

# [Respiratory syncytial virus (rsv) vaccine formulation](https://assignbuster.com/respiratory-syncytial-virus-rsv-vaccine-formulation/)

### Abstract

Respiratory syncytial virus (RSV) is the leading cause of upper and lower respiratory tract infections in both children and elderly people. In this study we evaluated the short- and long-term protective efficacy of the single intranasal immunization of RSV vaccine formulation consisting of the fusion (F) protein formulated with a polyIC, an innate defense regulator peptide and a polyphosphazene (Î” F/TriAdj). This vaccine induced significant systemic and local immunity, including RSV F-specific IgG1 and IgG2a, IgA and virus neutralizing antibodies, and IFN-Î³-secreting T cells in mice. Furthermore, this vaccination promoted the induction of RSV F 85-93 -specific CD8+ effector T cells with no virus replication in the lungs. To evaluate the duration of immunity induced by single intranasal vaccination, mice were challenged with RSV after five months of immunization. Significantly higher levels of IgG1, IgG2a and virus neutralizing antibodies were detected in Î” F/TriAdj vaccinated animals. Moreover, this vaccine formulation induced high local IgA secreting memory B cell development and B cell IgA production and conferred complete protection against subsequent RSV challenge without priming for enhanced disease. In conclusion, a single nasal vaccination of RSV Î” F protein formulated with TriAdj enhanced robust, long- term protective immune responses against RSV infection.

## Introduction

Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract disease in infants, older and immunocompromised individuals worldwide {Falsey, 2005 #586}. Many RSV vaccine candidates, including live attenuated strains, vectored and viral protein subunit vaccines are underway. Currently, there is no licensed RSV vaccine or specific treatments other than prophylaxis with passive antibody therapy (Palivizumab) {Group, 1998 #599}. The first RSV vaccine clinical trial with formalin-inactivated RSV (FI-RSV) failed to induce neutralizing antibodies and caused enhanced respiratory disease after natural RSV infection {Kim, 1969 #589}.

RSV is an enveloped virus, containing three transmembrane surface glycoproteins: the major attachment protein G, fusion protein F and small hydrophobic SH protein. The F protein is highly conserved and mediates fusion of the virus into host cells and subsequent formation of syncytia, making it a major target for subunit vaccine and antiviral drug development {Collins, 2008 #597}. However, mucosal vaccination with RSV F protein alone does not induce protective immunity {Vaux-Peretz, 1992 #648; Garlapati, 2012 #650} therefore, this protein needs to be combined with an adjuvant to induce strong, long-lasting protective immune responses.

Previously, we developed a novel combination adjuvant platform (TriAdj) comprising of three components, namely a polyI: C, innate defense regulator peptide (IDRs) and polyphosphazene (PCEP). Synthetic double-stranded RNA, polyIC, known to recognize TLR3 and retinoic acid-inducible gene 1(RIG-I) leading to induction of proinflammatory cytokines, which in turn activates various immune cells {Sivori, 2004 #581`, Longhi`, 2009 `#573; Longhi, 2009 #573}. To stabilize and protect the polyIC from degradation, two other immunmodulators an innate defense regulator (IDR) peptide and a polyphosphazene were added. Innate defense regulator (IDR) peptides are derivatives of natural host defense peptides with microbicidal, chemotactic properties {Yeung, 2011 #608}. Poly[di(sodium carboxylatoethylphenoxy)]-phosphazene (PCEP) is a synthetic biodegradable water-soluble polymer with immunostimulatory properties. It forms non-covalent complexes with antigens and/or other adjuvants to increase their stability and allow multimeric presentation {Andrianov, 2009 #618; Kovacs-Nolan, 2009 #621}. Previously, we developed a novel vaccine candidate against RSV (Î” F/TriAdj) consisting of a truncated version of the RSV fusion protein (Î” F) formulated with TriAdj. We have shown that double vaccination regime such as prime and boost intranasal immunization with this vaccine candidate establishes long-lasting humoral and cell-mediated protective immune responses without any evidence of immunopathology {Garg, 2014 #60; Garg, 2015 #63}{Garg, 2014 #60}{Garg, 2014 #60}{Garg, 2014 #60}{Garg, 2014 #60}{Garg, 2014 #60}.

In the present study we demonstrated that after single IN delivery with Î” F/TriAdj, vaccine elicits mucosal and systemic immune responses and offers complete protection from RSV challenge in mice. Furthermore, Î” F/TriAdj induced effective local CD8 + T cells, which is one of the hallmarks for successful vaccination against many viral infections. To evaluate the duration of immunity induced by this vaccine candidate after single intranasal vaccination, we carried out long-term trials. Five month after the vaccination, RSV Î” F formulated with TriAdj induced robust mucosal and systemic immune responses and complete protection from RSV challenge in mice. Furthermore, Î” F/TriAdj elicited long-lived local IgA secreting memory B cell development and B cell IgA production, as well as memory T cells.

