



Short Communication

Maternal antibodies by passive immunization with formalin inactivated respiratory syncytial virus confer protection without vaccine-enhanced disease



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ABSTRACT

Maternal immunization of mice with formalin inactivated respiratory syncytial virus (FI-RSV) resulted in the passive transfer of RSV antibodies but not cellular components to the offspring. The offspring born to FI-RSV immunized mothers showed serum RSV neutralizing activity, effectively controlled lung viral loads without vaccine-enhanced disease, did not induce pulmonary eosinophilia, and cytokine producing cells after live RSV infection. Therefore, this study provides evidence that maternal immunization provides an *in vivo* model in investigating the roles of antibodies independent of cellular components.

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1. Introduction

Respiratory syncytial virus (RSV), a family *Paramyxoviridae*, is the single most important viral cause of lower respiratory tract infection in infants and young children (Paramore et al., 2004; Thompson et al., 2003). The greatest hospitalization rates are in children within first 3 months of life and most children are infected during their first RSV season, for which maternal immunization could theoretically be beneficial (Glezen et al., 1986; Ochola et al., 2009; Siegrist et al., 1998). Formalin inactivated RSV vaccines (FI-RSV) were known to cause vaccine-enhanced disease in immunized children upon natural infection (Kapikian et al., 1969; Kim et al., 1969). However, the possible roles of antibodies induced by FI-RSV immunization in protection and disease have not been well understood yet although licensed drugs against RSV are based on antibodies prescribed for high-risk young infants (Alan et al., 2012; Hoopes et al., 2012; Resch et al., 2012). We hypothesized that maternal antibodies in pups born to vaccinated mothers would provide a proof-of-concept to investigate the roles of antibodies in inducing protection and vaccine-enhanced disease.

2. Immunization and challenge experiments

RSV (A2 strain) was harvested from infected HEp-2 cells, inactivated by a 1/10 volume of a 1:400 diluted 37% formaldehyde, purified by ultracentrifugation, and then absorbed onto aluminum hydroxide (4 mg/ml) as previously described (Prince et al., 2001). Female mice were intramuscularly primed and boosted with 2 and 1 µg FI-RSV (A2 strain) respectively in a 4-week interval ($n = 5$), mated and used as vaccinated mother mice. RSV specific antibodies were determined in immune sera of mother mice by using FI-RSV as an ELISA plate coating antigen as described previously (Quan et al., 2011). For RSV challenge, mice were intranasally infected with 1×10^6 plaque forming units (PFU).

3. Experimental methods

RSV plaque reduction and lung viral titer assays were performed to determine neutralizing activity and protective efficacy for viral clearance respectively as described (Quan et al., 2011). Cytokine secreting cell spots (ELISpot) were developed after stimulation of lung cells (1×10^5 cells/well) or spleen cells (5×10^5 cells/well) with inactivated RSV, and counted by an ELISpot reader (Song

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et al., 2010). Lung samples were fixed in neutral buffered formalin, embedded with paraffin blocks, thin-sectioned, and stained with hematoxylin and eosin (H&E), periodic acid-Schiff stain (PAS) or hematoxylin and congo red (H&CR) as described (Murawski et al., 2010). We used the scoring system for evaluating the histopathological severity of pneumonia, bronchioles, blood vessels and interstitial space on a scale of 0–3 by blinded observers (Mok et al., 2007). Stained bronchoalveolar lavage cells with CD45, CD11c, CD11b, and SiglecF antibodies were analyzed using LSRFortessa flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc.). Significant differences among treatments were evaluated by 1-way or 2-way ANOVA where appropriate. *P*-values of less than or equal to 0.05 were considered statistically significant.

4. Effective transfer of maternal antibodies but not cellular components to the offspring

FI-RSV immunized mother mice induced high levels of RSV specific total IgG as well as IgG1 and IgG2a isotype antibodies (Fig. 1A, B, and C). Pups (*n* = 5–10) during the 3-week period of milk suckling also showed high levels of serum IgG, and isotype antibodies

comparable to those in mothers (Fig. 1A, B, and C). This breast feeding is important because maternal IgG is transferred to infant mice via milk sucking in mice (Van de Perre, 2003). A progressive decline in the levels of maternal antibodies was observed in pups and antibody levels were decreased to half approximately 2–3 weeks after weaning (Fig. 1A, B, and C). By age of 12 weeks old, all mice born to immunized mothers showed no detectable levels of antibodies, similar to naïve mice. In contrast, all mother mice maintained high levels of RSV specific antibodies for over 19 weeks (Fig. 1A, B, C). As expected, these results suggest that antibody-secreting cells were not transferred to pups.

