 1 min, and serial dilutions of the supernatants were added to subconfluent Hep-2 cells. The supernatants were removed after 2 h incubation at 37 °C and overlaid with 1.6% low-melting agarose in MEM. The overlay medium was removed after five days, and the cells were fixed and stained with 0.5% Crystal Violet to visualize the plaques. Results are expressed as p.f.u. g⁻¹ lung tissue.

**Statistical analysis.** All data were analysed using GraphPad PRISM version 5 for Windows (GraphPad Software). Differences among all groups were examined using one-way ANOVA, followed by a Newman–Keuls post test. When 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 at P<0.05.
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