It is well known that an RSV vaccine needs to induce a balanced Th1/Th2 adaptive immune response, which stimulates the generation of high-affinity neutralizing antibodies with effector cytotoxic CD8 + T cells {Graham, 2011 #642}.

## Results

Single mucosal vaccination with Î” F/TriAdj induces strong systemic immune responses: Antibodies play a major role in protection against many viruses. To characterize the quality of the humoral immune responses induced by single vaccination of Î” F/TriAdj, IgG1 and IgG2a titers were measured in the serum. As shown in Fig. 1A & B, the mice immunized with Î” F/TriAdj developed significantly higher IgG1 and IgG2a levels, than placebo groupsboth before and after challenge with RSV. These results indicate that formulation of Î” F protein with TriAdj promotes a balanced humoral immune response.

Neutralizing antibody plays a protective role against viral infections. In order to evaluate the biological function of the Î” F-specific serum antibodies, Virus neutralization (VN) titers were determined. Mice immunized with Î” F/TriAdj developed significantly higher neutralizing antibody titers than the ones immunized with PBS both before and after challenge with RSV (Fig 1C).

To investigate the phenotype of the cellular immune response, the Î” F-induced secretion of IFN-Î³ and IL-5 by splenocytes was measured four days after challenge. Significantly higher numbers of IFN-Î³ secreting cells were generated from mice vaccinated with Î” F/TriAdj formulation, while the number of IL-5 secreting cells was low in vaccinated mice (Fig 1D). This suggests the addition of RSVÎ” F protein to the TriAdj resulted in a stronger, Th1-biased immune response, which is in also agreement with the enhanced IgG2a production.

Intranasal vaccination with single dose of Î” F protein formulated with TriAdj induces robust protective mucosal immune: Mucosal immune response play a crucial role in first line defense of host by blocking the spreading of RSV infection in the lungs. Thus, an effective RSV vaccine should induce local IgA antibodies in the respiratory tract. To evaluate the mucosal immune responses induced by Î” F/TriAdj, we measured Î” F-specific IgA antibody levels in the lungs. Significantly higher IgA production was observed in mice immunized withÎ” F/TriAdj in comparison with mice immunized with PBS (Fig 2A). To determine whether single IN vaccination with the Î” F/Triadj formulations would affect viral clearance, all mice except those in one of the two Placebo groups were challenged IN with RSV. No infectious virus was recovered in lungs of mice immunized with Î” F/TriAdj, showing full protection from infection (Fig 2B).

In order to investigate whether protection is correlated with higher cross-presentation and cell-mediated immune responses, we evaluated RSV F-specific CD8 + T cells infiltration with RSV F 85-93 pentamers in the lungs after RSV challenge. The Î” F/TriAdj induced a significantly higher number of F 85-93 specific CD8 + T cells influx in the lungs (Fig 6A), which suggests that vaccination with Î” F/TriAdj promotes a cytolytic CD8 + T cell response to RSV infection. Virus-specific effector CD8 + T-cell response has been shown to play a critical role in RSV clearance {Graham, 2011 #642}. Therefore, we analyzed the effecter function of the CD8 + T cells according to their expression of the IFN-Î³ by ï¬‚ ow cytometry. After RSV challenge, the Î” F/TriAdj-immunized group showed higher frequencies of IFN-Î³-expressing CD8 + T cells than PBS-immunized, RSV-challenged mice in the lungs (Fig 6). This suggests that Î” F/TriAdj promotes a stronger effector CD8 + T cell response to RSV.

Single nasal vaccination with Î” F/TriAdj induces Long-term systemic immune responses: To assess the long term memory effect induced by single intranasal vaccination with Î” F/TriAdj, serum IgG and VN antibody levels were measured periodically over five months. The mice immunized with Î” F/TriAdj developed strong IgG and VN titers compare to placebo control group for approximately 25 weeks (Fig 3A & B). We next examined whether IN vaccination promoted the induction of long term Î” F-specific serum IgG1 and IgG2a. The Î” F/TriAdj- vaccinated mice developed significantly higher Î” F-specific serum IgG1 and IgG2a titers than placebo groups both before and after challenge with RSV (Fig 4A & B). VN titers were also determined to evaluate the functional quality of the Î” F-specific serum antibodies after RSV challenge. Mice immunized with Î” F/TriAdj developed significantly higher neutralizing antibody titers compared to PBS group (Fig 4C). To further define the phonotype of the immune response, the Î” F-induced secretion of IFN-Î³ and IL-5 by splenocytes was measured four days after challenge with RSV. The Î” F/TriAdj formulation generated a significantly higher frequency of IFN-Î³ secreting cells compared to the placebo. The numbers of IL-5 secreting cells were low in all vaccinated mice (Fig 4D), which suggests Th1 bias immune responses.