5. Passively transferred maternal antibodies from FI-RSV immunized mother mice confer protection

The levels of RSV plaques were significantly lowered in sera from 3 weeks old pups, similar to those from immunized mothers (3wk pups, Fig. 1D). The sera from pups with 8 weeks old showed substantial reductions in the plaque forming units at lower dilutions but no reduction with over 160 dilutions.

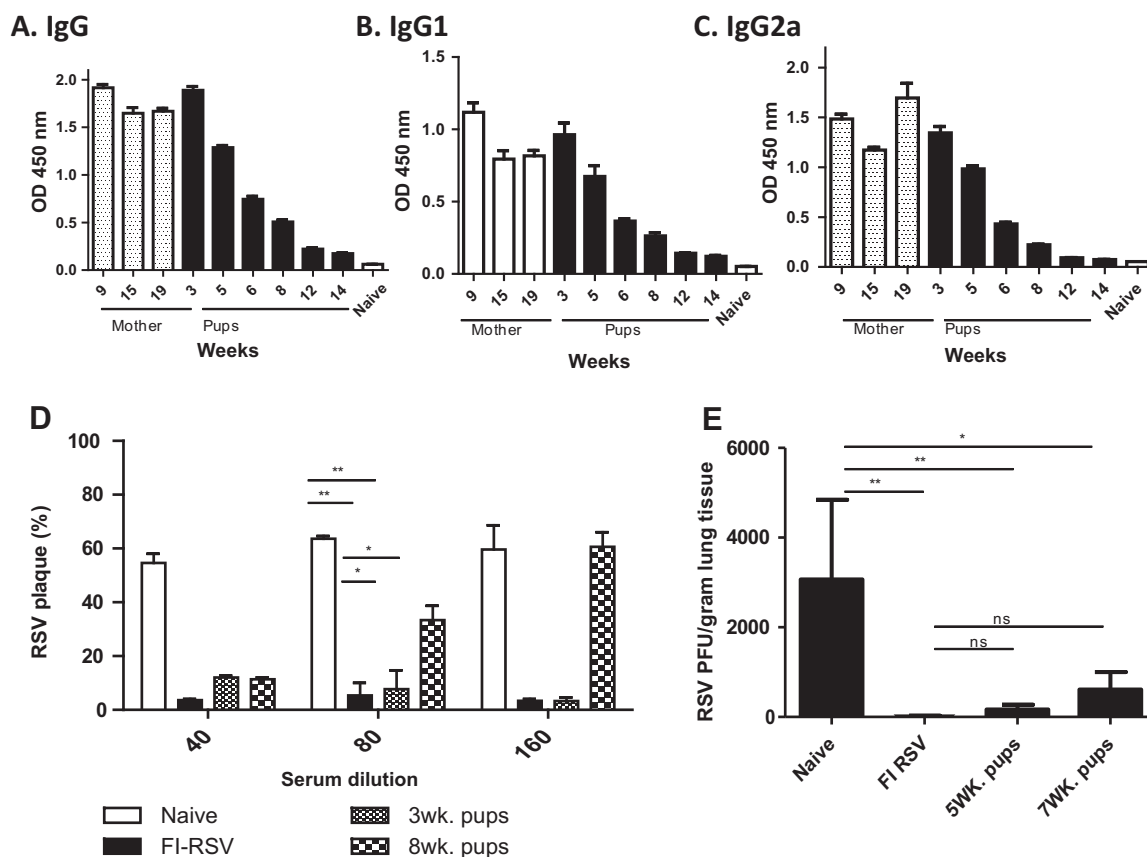


Fig. 1. Decay kinetics and protective roles of passively transferred maternal antibodies. (A–C) Decay kinetics of passively transferred maternal antibodies. Levels of RSV specific IgG and isotype antibodies are presented at different ages of pups (*n* = 5–10) born to FI-RSV immunized mice (*n* = 5). For comparison of antibody maintenance, antibody levels of immune mothers were included at 9, 15, and 19 weeks post immunization. The ages of pups are indicated as weeks (3, 5, 8, 12, and 14). (A) Total IgG antibodies specific to RSV. (B) IgG1 isotype antibodies specific to RSV. (C) IgG2a isotype antibodies specific to RSV. (D) RSV neutralizing activity of immune sera (*n* = 5–10). Serially diluted sera after heat-inactivation were used to determine their capacity to reduce RSV plaque formation. Naive: sera of naïve mice, FI-RSV: immune sera of FI-RSV immunized mice (8–10 weeks old, *n* = 5). 3wk pups: sera of 3-week old pups born to FI-RSV immunized mothers, 8wk pups: sera of 8-week old pups born to FI-RSV immunized mothers. Statistical significances (GraphPad InStat software) are indicated between naïve sera and FI-RSV immune sera or passively transferred antibody sera (3wk.pup or 8wk pups). (**P* < 0.05; ***P* < 0.01). (E) RSV loads in lungs after challenge. Lungs from individual mice in a different set of experimental groups (*n* = 5–10) from the panel D were collected on day 5 post challenge (1×10^6 PFU/mouse i.n.), and lung virus loads (PFU/g lung tissues) in each mouse were determined in HEP2 cells. Naive: Unimmunized mice infected with RSV, FI-RSV: FI-RSV prime boost immunized mice born to naïve mothers at day 5 post RSV challenge, 5wk pups: 5-week old pups at day 5 post RSV challenge, 7wk pups: 7-week old pups at day 5 post RSV challenge. Each value represents the mean $\pm$ SD (standard deviation) in triplicates. Statistical significances (GraphPad InStat software) are indicated between FI-RSV and FI-RSV pups and between FI-RSV and FI-RSV pups. Bars indicate significant differences between groups (ns; not significant; **P* < 0.05).

