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A novel RSV F-Fc fusion protein vaccine reduces lung injury induced by respiratory syncytial virus infection

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ABSTRACT

Respiratory syncytial virus (RSV) infection causes significant disease in the lower respiratory tract of young children, and there is currently no licensed vaccine to prevent RSV infection. The F glycoprotein is considered a major antigenic target for RSV vaccine development. Recent evidence indicates that the pre-fusion F state, compared with the post-fusion F state, is a superior antigen for generation of neutralizing antibodies. In this study, we developed a novel vaccine antigen, RSV glycoprotein F fused with an IgG Fc fragment (F-Fc). The F-Fc fusion protein is predominantly a hexamer and could be recognized by the pre-fusion F-specific monoclonal antibody D25. Intranasal immunization with the F-Fc fusion protein promoted a protective Th1-biased cellular immune response relative to that promoted by immunization with the F protein. This immunization strategy significantly reduced the lung viral load in mice. Furthermore, immunization with F-Fc reduced lung pathology and the production of pro-inflammatory cytokines and chemokines in the lung after RSV infection. These results suggest that the F-Fc protein may be a safe and effective RSV vaccine candidate.

1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of viral bronchiolitis and pneumonia in infants worldwide (Hall et al., 2013). Nearly all children are infected with RSV at some point (Glezen and Denny, 1973), and individuals can be repeatedly re-infected with RSV throughout life. RSV infection can cause severe respiratory tract inflammation in susceptible young children, the elderly and immunosuppressed individuals, resulting in an appreciable global economic burden (Gonzalez et al., 2012; Nair et al., 2010). One of the main target populations of RSV vaccines is younger individuals, and a formalin-inactivated whole-virus vaccine (FI-RSV) given intramuscularly resulted in enhanced respiratory disease (ERD), particularly in the youngest age cohort, immunized between 2 and 7 months of age (Graham et al., 2015). Development of an effective RSV vaccine has faced many obstacles and challenges (Fretzayas and Moustaki, 2010; Roberts et al., 2016; Vekemans et al., 2018). Despite decades of intensive research, there is still no licensed vaccine. To develop safe vaccines and avoid failure such as that of the FI-RSV vaccine, it is critical to understand the mechanisms that led to ERD. Subsequent studies showed that the increased severity of lung disease caused by FI-RSV was due to low antibody avidity and an exaggerated Th2-biased immune response (Delgado et al., 2009; Graham et al., 1993).

Various prototype vaccines have been developed in recent years; 19 vaccine candidates and mAbs for different target populations are in clinical trials. The majority of vaccines (11 of 18) in clinical trials are based on the F glycoprotein (Mazur et al., 2018), including a nanoparticle vaccine which is currently in a phase 3 clinical trial in pregnant women (August et al., 2017; Mazur et al., 2018). Glycoprotein F mediates viral entry and contains important neutralizing epitopes (Graham et al., 2015). A commercially available monoclonal antibody (targeting glycoprotein F), palivizumab, has been used to treat children with RSV infections (Scott and Lamb, 1999). During intracellular maturation, the RSV F precursor (F_0) is cleaved by a furin-like protease, producing disulfide-linked F1 and F2 fragments. At the time of virus-host cell membrane fusion, the F protein rearranges from a pre-fusion conformation to a post-fusion conformation. According to previous research, most neutralization-sensitive sites recognized by the most potent neutralizing antibodies are only present on pre-fusion F (Graham et al., 2015). To induce high titres of neutralizing antibodies, a few pre-fusion F proteins were engineered (Blais et al., 2017; Krarup et al., 2015; McLellan et al., 2013a; Swanson et al., 2014).

In addition to enhancing neutralization activity by a structure-based approach, the RSV vaccines were also designed to improve the Th1-

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biased immune response induced by the F antigen. Studies in animal models suggest that a safe RSV vaccine should combine a high neutralizing-antibody response with a cellular response that is Th1 biased (Christiaansen et al., 2014; Shaw et al., 2013). A previous report also suggests that promoting a balanced, RSV-specific, Th1-biased immune response could clear viral infection without excessive or damaging inflammation of the infected tissues (Bueno et al., 2008). The chimeric vaccine rBCG-N-hRSV (recombinant BCG strains expressing RSV N protein), which protected mice from RSV infection through promotion of a Th1-biased type immune response, is in a clinical trial (Cautivo et al., 2010; Mazur et al., 2018). Live-attenuated vaccines against RSV induce a Th1-biased response, and clinical study results show that these candidates do not prime for ERD following subsequent exposure to wildtype RSV after vaccination (Karron et al., 2015; Mazur et al., 2018; Wright et al., 2007).

A novel mucosal vaccination strategy is the use of an IgG Fc fragment fused to an antigen (Ye et al., 2011). This vaccine strategy depends on enhancing or inhibiting interactions with a narrow subset of Fc receptors (FcRs) (Czajkowsky et al., 2012). In recent years, this vaccine strategy has been used to produce vaccine candidates against infectious agents such as influenza (HA-HuFc) (Loureiro et al., 2011), herpes simplex virus (HSV) (gD-Fc) (Ye et al., 2011), Ebola (ZEBOVGP-Fc) (Konduru et al., 2011), human immunodeficiency virus (HIV) (Gag-Fc) (Lu et al., 2011), and tuberculosis (ESAT6:HspX:Fc) (Soleimanpour et al., 2015). In particular, the HIV gp120-Fc fusion protein vaccine showed a notable antibody response in rhesus macaques (Shubin et al., 2017). These Fc fusion protein vaccines increased Th1-biased immune responses and neutralizing antibody titres, which are crucial for a successful RSV vaccine. Therefore, we decided to apply this promising mucosal vaccination strategy to RSV vaccine design.

We constructed an F-Fc fusion protein and found that though the primary sequence of F in F-Fc is the same as that of post-fusion F, the F-Fc fusion protein can be recognized by the conformation-specific antibody D25 (McLellan et al., 2013b). This finding suggests that the F-Fc fusion protein may be a novel antigen for an RSV subunit vaccine.

The TLR4 agonist monophosphoryl lipid A (MPL) has been found to be a safe and effective adjuvant for mucosal RSV vaccine application in cotton rats (Blanco et al., 2014), and intranasal delivery is the most effective route for inducing potent and broad mucosal immune responses at multiple mucosal sites compared with those resulting from delivery via other mucosal delivery routes (Yang and Varga, 2014). Therefore, in this study, we intranasally (i.n.) immunized mice with an F-Fc fusion protein combined with the adjuvant MPL.

In our study, compared with that of post-fusion F vaccination, the F-Fc vaccine strategy could further benefit RSV-specific neutralizing antibody generation and improve protective Th1-biased cellular immune responses. These immune responses conferred resistance to viral replication, reduced lung pathology and effectively reduced pro-inflammatory cytokines and chemokines in the lung after RSV challenge. In summary, our study suggests that the F-Fc fusion protein could be a safe and protective vaccine candidate for reducing lung injury caused by RSV infection.

2. Materials and methods

2.1. Cells and virus

FreeStyle™ 293-F cells were purchased from Invitrogen (R790-07). The HEp-2 cell line and RSV A2 strain were gifts from Dr. Zishu Pan (Wuhan University). RSV A2 was prepared and maintained by ultracentrifugation as previously described (Krzyzaniak et al., 2013; McGinnes et al., 2011).