Mucosal vaccination with Single dose of Î” F/TriAdj induces Long-term protective mucosal immune response: Local antibody play a major role in protection from respiratory viruses such as RSV in the lung. To access the long term local immune responses with Î” F/TriAdj vaccination, induction of Î” F-specific IgA in the lungs was examined by ELISA. Mice immunized with Î” F/TriAdj developed significantly higher IgA levels when compared to placebo and placebo virus challenged groups (Fig 5A). Furthermore, we checked the development of IgA-secreting memory B cells in lungs after RSV challenge. Î” F/TriAdj formulations generated a significantly higher frequency of RSV Î” F-specific IgA-secreting memory B cells in lungs in comparison to PBS (Fig 5B). In summary, single dose of Î” F/TriAdj via IN vaccination stimulated strong IgA-secreting memory B cell development and B cell IgA production.

To determine viral clearance after single vaccination with Î” F/TriAdj, all mice except those in one of the two placebo groups were challenged IN with RSV on day 150. The mice were sacrificed after four days, and virus titers in the lungs were determined. No infectious virus particle was recovered from mice vaccinated with Î” F/TriAdj, showing that these mice still had sufficient immunity to be completely protected from challenge virus replication in the lungs (Fig 5C).

## Discussion

RSV is one of the major global burdens of causing a broad spectrum of respiratory illnesses in children and older population worldwide. There is still no licensed RSV vaccine, in part due to the disastrous outcomes observed following vaccination of naÃ¯ve children with FI-RSV {Kim, 1969 #589}. Natural infection with RSV fails to protect against subsequent infection because it stimulate modest immunogenicity and short-lived immunological memory against virus. Similar to natural RSV infection, many promising RSV vaccine have failed to generate long-lasting, protective immune responses {Power, 2008 #769; Hall, 2001 #789}. For wide clinical application, RSV vaccine will need to be safe, easy to administer and must stimulate strong long-term protective immunity than natural RSV infection {Pulendran, 2011 #796}. Protein subunit vaccines have a high safety profile, but are generally poorly immunogenic and induce short-lived humoral and cellular immunity {Graham, 2011 #642}. So adjuvants are added to subunit vaccines to stimulate faster, stronger, and long-lasting immune responses to vaccines. At the clinical level, multi dose vaccination is expensive and has potential safety problems. Whereas, single vaccination formats are cost effective, safe (no risk for vaccine contamination) and more convenient to health worker. In the present study, we demonstrated the short as well as long term protective efficacy including stable systemic IgG, local IgA production and neutralizing antibody titers of a RSV Î” F protein formulated with TriAdj in mice after single IN immunization. Furthermore, Î” F/TriAdj promoted an effector CD8 + T cells with compete protection against RSV infection without inducing vaccine-enhanced pathology in the lungs. A vaccine requiring only a single administration to be effective would be highly practical, as it would improve patient compliance Therefore, single intranasal immunization with RSV Î” F protein formulated with TriAdj is a promising approach to induce a long-lasting protective RSV-specific immune response.

Mucosal immunization via IN route is invasive and suitable for generation of both local and systemic immunity. RSV infect through respiratory tract, therefore mucosal antibodies specifically IgA play an important role in prevention and clearance of virus. Previously it has been shown that protection against RSV infection is more correlated to the levels of mucosal IgA rather than to systemic antibody in human {Watt, 1990 #687}. In mice, single RSV infection induces short-lived neutralizing antibodies with absence of antibody-secreting memory B cells {Schmidt, 2012 #869}. Interestingly, we have recently demonstrated that the RSV infected mice were showed significantly lower level of local B cell IgA production and IgA secreting memory B cell development that indicates an impaired local antibody response that allows RSV re-infection and explain the short-term protective immunity with natural infection with RSV{Garg, 2016 #1340}. In contrast, intranasal vaccination with single dose of RSV Î” F protein formulated with TriAdj promotes the induction of long-lasting local IgA secreting memory B cell development and B cell IgA production which is a major criterion for an effective RSV vaccine.

Previously, we and others have reported that strong, effective and protective immune responses were induced only after a booster immunization with RSV F protein with adjuvant {Garg, 2014 #891}{Blanco, 2014 #1423}{Lambert, 2015 #1442}. A single mucosal vaccination of adenovirus-based RSV vaccine had also been proved effective in short term {Kim, 2010 #1443}. But this is the first report showing the long term protective efficacy of single mucosal vaccination of an adjuvanted RSV F protein subunit vaccine in mice.