To determine a protective role of maternally transferred antibodies, adult mice and pups born to vaccinated mothers were intranasally infected with 1×10^6 plaque forming units (PFU) of RSV at ages between 5 and 7 weeks old, when their antibody levels were approximately 70% and 30% of their mothers (Fig. 1). Lung viral loads at day 5 post challenge were significantly lower or below the detection limit in the groups of vaccinated adult mice (FI-RSV, Fig. 1E), 5 or 7 week-old pups (5wk pups, 7wk pups, Fig. 1E) born to vaccinated mothers compared to those in naïve mice born to unimmunized mother mice (Naïve, Fig. 1E). We observed a tendency of increasing lung viral loads as pups were becoming aged (5wk pups, 7wk pups, Fig. 1E) although there were no statistical significant differences, which seems to reflect the levels of RSV neutralizing antibodies among different groups (Fig. 1D). However, adult mice (over 12 weeks old) of the offspring born to FI-RSV immune mothers showed high levels of lung viral loads similar to those in naïve adult mice (data not shown). These results indicate that passively transferred antibodies by FI-RSV maternal immunization have the capacity to neutralize RSV and to control lung viral loads after RSV challenge.

6. Offspring with passively transferred maternal antibodies do not show vaccine-enhanced pulmonary disease

Lung histopathology is a key aspect in assessing the protection against RSV. Histopathology of fixed lung samples at day 5 post RSV challenge was examined by staining with hematoxylin and eosin (H&E), PAS, or H&CR as described in experimental methods. Naïve mother-born mice that were actively vaccinated with $1 \mu\text{g}$ of FI-RSV and then infected with live RSV at the age of 7 weeks old showed a high degree of inflammation as evidenced by infiltrates in the airways, blood vessels, and interstitial spaces as well as PAS positive mucus production and H&CR positive eosinophils upon RSV challenge (FI-RSV, Figs. 2 and 3). However, when pups born to FI-RSV vaccinated mothers were challenged with RSV at

the age of 5 or 7 weeks old, they did not show such inflammatory sign of lung histopathology, as determined by low levels of H&E staining infiltrates, PAS positive mucus production, and H&CR positive eosinophils (5wk pups, Figs. 2 and 3).