2.2. Vaccine formulations

The cDNA encoding the extracellular domain of RSV A2 glycoprotein F (26–513 aa, with a deletion of the fusion peptide residues 137 to 146) was amplified by PCR from plasmid pLEXm-RSVFΔFP gifted by Dr. Peter D. Kwong (Vaccine Research Center, NIAID/NIH) (McLellan et al., 2011). Mouse IgG2a was used instead of IgG1, because mouse IgG2a is capable of binding mouse FcγRI, a high-affinity IgG receptor. Oligonucleotide site-directed mutagenesis was used to replace the C1q-binding residues Glu318, Lys320, and Lys322 with Ala residues to construct a nonlytic Fc fragment (Ye et al., 2011). Between F and the Fc fragment, we used the peptide sequence GSSGGGSSGGSSS as a linker. These DNA fragments were ligated into an engineered pCDNA3.1 vector carrying a CD5 protein secretion signal sequence (Lu et al., 2011). Plasmids containing the chimeric F-Fc protein were transfected into FreeStyle™ 293-F cells with polyethylenimine (PEI, the ratio of the amount of plasmid DNA to the amount of PEI was 1:4.), and after 7 days, proteins were collected from cell supernatants and purified by protein G (GE). The F-Fc protein was eluted with 0.1 M glycine buffer pH 2.7. The main products were eluted at pH 4.5 (detected by pH sensor), and immediately neutralized with Tris-HCl pH 8.8. To generate a soluble post-fusion F protein, we also amplified a fragment from the pLEXm-RSVFΔFP (1–513 aa, with a deletion of the fusion peptide residues 137 to 146) and inserted it into the pSecTag2A vector. The pre-fusion-stabilized RSV F protein was assembled by incorporating the amino acid changes described by McLellan et al. and contains the foldon oligomerization domain (GSGYIPEAPRDGQAYVRKDGEWVLLSTFL) used by McLellan et al. (2013a). Post-fusion and pre-fusion F proteins were expressed in the same way as the F-Fc protein and purified by Ni^{2+}-nitrilotriacetic acid (NTA) resin, as previously described (McLellan et al., 2011, 2013a). FI-RSV vaccine was prepared as previous reported (Olszewska et al., 2004). Briefly, RSV A2 was grown in HEp-2 cells. Infected cells were incubated for 4 days at 37 °C, and then cells were harvested by three freeze-thaw cycles. The resulting cell lysates were clarified by centrifugation for 10 min at 1000 rpm, and the supernatant was collected. Formalin was added (final concentration 1:4000) for 72 h at 37 °C. The pre-cleared cell supernatant was centrifuged at 50000 × *g* (SW32 Ti rotor, Beckman Optima90-K ultracentrifuge) for 1 h at 4 °C. Pellets were gently washed and reconstituted in PBS, and further four-fold concentration was achieved after 30 min of precipitation on alum (4 mg/ml), followed by centrifugation for 30 min at 1000 × *g*.

Monophosphoryl lipid A (MPL) was obtained from Sigma (L6895). A 2 mg/ml stock was prepared in saline/0.2% triethylamine, and heated in a 65 °C water bath for 5 min, as previously described (Baldridge and Crane, 1999; Blanco et al., 2014).

2.3. Mouse vaccination and challenge protocols

Specific pathogen-free (SPF) female BALB/c mice at the age of 6–8 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were housed in an SPF environment under standard conditions. All mouse experiments were approved by the ethics committee of the Wuhan Institute of Virology, Chinese Academy of Science (permit number WIVA25201407). Six-to-eight-week-old female BALB/c mice (6 animals per group) were anaesthetized with pentobarbital sodium (1 mg/20 g) and immunized intranasally (i.n.) with 0.187 nmol protein (10 μg of glycoprotein F (post-fusion or pre-fusion); 15.15 μg of F-Fc, the molecular weight of F-Fc was calculated by the ExPASy ProtParam tool.) in combination with 5 μg of MPL (Sigma) (per mouse) at weeks 0 and 2. Mice in the control group were immunized with 20 μl of phosphate-buffered saline (PBS) in combination with 5 μg (per mouse) of MPL. For immunization with FI-RSV, mice were intramuscularly (i.m.) immunized with 100 μl of FI-RSV (per mouse) at weeks 0 and 2. Twenty-eight days after the first vaccination, mice were challenged i.n. with 6×10^6 pfu of the RSV A2 strain.

Method details for size exclusion chromatography, binding assay, competition ELISA, determination of antibody titres by ELISA, antibody

neutralization assay, cytokine secretion by RSV-specific T cells, intracellular cytokine staining, lung virus and inflammation cytokines and chemokine measurements, histology, and antigen uptake and presentation assay can be found in the supplementary material methods section.

2.4. Statistics

Data were analysed with GraphPad 5 software using one-way ANOVA (analysis of variance) and Student's *t*-test.

3. Results

3.1. Design and characterization of soluble post-fusion F, pre-fusion F, and F-Fc fusion proteins in this study

The soluble RSV post-fusion F protein spanned amino acids 26–513 of the protein ectodomain with a deletion of fusion peptide residues 137 to 146. Pre-fusion F protein was obtained by introducing stabilising mutations and insertion of a foldon trimerization domain at the C-terminus of the RSV A2 F protein ectodomain (1–513), as previous described (McLellan et al., 2013a). The F-Fc fusion protein was generated by cloning glycoprotein F ectodomain 26-513 without the fusion peptide in frame with the mouse IgG2a Fc fragment (the complement C1q-binding motif was removed from mouse IgG2a to produce a nonlytic fusion protein (Ye et al., 2011)) (Fig. 1A). Purified post-fusion F, pre-fusion F, F-Fc proteins were characterized by SDS-PAGE (Fig. 1B). Post-fusion and pre-fusion F proteins were cleaved, producing F1 and F2 fragments, as previously reported (McLellan et al., 2011). The F-Fc fusion protein was also cleaved at the same site, producing F1-Fc and F2 fragments. To clarify the oligomerization states of these proteins, gel filtration chromatography with standard protein markers was conducted. As judged by molecular weight markers, the majority of post-fusion and pre-fusion F proteins eluted from the gel filtration column as a trimer. However, with the addition of a dimer Fc domain at the C-terminus of the F protein, the F-Fc fusion protein eluted from the gel filtration column as a hexamer (Fig. 1C).

3.2. The F-Fc fusion protein could be recognized by the pre-fusion-specific monoclonal antibody D25

To determine the antigenic properties of the post-fusion F protein, pre-fusion F protein, and F-Fc fusion protein, they were tested in an ELISA for reactivity with conformation specific antibodies (Abs). D25 is described as an Ab that recognizes an epitope of antigenic site Ø, which is specific for the pre-fusion F protein (McLellan et al., 2013b). The Ab palivizumab recognizes an epitope of antigenic site II shared by pre-fusion and post-fusion F protein (Palomo et al., 2016). D25 reacts efficiently with the F-Fc fusion protein at low dose (10 ng/well) but reacts much more weakly with the post-fusion F protein at same dose (Fig. 2A). The post-fusion F and F-Fc fusion proteins showed similar patterns of reactivity with palivizumab. The reactivity of palivizumab to the F-Fc fusion protein was approximately 2.2-fold higher than that to post-fusion F protein (Fig. 2B). The binding of D25 to the post-fusion F, pre-fusion F, and F-Fc fusion proteins was further measured with an Octet instrument (Fig. 2C, D and E). The K_D(M) for the post-fusion F protein -D25 Ab interaction was 4.05×10^{-7}. The K_D(M) for the pre-fusion F protein -D25 Ab interaction was 1.89×10^{-9}. The K_D(M) for the F-Fc fusion protein -D25 Ab interaction was 8.69×10^{-9}, which is lower than that for the post-fusion F protein-D25 Ab interaction but higher than that for the pre-fusion F protein-D25 Ab interaction. These results suggested that the F-Fc fusion protein could bind D25 with high affinity and maybe contains the pre-fusion conformation.