Infiltration of eosinophils ($\text{CD45}^+\text{CD11c}^-\text{CD11b}^+\text{SiglecF}^+$) is an important parameter in assessing inflammatory disease after RSV infection (Castilow et al., 2008a; Elliott et al., 2004). FI-RSV actively vaccinated mice that were originally born to unimmunized naïve mothers displayed a high level of eosinophil phenotypic cells in BALF samples as determined by flow cytometry (Fig. 4A). However, eosinophil-like phenotypic cells were not observed in 5 or 7-week-old pups born to vaccinated mothers after RSV challenge, indicating no RSV vaccine-enhanced disease (5wk pups, Fig. 4A).

7. Pups born to FI-RSV immunized mother mice do not induce local and systemic cytokine producing cells

To determine cellular immune responses, cytokine secreting cell spots were determined on Multi-screen 96 well plates after stimulation of lung cells (1×10^5 cells/well) or spleen cells (5×10^5 cells/well) with inactivated RSV at day 5 post RSV challenge as previously described (Song et al., 2010). As a control, a group of mice was actively immunized with FI-RSV and then challenged with live RSV. This active immunization of naïve mice resulted in highest levels of IL-4 cytokine secreting spots in both spleen and lung cell samples upon RSV challenge (Fig. 4C, E). Also, significant numbers of IFN- γ secreting cell spots were observed from the spleen and lung cells of FI-RSV immunized mice (Fig. 4B, D). In contrast, pups born to vaccinated mothers did not show such cytokine-secreting cell responses. As expected, these results indicate that antibody producing cells and cellular immune components were not passively transferred to the offspring since most of immunity is transferred via breast milk. Therefore, maternal immunization could separate humoral immunity from cellular components in the offspring.

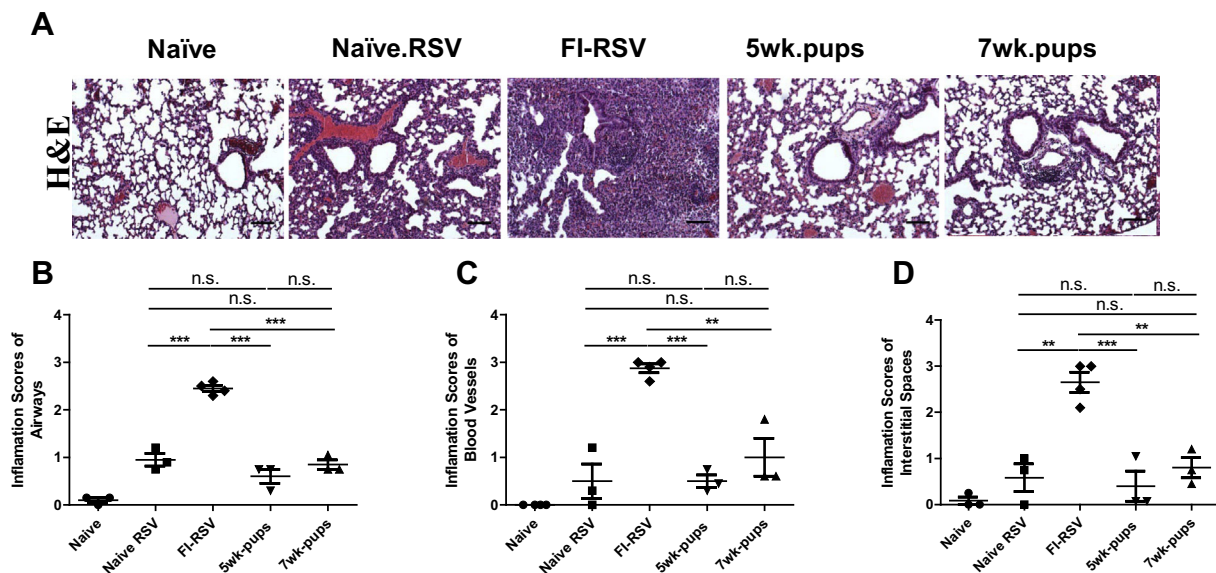


Fig. 2. Maternal antibodies confer protection without pulmonary histopathology. H&E staining shows a degree of pneumonia in airways, blood vessels, and interstitial spaces (the scale bars, 100 μm) at day 5 post RSV challenge. (A) A representative of H&E stained tissue sections from each group of mice. (B–D) Inflammation scores on a scale of 0–3 as diagnostic criteria ($n = 5$ per group). (B) Airways. (C) Blood vessels. (D) Interstitial spaces. Naïve: Naïve mice without RSV challenge, Naïve RSV: Naïve mice (8 weeks old) with RSV infection, FI-RSV: FI-RSV immunized mice (8–10 weeks old) born to naïve mothers at day 5 post RSV challenge, 5wk pups: 5-week old pups born to FI-RSV immunized mothers at day 5 post RSV challenge, 7wk pups: 7-week old pups born to FI-RSV immunized mothers at day 5 post RSV challenge. Each value represents the mean $\pm$ SE. Bars indicate significant differences between groups (** $P < 0.01$; *** $P < 0.001$).