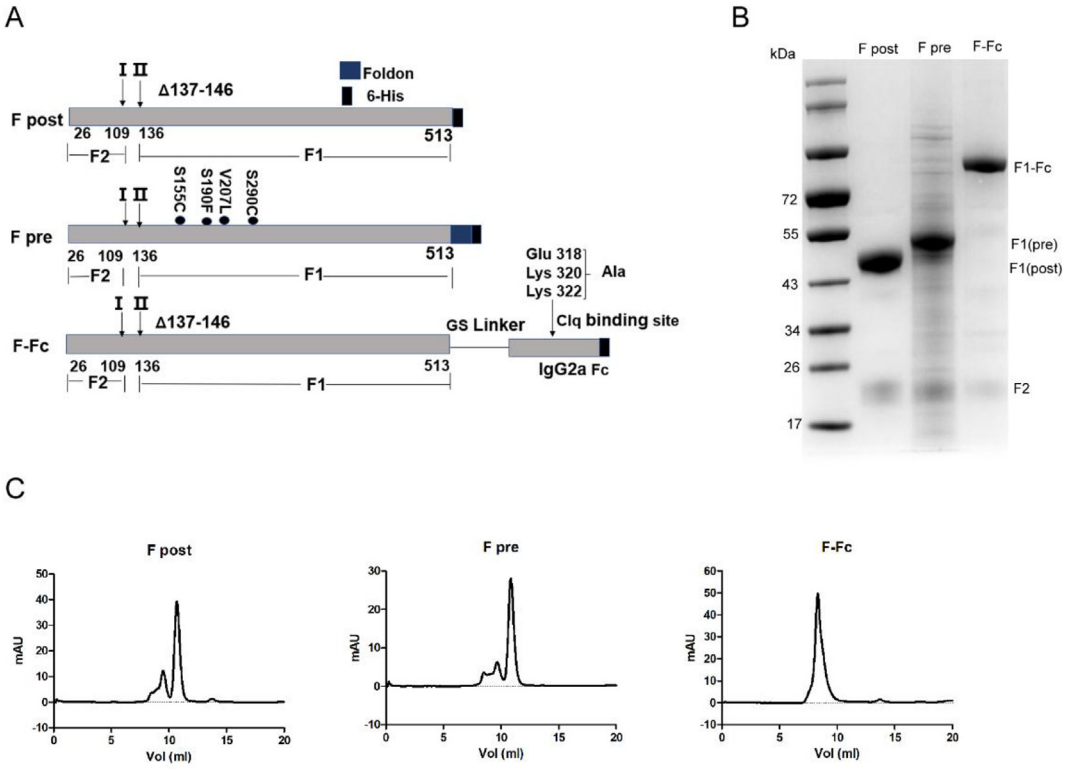
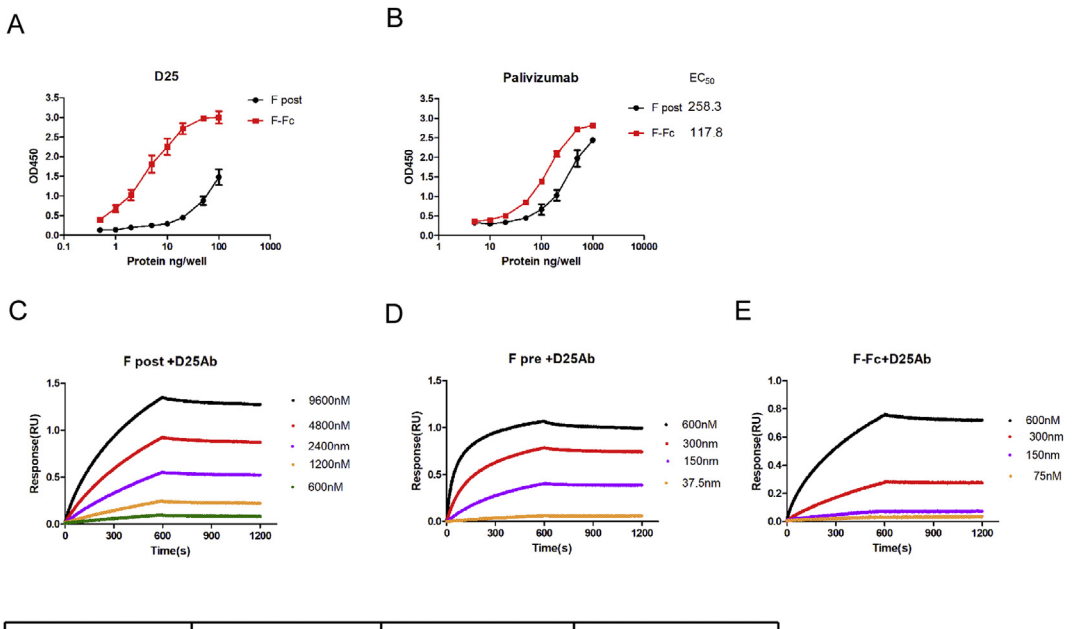


Fig. 1. Design and characterization of the post-fusion F, pre-fusion F, and F-Fc fusion proteins (A) Domain structure of the post-fusion F, pre-fusion F, and F-Fc proteins. The mouse IgG2a Fc fragment contains hinge, CH2, and CH3 domains. To produce a nonlytic fusion protein, the complement C1q-binding residues Glu318, Lys320, and Lys322 were replaced with Ala. The post-fusion F, pre-fusion F, and F-Fc proteins contain a His Tag at the C-terminus. (B) Analysis of the purified post-fusion F, pre-fusion F, F-Fc proteins by SDS-PAGE/Coomassie under reducing conditions. (C) Gel filtration chromatograms of the post-fusion F, pre-fusion F, and F-Fc proteins, with bovine serum albumin (BSA, 68 kDa) and ferritin (440 kDa) as protein markers (Fig. S1).



Binding pair	kon(1/Ms)	kids(1/s)	K_D(M)
F post+D25Ab	2.39E+02	9.69E-05	4.05E-07
F pre+D25Ab	1.93E+04	3.65E-05	1.89E-09
F-Fc+D25Ab	5.37E+03	4.67E-05	8.69E-09

Fig. 2. Antigenic properties of the post-fusion F, pre-fusion F, and F-Fc fusion proteins. (A–B) ELISA of the post-fusion F, and F-Fc proteins with conformation specific monoclonal antibodies. (C–E) Binding kinetics of the post-fusion F, pre-fusion F, and F-Fc proteins to the D25 antibody detected with Fortebio Octet RED. The D25 antibody was immobilized with 50 μg/ml. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. F-Fc immunization induced an effective antibody response in mice

To determine whether the F-Fc protein efficiently elicits antibodies specific to RSV glycoprotein F, mice were intranasally immunized with F-Fc, post-fusion F, pre-fusion F or PBS as a control in combination with the adjuvant MPL and boosted 2 weeks later. Antibody responses were assessed in the serum of immunized animals, including PBS control mice, 14 days after the boosting immunization. RSV infection can cause severe inflammation of the respiratory tract and is a problem especially in the bronchioles. Local antibody responses in the lungs also play a key role in protecting against RSV infection (Garg et al., 2016). We also detected glycoprotein F-specific IgG and IgA in lung homogenates. The levels of post-fusion and pre-fusion F-specific IgG and IgA antibodies in serum and lung homogenates were significantly higher in mice immunized with F-Fc than in mice immunized with post-fusion F ($p < 0.001$) (Fig. 3A–D). The levels of pre-fusion F-specific IgG and IgA antibodies in serum were significantly higher in mice immunized with pre-fusion F than in mice immunized with F-Fc ($p < 0.001$) (Fig. 3 A and B). However, the levels of pre-fusion F-specific IgG and IgA antibodies in lung homogenates were significantly higher in mice immunized with F-Fc than in mice immunized with pre-fusion F ($p < 0.001$) (Fig. 3 C and D). As stated before, the binding of the D25Ab to F-Fc is stronger than that to the post-fusion F protein but weaker than that to the pre-fusion F protein (Fig. 2). Consistent with this result, the neutralization assay indicated that serum from F-Fc-immunized mice exhibited higher neutralizing activity than the serum from post-fusion F-immunized mice (2–3-fold) but lower activity than the serum from pre-fusion F immunized mice (2–3-fold) (Fig. 3E). A competition ELISA demonstrated that pooled sera of mice immunized with the pre-fusion F and F-Fc antigens (but not sera of post-fusion F-immunized mice) inhibit D25 binding (Fig. 3F).

3.4. F-Fc immunization improves the Th1-biased cellular immune response and promotes the production of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells in the spleen

To determine whether adaptive cellular immunity is enhanced by F-Fc immunization, we evaluated cytokine secretion by T cells obtained from the spleen. Spleens were harvested from immunized mice on day 14 after boosting and stimulated with purified RSV glycoprotein F or medium alone, and cytokine secretion was evaluated in the supernatant. Cytokine responses were mainly determined by assessing IFN-γ, IL-2, IL-10, and IL-4 levels. Both IFN-γ and IL-2 are important Th1-type cytokines, and IL-10 is an immunomodulator that plays a crucial role in controlling disease severity in RSV infection, as previously reported (Loebbermann et al., 2012). The ratio of IgG2a to IgG1 during infection or immunization has been used to judge the Th bias of the immune response (Markine-Goriaynoff and Coutelier, 2002). Therefore, we also examined this ratio in post-fusion F-, pre-fusion F- and F-Fc-immunized mice to evaluate the bias of the T cell immune response elicited. Compared with the samples from mice immunized with post-fusion F and pre-fusion F, cell suspensions obtained from mice vaccinated with F-Fc secreted higher levels of the Th1-type cytokines IFN-γ and IL-2 and of IL-10 ($p < 0.001$) (Fig. 4A, B and D). Low levels of the Th2-type cytokine IL-4 (under 10 pg/ml) were observed in cultures of spleen cells from the three groups of mice (Fig. 4C). Consistent with these results,

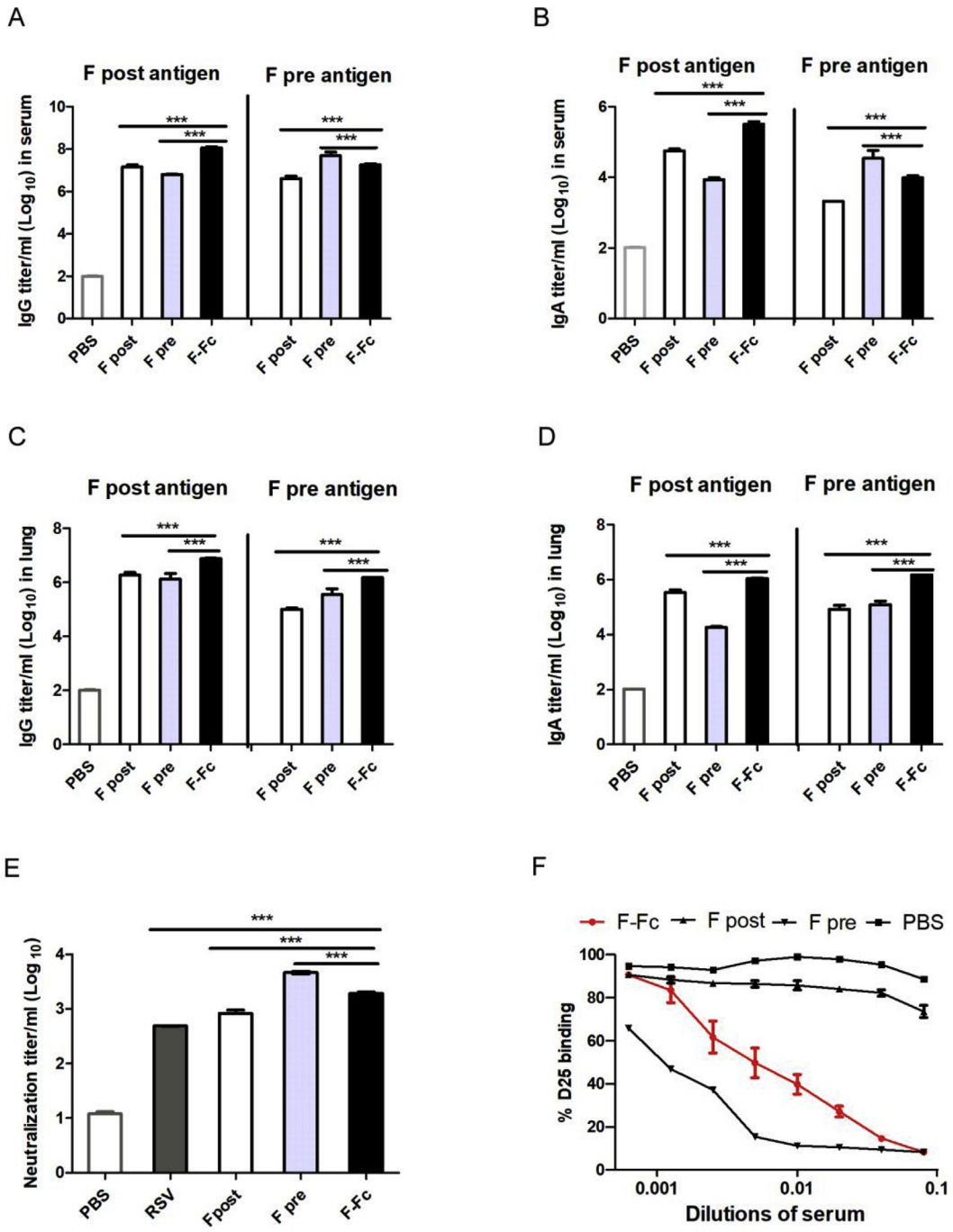


Fig. 3. Antibody responses to the post-fusion F, pre-fusion F, and F-Fc fusion proteins in this study. Serum and lung tissues were harvested 14 days after the booster immunization and pooled. (A–B) Measurement of glycoprotein F-specific IgG and IgA antibodies in serum by ELISA. Purified post-fusion F and pre-fusion F were the target antigens. The data are shown as the mean ± SD of n = 6 mice per group (n = 5 mice for the pre-fusion F group), and three independent experiments were analysed. The data were analysed by one-way analysis of variance and the Bonferroni multiple comparison test. ***P < 0.001. (C–D) Glycoprotein F-specific IgG and IgA antibodies in lung homogenates were measured by ELISA. Purified post-fusion F and pre-fusion F were the target antigens. The data are shown as the mean ± SD of n = 6 mice per group (n = 5 mice for the pre-fusion F group), and three independent experiments were analysed. The data were analysed by one-way analysis of variance and the Bonferroni multiple comparison test. ***P < 0.001. (E) Serum from mice immunized with the F-Fc fusion protein showed enhanced titres of neutralizing antibodies. To obtain a positive serum for the neutralization assay, mice were anaesthetized with pentobarbital sodium (1 mg/20 g) and then infected by intranasal (i.n.) inoculation of RSV (6×10^5 pfu/mouse in 50 μl) at weeks 0 and 2. Serum was harvested 14 days after the booster infection. Neutralizing antibody titres, defined as the dilution resulting in a 60% reduction in virus titres, were determined as described in the Materials and Methods. The data are shown as the mean ± SD of n = 6 mice per group (n = 5 mice for the pre-fusion F group), and three independent experiments were analysed. The data were analysed by one-way analysis of variance and the Bonferroni multiple comparison test. ***P < 0.001. (F) Inhibition of D25 binding to the F-Fc fusion protein, pre-fusion F protein, and post-fusion F protein by pooled sera from mice immunized with the F-Fc antigen, pre-fusion F antigen, or post-fusion F antigen, or mice immunized with PBS. For analysis, 100% D25 binding was defined as the binding level in the absence of competing sera.