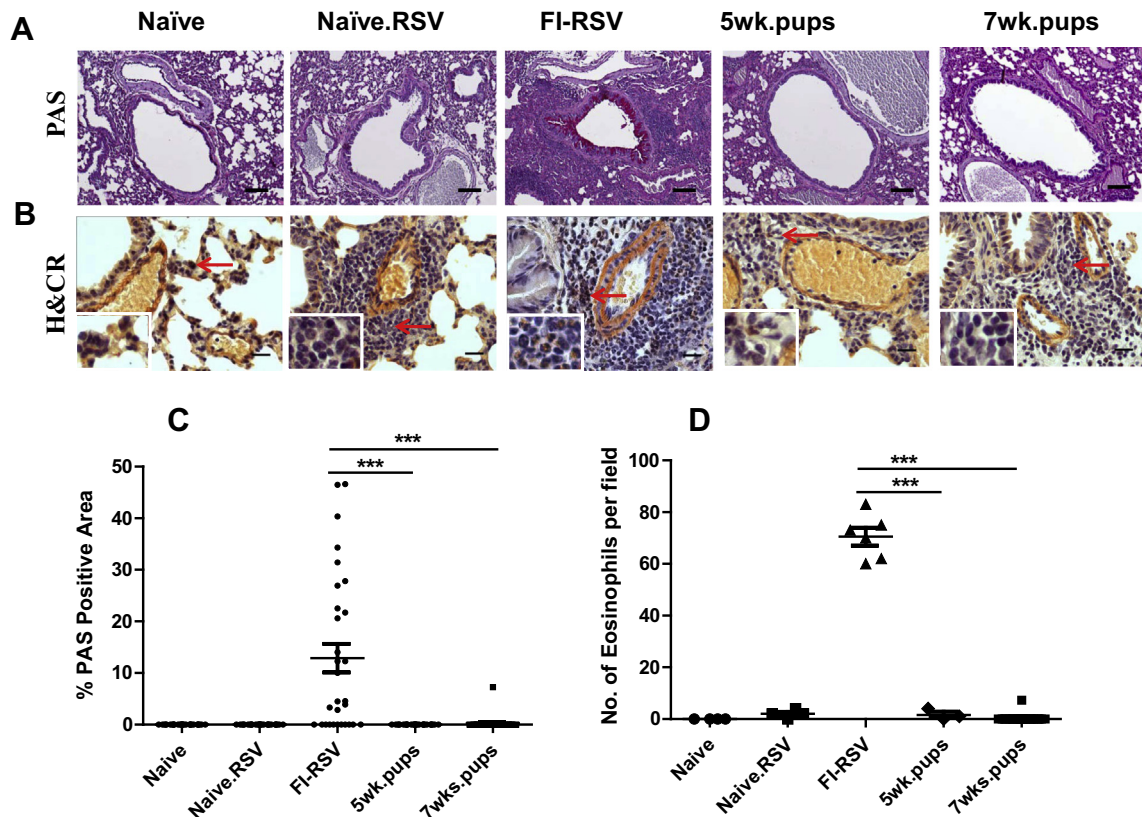


Fig. 3. Maternal antibodies do not induce mucus production and pulmonary eosinophilia upon RSV challenge. (A) Periodic acid Schiff (PAS) staining (Scale bars, 100 µm). (B) Hematoxylin and Congo Red (H&CR) staining (Scale bars, 20 µm). The inserts in H&CR images are details of arrow areas with eosinophil infiltration in lungs. (C) Scores for bronchiolar PAS positive mucus production. (D) Pulmonary eosinophils. Each symbol in mucus production percentage represents one airway of 10 individual airways in each mouse ($n = 5$). The degrees of pulmonary eosinophilia were indicated by an H&CR stains and expressed as numbers of eosinophils present per 400× field. Each value represents the mean $\pm$ SE. Bars indicate significant differences between groups (*** $P < 0.005$). The groups are the same as described in the Fig. 2.

8. Discussion and summary

In humans, maternal antibodies are transplacentally transferred to babies by active transcytosis which is facilitated by IgG Fc receptor-like molecules on placentas (Van de Perre, 2003). Different from humans, mother mice mostly transfer IgG antibodies to pups via breast milk feeding across the neonatal intestinal epithelium where enterocytes express a surface membrane receptor recognizing Fc of IgG and facilitating transcytosis of antibodies (Van de Perre, 2003). These differences in the mechanisms of antibody transfer between mice and humans need to be considered in interpreting mouse data and designing maternal immunization studies in mouse models. Neonatal protection before weaning and antibody decline kinetics might not faithfully represent the real cases in humans. Cross-fostering of pups with immunized or unimmunized mothers would be informative in better deciphering the difference in transfer mechanisms. Guinea pig would be an alternative appropriate animal model for use in maternal immunization studies (Chatterjee et al., 2001). Nonetheless, our *in vivo* model of maternal immunization provides evidence that pups with maternal antibodies induced by FI-RSV immunization have neutralizing activity and to control lung viral loads after RSV challenge without vaccine-enhanced disease. Consistent with the results in this study, it was reported that antibody-mediated or immune complex deposition enhancement of disease has not been observed with passively acquired antibodies (licensed drugs) (Alan et al., 2012; Gimenez et al., 1996; Graham, 2011; Hoopes et al., 2012; Resch et al., 2012).

In summary, maternal antibodies transferred to the offspring from FI-RSV immunized mother mice were found to be effective

in lowering lung viral titers without causing RSV vaccine-enhanced lung disease. Thus, maternal immunization could be an approach in investigating the roles of vaccine-induced antibodies in the *in vivo* system. Consistent with results in this study, previous studies demonstrated that FI-RSV vaccinated cotton rats and mice were found to effectively clear lung viral loads (Boelen et al., 2000; Kamphuis et al., 2012; Li et al., 2000; Prince et al., 2001, 1986; Waris et al., 1997, 1996). The induction of neutralizing and non-neutralizing antibodies by FI-RSV immunization might be variable depending on the vaccine dose and immunization protocol. FI-RSV immunized mothers showed severe lung disease upon RSV infection as determined by lung histopathology, mucus production, and infiltration of eosinophils. The main features of enhanced RSV disease are the induction of T helper type 2 responses including high levels of IL-4 cytokine and infiltrates of eosinophils (Castilow et al., 2007; Weiss et al., 2011). Excess INF- γ was shown to contribute to clinical signs of systemic disease after RSV challenge (Castilow et al., 2008b). Therefore, cellular immune components primed during FI-RSV immunizations were major determinants responsible for causing RSV vaccine-enhanced lung disease but not humoral RSV specific antibodies. Because of potential lung disease by FI-RSV immunization and RSV infection in mice and other animal models, it is very unlikely to be considered for the use of FI-RSV vaccines in humans for maternal immunization. It is important to test candidate RSV vaccines such as live attenuated virus or subunit RSV F (or G) vaccines for maternal immunization studies and early protection in young infants. This study demonstrates the independent contribution of humoral antibodies and T cellular components to protection and disease, respectively.