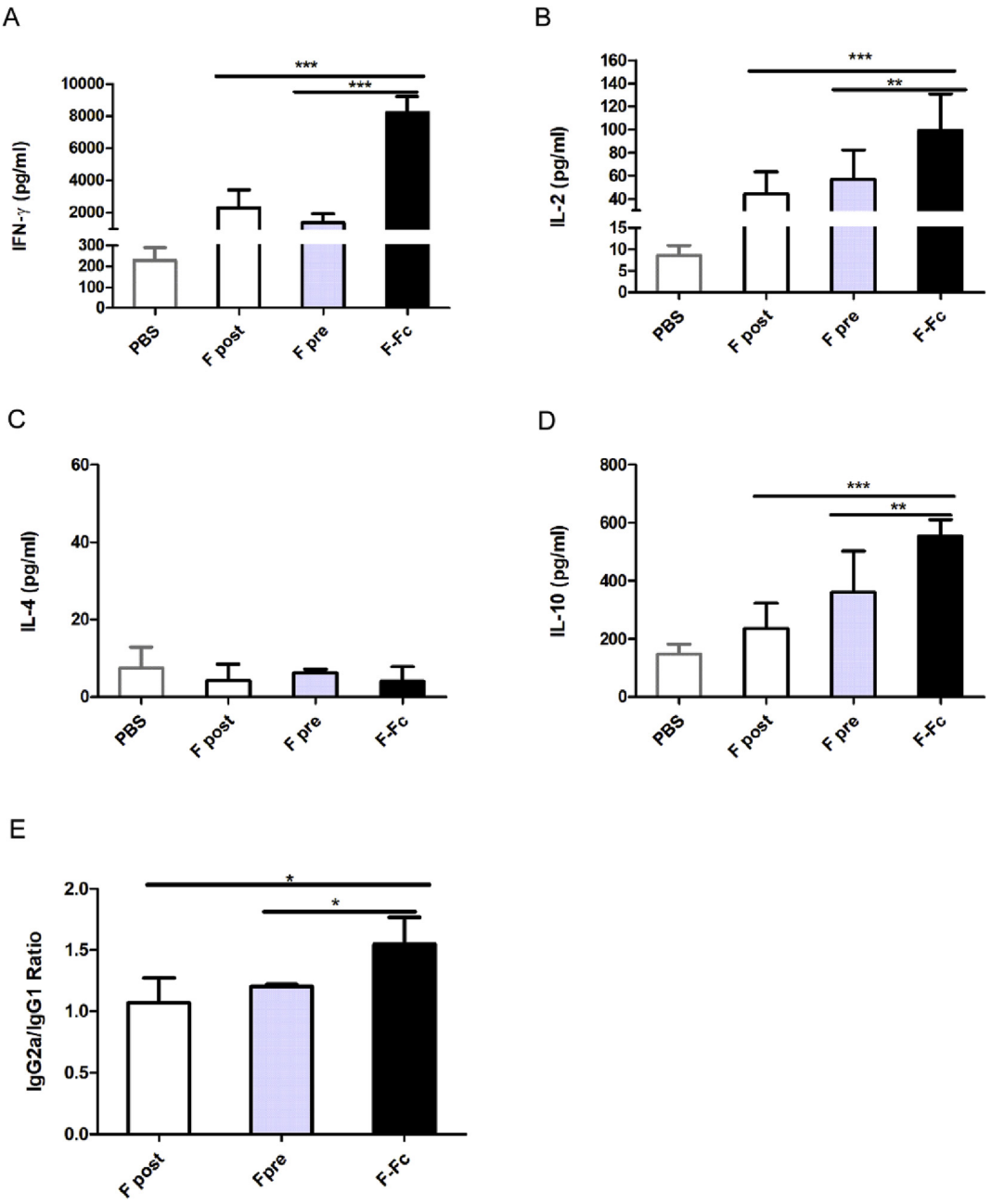


Fig. 4. Immunization with the F-Fc protein improves the production of Th1-type cytokines and the cytokine IL-10 and increases the ratio of glycoprotein F-specific IgG2a to IgG1 antibodies. (A) Spleens were collected from immunized mice 14 days after booster immunization (n = 6; n = 5 mice for the pre-fusion F group). A total of 5×10^5 cells was stimulated *in vitro* with purified glycoprotein F (20 μg/ml) for 24 h. The cytokines (A–D) IFN-γ, IL-2, IL-10 and IL-4 in the culture supernatant were detected by ELISA. The detection limits of these cytokines were 15.6, 3.1, 7.8 and 3.1 pg/ml, respectively. The data represent the means ± SD. The data were analysed by one-way ANOVA (analysis of variance). The data were analysed for two independent vaccination experiments. (E) The ratios of F-specific IgG2a/IgG1 in the serum of mice immunized with the post-fusion F, pre-fusion F, and F-Fc fusion proteins were measured by ELISA. The data represent the means ± SD. The data are analysed for three independent experiments. The data were analysed by one-way ANOVA (analysis of variance). *P < 0.05, **P < 0.01, ***P < 0.001.

immunization with the F-Fc protein improved the glycoprotein F-specific IgG2a/IgG1 antibody ratio (Fig. 4E). Taken together, these results demonstrate that F-Fc immunization improves the Th1-biased cellular immune response compared with that of soluble F protein vaccination.

To evaluate T cell activation, we measured the amount of IFN-γ-producing T cells by flow cytometry. IFN-γ produced by RSV-specific $CD8^+$ T cells is critical for virus elimination. We detected increased numbers of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in response to the F protein in mice immunized with the F-Fc protein (approximately 2–3-fold higher than those in the post-fusion F- and pre-fusion F- vaccinated groups) (Fig. 5). We conclude that F-Fc immunization induces a more robust RSV-specific T cell immune response with a Th1-like pattern of cytokine secretion.

3.5. F-Fc vaccination reduces RSV replication and associated morbidity in mice

To determine whether F-Fc vaccination could protect mice from RSV infection, all mice were challenged with 6×10^6 pfu of the RSV A2 strain 14 days after the boosting immunization. Four days after infection, lung tissue was harvested, and plaque assays were used to detect the viral load in the supernatant of lung homogenates (Fig. 6A). Control

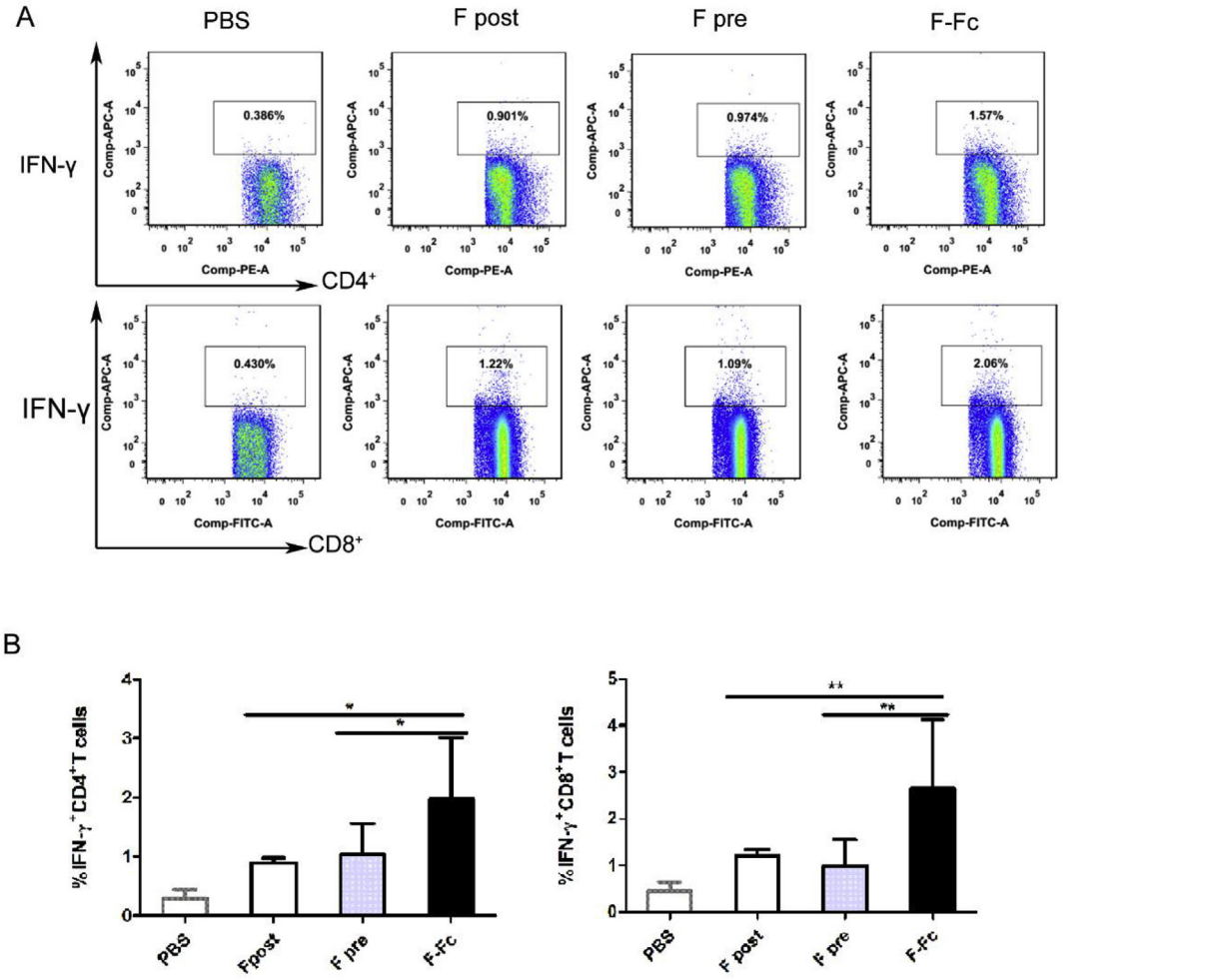


Fig. 5. Immunization with the F-Fc protein increases the percentages of IFN-γ-producing T cells in the spleen. Spleen cells were harvested from immunized mice 14 days after the boosting immunization and stimulated for 10 h with purified glycoprotein F or culture medium as a control. Lymphocytes were gated by forward and side scatter and T cells were labeled with anti-CD3 antibodies and identified by their respective surface markers CD4 and CD8, and intracellular IFN-γ staining. Immunization conditions are displayed at the bottom. (A) The cells in the rectangle insert are representative dot plots for $CD4^+$/IFN-γ^+, $CD8^+$/IFN-γ^+ T cells, and (B) the graph summarizes the percentages of $CD4^+$/IFN-γ^+, $CD8^+$/IFN-γ^+ T cells. The graphs are representative of two independent vaccination experiments (n = 6, n = 5 mice for the pre-fusion F group). The data represent the means ± SD. The data were analysed by one-way ANOVA (analysis of variance) and the Bonferroni multiple comparison test. **P < 0.01.

(PBS-vaccinated) mice had sizeable amounts of RSV in their lungs approaching a level of 10^5 pfu. Most of the mice (4/6) immunized with post-fusion F were totally protected from RSV infection, with no detectable virus in their lungs; however, some of the mice (2/6) still exhibited a level of approximately 10^2 pfu of virus in their lungs. The post-fusion F protein immunization could significantly reduce viral load after RSV infection compared with that of PBS immunization ($P < 0.001$), which is consistent with a previous report (Palomo et al., 2016). In the pre-fusion F and F-Fc immunization groups, none of the mice had detectable virus in their lungs, based on plaque assays. However, in general, the viral load in mice immunized with pre-fusion F or the F-Fc protein was not significantly different from that in post-fusion F controls.

All mice started to lose weight after RSV challenge. From day four after infection onwards, mice immunized with the post-fusion F, pre-fusion F, and F-Fc fusion proteins started to recover, whereas mice treated with PBS continued to lose weight (Fig. 6B and C). These results indicate that immunizations with post-fusion F, pre-fusion F, and F-Fc were protective upon RSV challenge.

3.6. F-Fc immunization reduces lung pathology and pro-inflammatory cytokines and chemokines in homogenized lung tissues after RSV infection

A challenge in the development of RSV vaccine candidates is the safety aspect of ERD after RSV infection (Kapikian et al., 1969; Kim et al., 1969); we also evaluated the lung pathology of the challenged mice by histochemical analysis. Seven days after challenge, lung tissues were removed and stained with haematoxylin/eosin (H&E) and periodic acid-Schiff (PAS) to evaluate the lung histopathology. Positive controls for ERD were mice immunized i.m. and boosted with FI-RSV. After RSV infection, mice immunized with PBS and FI-RSV displayed the severe inflammation with alveolar inflammatory patches. PAS-positive mucus production was only observed in FI-RSV-immunized mice. By contrast, we observed only slight lung infiltration in pre-fusion F- and F-Fc-immunized mice. Mice immunized with post-fusion F showed moderate lung infiltration compared with that of pre-fusion F- and F-Fc-immunized mice (Fig. 7). This result demonstrates that vaccination with the F-Fc protein induces protective immunity in mice, which reduces lung injury after RSV infection.

As a measure of disease severity in mice following RSV challenge, cytokines (IFN-γ, IL-6, IL-4, and IL-10) and chemokines (MIP-1α) correlated with disease severity were examined in homogenized lung

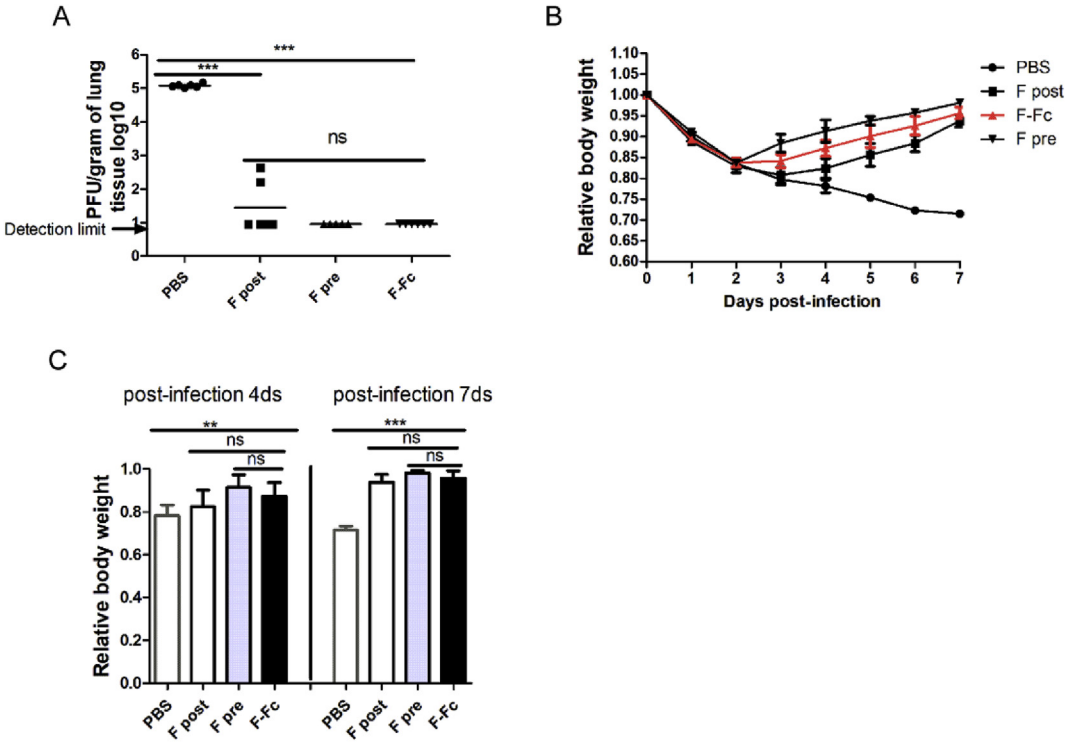


Fig. 6. Immunization with the F-Fc protein reduces the presence of virus in lung tissues and body weight loss after RSV infection. (A) Four days after infection, lungs were removed. The virus titres in the supernatant of homogenates from lung tissues were determined by a plaque assay. Each data point represents a single animal, with the value calculated as an average of triplicate measurements. The bar for each cohort represents the group mean (n = 6; n = 5 mice for the pre-fusion F group). The data were analysed by one-way ANOVA (analysis of variance) and the Bonferroni multiple comparison test. ***P < 0.001, ns represents not significant. (B) Fourteen days after the booster immunization, mice were infected with 6×10^6 pfu of RSV, and body weight was determined daily for 7 days after infection. The graph represents the average relative (compared with day 0) body weight ± SD of all mice in each group. (C) The data for body weight for 4 and 7 days after infection were analysed by one-way ANOVA (analysis of variance) and the Bonferroni multiple comparison test. ***P < 0.001, **P < 0.01, ns represents not significant.