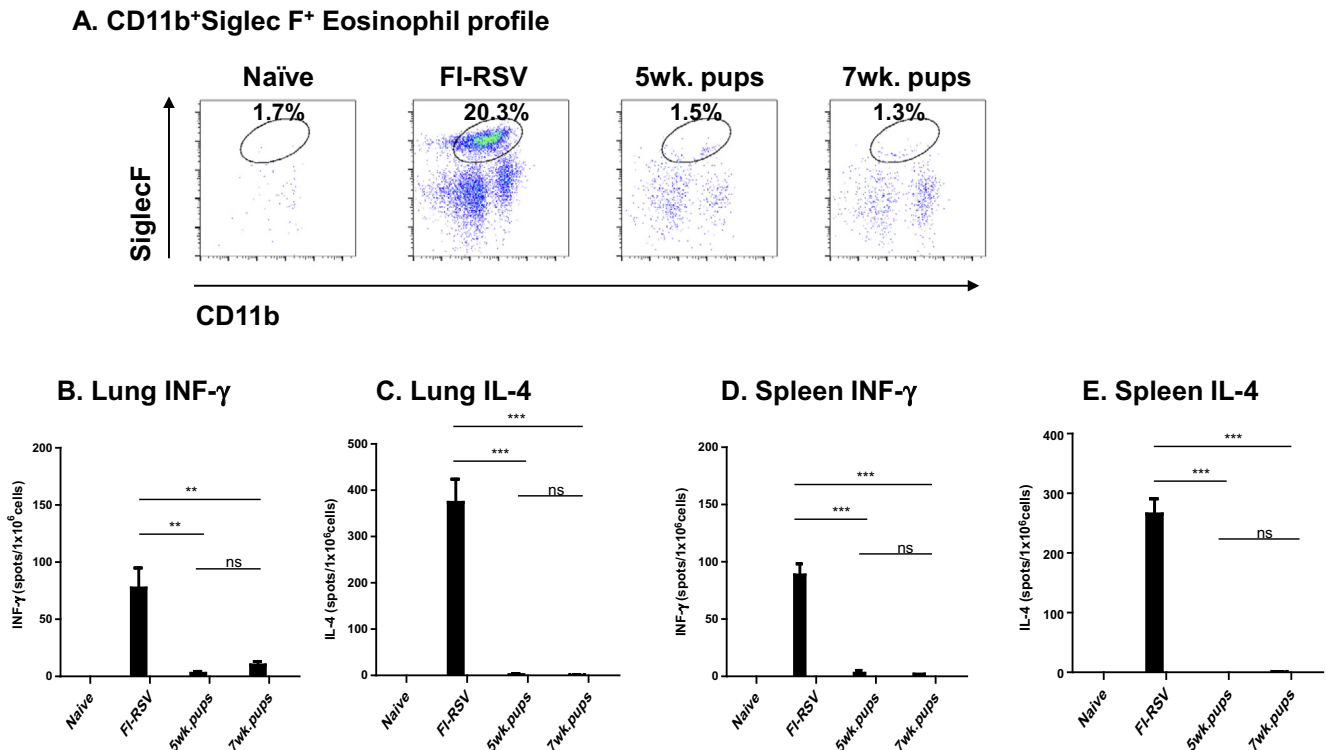


Fig. 4. Eosinophils in BAL fluids, and cytokine-secreting cellular responses in lungs and spleens. (A) Flow cytometry profiles of eosinophils in bronchoalveolar lavage (BAL) fluids. BAL cells were first gated by CD45⁺ leukocytes and CD11c⁺ granulocytes. The CD11b⁺ and SiglecF⁺ were then gated to represent the phenotypes of eosinophils. The circled percentages indicate eosinophils in BAL fluids. BAL cells were pooled from 5 mice per group. A representative is shown out of 2 independent sets of experiments. The groups are the same as described in the Fig. 2. (B) IFN-γ secreting lung cell ELISpots ($n = 5$). (C) IL-4 secreting lung cell ELISpots ($n = 5$). (D) IFN-γ secreting spleen cell ELISpots ($n = 5$). (E) IL-4 secreting spleen cell ELISpots ($n = 5$). The spot numbers are the same as described in the Fig. 2. Each value represents the mean $\pm$ SE. Statistical significant differences are indicated between groups (ns, not significant; ** $P < 0.01$; *** $P < 0.001$).

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