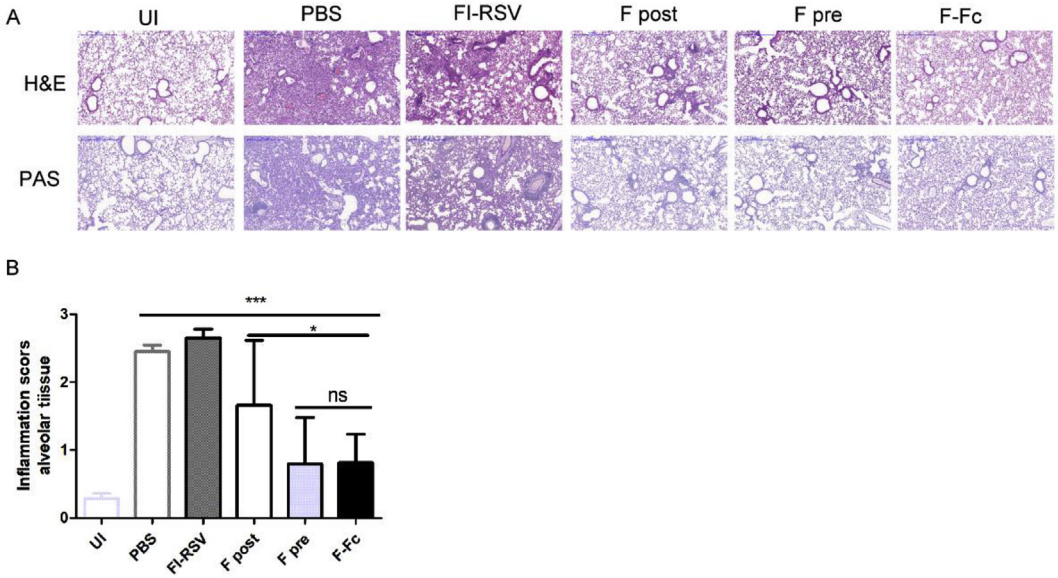


Fig. 7. Immunization with the F-Fc protein reduces lung injury. (A) Seven days after RSV challenge, lungs were removed and fixed with 4% paraformaldehyde and stained with periodic acid-Schiff (PAS) and haematoxylin/eosin (H&E) to assess pulmonary histopathology (n = 5 per group, except the FI-RSV and uninfected groups, in which n = 3). Scale bars represent 500 μm. UI, uninfected. (B) Scoring of pulmonary inflammation after RSV challenge of immunized mice. Scores ranged from 0 (normal) to 4 (severe). The degree of inflammation in the alveolar tissue was graded as follows: 0, normal; 1, increased thickness of the interalveolar septa (IAS) by oedema and cell infiltration; 2, increased thickness of the IAS with the presence of luminal cell infiltration; 3, abundant luminal cell infiltration; and 4, inflammatory patches formed. The data were analysed by one-way ANOVA (analysis of variance) and the Bonferroni multiple comparison test. ***P < 0.001, *P < 0.05, ns represents not significant. The data are representative of two independent vaccination experiments.

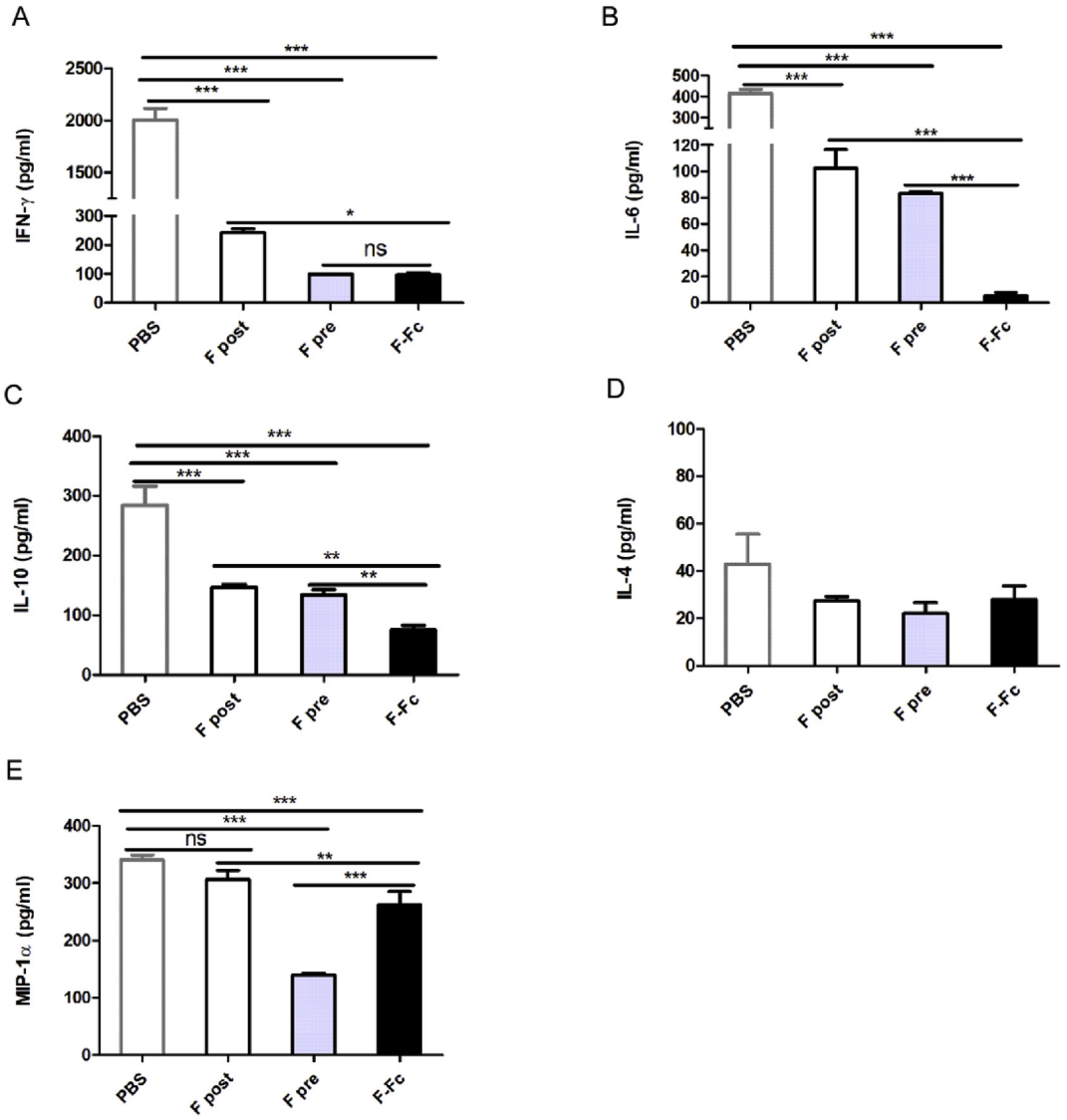


Fig. 8. Immunization with the F-Fc protein reduces the production of cytokines and chemokines in lung tissues after RSV infection. (A–E) Seven days after RSV challenge, lungs were removed, and the lung homogenates were evaluated by ELISA to measure the levels of the cytokines IFN-γ, IL-6, IL-10, IL-4, and MCP-1α. The detection limits of these cytokines and chemokines were 15.6, 7.81, 7.8, 3.9, and 15.63 pg/ml, respectively. Each bar represents a pool of five mice from the same treatment group. The data represent the means ± SD. The data were analysed by one-way ANOVA (analysis of variance) and the Bonferroni multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, ns represents not significant.

tissues at day 7 after RSV challenge (Barends et al., 2004; Blanco et al., 2002). Although concentrations of IL-6 and IL-10 in mice immunized with the post-fusion F, pre-fusion F or F-Fc proteins were all significantly lower compared to those of the PBS control, the F-Fc fusion protein-immunized group had lower levels relative to those of post-fusion F- and pre-fusion F-immunized mice (Fig. 8 B and C). In particular, the concentrations of IL-6 in mice immunized with F-Fc antigen were under the limit of detection (Fig. 8B). Concentrations of IFN-γ in mice immunized with F-Fc and pre-fusion F were similar and were lower than those in mice immunized with post-fusion F (Fig. 8A). Concentrations of MIP-1α in mice immunized with F-Fc were higher than those in the pre-fusion F-immunized group but lower than those in the PBS group and post-fusion F group, whereas no significant differences were observed in mice immunized with the post-fusion F protein compared with those in the PBS control group (Fig. 8E). Concentrations of IL-4 were low in mice of all groups (under 50 pg/ml), and no significant differences was observed among mice from each group (Fig. 8D). These data demonstrate that F-Fc immunization efficiently reduced the level of virus-related inflammation *in vivo*.

4. Discussion

Recently, various types of RSV vaccines based on glycoprotein F have been shown to induce effective neutralizing antibodies, including purified protein vaccines (Capella et al., 2017; McLellan et al., 2013a; Swanson et al., 2011), virus-like particles (Blanco et al., 2018; McGinnes Cullen et al., 2015), nanoparticles (August et al., 2017; Francica et al., 2016), viral vector vaccines (Green et al., 2015; Phan et al., 2017), live attenuated vaccines (Karron et al., 2013, 2015; Wright et al., 2007) and so on. These vaccines contain the pre-fusion F conformation or post-fusion F conformation. Recent evidence indicates that compared to the post-F state, the pre-fusion F state is a superior target for neutralizing antibodies. The conformation change from pre-fusion F to the post-fusion F conformation occurs spontaneously. It is difficult to maintain a stabilized pre-fusion F conformation. A few constructs preserve the pre-fusion F-specific epitopes by stabilising mutations. In this study, we found that pre-fusion F-specific epitopes were also preserved by the addition of an Fc domain at the C-terminus of the F protein (the F-Fc fusion protein can be recognized by the conformation specific

antibody D25). Since Fc fusion could improve the stability of the fusion proteins (Levin et al., 2015), this funding suggest that the stabilized F-Fc also helped the presentation of antigen site Ø.

The F-Fc fusion protein binds to the D25 antibody with high affinity compared with that of post-fusion F (F-Fc K_D(M): 8.69×10^{-9}; post-fusion F K_D(M): 4.05×10^{-7}), which indicates that antigen site Ø is preserved on F-Fc. Antigen site Ø is considered the outstanding target for a potent neutralizing antibody (Graham et al., 2015). However, this affinity is weaker than that of the interaction of the pre-fusion F protein-D25 Ab (K_D(M): 1.89×10^{-9}). In our study, the titres of neutralizing antibodies in mice immunized with the pre-fusion F protein, were 5–6-fold higher than those elicited by post-fusion F alone, which is comparable with previous work using pre-fusion F immunization (Krarup et al., 2015; Palomo et al., 2016). The titres of neutralizing antibodies in mice immunized with the F-Fc fusion protein were 2–3-fold higher than those in mice immunized with the post-fusion F protein. These results suggested that the F-Fc fusion protein may contain mixed forms of confirmations of F. Post-fusion F, which has the advantage of being highly stable, could also induce sizeable titres of neutralizing antibodies and reduce the viral load after RSV infection, as previously reported (McLellan et al., 2011; Palomo et al., 2016). However, with the addition of the Fc domain, F-Fc immunization combined the enhanced antibody response with promotion of a Th1-biased cellular immune response. Therefore, on aggregate, the lung injury was the least in mice immunized with F-Fc compared with that of the post-fusion F and PBS groups. Pre-fusion F immunization induced the highest levels of neutralizing antibodies, the lung injury after RSV challenge was also slight, and no significant differences in pulmonary inflammation were observed between mice immunized with pre-fusion F and mice in the F-Fc-immunized group. Compared with pre-fusion F immunization, F-Fc immunization promoted Th1-biased cellular immune responses and induced increased pre-fusion F-specific antibodies in the lung. RSV infection caused enhanced expression of cytokines and chemokines in lung. These processes result in viral clearance, and at the same time, elicit histologic inflammation in the lung (Blanco et al., 2002). Immunization with pre-fusion F and F-Fc fusion proteins significantly reduced the viral load in the lung after RSV challenge. Therefore, the cytokine and chemokine levels were low after RSV challenge in the lungs of mice immunized with the pre-fusion F and F-Fc fusion proteins. Mice immunized with the F-Fc fusion protein induced higher titres of pre-fusion F-specific antibodies in the lung than those in the lungs of mice immunized with the pre-fusion F protein. Therefore, the concentrations of IL-6 and IL-10 in mice immunized with F-Fc were lower than those in mice in the pre-fusion F-immunized group. The concentrations of MIP-1α in mice immunized with pre-fusion F were lower than those in mice in the F-Fc-immunized group, whereas the mechanism is unclear.

Most antigen-presenting cells (APCs) express Fc-gamma receptors (FcγRs) on their surface; thus, it is expected that the optimal binding of Fc-fusion proteins to one or more of these receptors will enhance uptake of antigen by APCs (Czajkowsky et al., 2012). APCs take up antigen and then process and present or cross-present them to T cells. This process is crucial to the adaptive immune response. The uptake and presentation of antigens by bone marrow-derived dendritic cells (BMDCs) *in vitro* indicated that the F-Fc protein is more efficiently taken up by APCs and presented to T cells than F protein (Fig. S2A). Meanwhile, the use of an Fc fusion antigen as a vaccine is complicated by the ability of the Fc domain itself to bind to FcRs (Fc receptors), as this would cause unwanted positive binding signals or biological effects. Therefore, the interactions of Fc-FcRs need to be critically evaluated (Levin et al., 2015).

As professional APCs, dendritic cells (DCs) take up antigen and secrete IL-12, which in turn activates STAT4, promoting the expansion and differentiation of committed Th1 cells. Our data show that vaccination with F-Fc promoted the secretion of the Th1-type cytokines IFN-γ and IL-2 by RSV-specific T cells residing in the spleens of vaccinated mice and increased the ratio of glycoprotein F-specific IgG2a to IgG1 antibodies (Fig. 4 and Fig. S2B). This Th1-skewed immunity helps clear pathogens with no evident induction of inflammation or lung injury (Roopenian and Akilesh, 2007; Ward and Ober, 2009). As a result, lung injury induced by RSV infection was reduced with F-Fc fusion protein immunization. These consequences were in accordance with previous studies in other viruses, such as TB subunit vaccines (ESAT6-Fc, HspX-Fc), an HSV subunit vaccine (gD-Fc) and an HIV subunit vaccine (gag-Fc) (Lu et al., 2011; Soleimanpour et al., 2015; Ye et al., 2011). Based on the abovementioned studies and our work, we suggest that an Fc fusion protein can promote Th1-skewed immune responses, which may be especially beneficial in RSV vaccine design.

In this study, we chose the intranasal administration route. Previous reports indicated that an Fc fusion protein is more efficiently transported across this epithelial surface, which depends on the neonatal Fc receptor (FcRn). As a major histocompatibility complex (MHC) class I-related molecule, FcRn could transport IgG antibody and Fc fusion protein across mucosal surfaces in adults (Lu et al., 2011; Ye et al., 2011). Therefore, the protective effect of F-Fc immunization may be partially due to FcRn/IgG transport pathway. Furthermore, due to the FcRn/IgG transport pathway, the immune activity of the F-Fc fusion protein could be prolonged by the increased plasma half-life and reduced renal clearance of large molecules, which can decrease the frequency of administration (Roopenian and Akilesh, 2007). Additionally, FcRn has been shown to mediate the pulmonary delivery of an erythropoietin-Fc fusion protein in nonhuman primates (Bitonti et al., 2004); therefore, it will be of interest to further determine whether F-Fc immunization can elicit protective immune responses after RSV infection in a rhesus macaque model in future work.

Overall, our study demonstrates that the F-Fc fusion protein binds the pre-fusion F- specific antibody D25. Immunization with the F-Fc protein plus MPL adjuvant enhances the production of neutralizing antibodies relative to that of immunization with post-fusion F/MPL and improves the Th1-biased cellular immune response, protecting mice from serious lung injury after RSV infection. In conclusion, this novel F-Fc/MPL subunit vaccine may be a candidate for the development of a safe and efficient RSV vaccine for reducing lung injury induced by RSV infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.02.017.

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