Clinical trial with FI-RSV failed to induce protection against RSV due to generation of poor neutralizing antibodies and TLR activation that led to vaccine-associated enhanced disease {Delgado, 2009 #513}. The inclusion of polyI: C as TLR ligand in vaccine formulation, is expected to overcome the limitations of inactivated RSV vaccines. The adjuvant effect such as long-lasting T cell immunity of polyI: C is likely caused by the direct interaction with PRRs such as TLR3, MDA5 and RIG-I, leading to production of pro-inflammatory cytokines, and chemokines. Interestingly, it has been shown that alveolar macrophages and lung DCs induced strong IgA and IgG antibodies by addition of TLR3 ligands, which were probably linked to secretion of BAFF/APRIL cytokines and activated B cells {Xu, 2008 #686}. However, we and others have been shown that IN administration of higher doses of polyIC caused marked production of inflammatory cytokines/chemokines accompanied by impaired lung function {Stowell, 2009 #682; Boukhvalova, 2010 #685; Aeffner, 2011 #750} suggesting excessive stimulation of local immune responses can result in detrimental effects. In contrast, Î” F/TriAdj has an advantage over polyIC alone as it has shown full protection form RSV infection without any exacerbation of chronic pulmonary disease. This demonstrates that a combination of polyIC, IDR and PCEP mediates optimal enhancement of the RSV-specific immune response without vaccine enhanced disease. We believe that IDRs is one of the major anti-inflammatory components in our vaccine formulation as it has been previously shown to enhance cell-mediated immune responses and to modulate excessive consequences of TLR signaling {Bowdish, 2005 #766; Hancock, 2006 #767}, whereas polyphosphazenes enhance antigen-specific humoral immunity through the formation of non-covalent complexes with protein {Andrianov, 2005 #667}, suggesting major roles from both of these compounds in this vaccine formulation.

An important factor for successful vaccine against RSV requires the generation of effective CD8 + T cell in the lungs. Previously, it has been shown that mild to severe infection with RSV failed to induce virus-specific IFN-Î³ recall responses in the infants {Lee, 2007 #806}. To define the stimulation of local effective CD8 + T cell responses induced by Î” F/TriAdj, RSV F specific pentamer staining demonstrated marked induction of IFN-Î³ secreting RSV-specific CD8 + T cells in lungs. Similarly, exogenous IFN-Î³ expression protects against RSV infection in the lungs of BALB/c mice {Kumar, 1999 #805}. This evidence suggests that complete protection against RSV was associated with the presence of IFN-Î³ + RSV-specific CD8 + T cells.

In summary, our data show that RSV Î” F protein formulated with TriAdj vaccine represents a safe, effective and promising RSV vaccine candidate. The major advantages associated with this mucosal RSV vaccine is to induction of long-term protective immunity with a single mucosal vaccination by stimulating long-lived RSV-specific neutralizing antibodies, memory B and CD8 + T cells, therefore warranting additional evaluation as a vaccine against RSV in clinical trials.

## MATERIALS AND METHODS

Virus and vaccine formulation: The RSV (A2 strain) was propagated in Hep-2 cells (American Type Culture Collection, VR-1540). The RSV Î” F protein with his-tag was produced and purified as described previously {Garlapati, 2012 #650}. Briefly, HEK-293T cells were transfected with an episomal vector expressing the Î” F protein using Turbofect (Fermentas, R0534). The Î” F protein with his-tag was purified using TALON Superflow resin (Clontech,) according to the manufacturer’s instructions. The Î” F protein was formulated with 10 µg poly (I: C) (Invivogen, tlrl-picw), 20 µg IDR1002 (Genscript, 818360) and 10 µg PCEP (Idaho National Laboratory) in PBS (Î” F/TriAdj) as described previously.

Animals, immunizations and challenge: Six to eight week old female BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were immunized once IN with Î” F/TriAdj. Two additional groups of mice received PBS IN (Placebo control). Three week (short-term trial) and twenty weeks (long term trial) post-vaccination, all except one of the Placebo groups were challenged IN with RSV strain A2 (5 x 10 5 PFU/50µl). Mice were euthanized four days after RSV challenge for detection of immune response and virus in the lungs. Blood samples were drawn by cardiac puncture under anesthesia prior to challenge and at regular intervals afterwards. All procedures were approved by the University Animal Ethics Committee in accordance with the standards stipulated by the Canadian Council on Animal Care.

Lung fragment cultures and ELISA: Lavaged lungs of euthanized mice were cut into small pieces and lung fragment cultures were prepared as described previously {Mapletoft, 2008 #591}. ELISA was performed as described previously {Garg, 2014 #942}. RSV Î” F-specific IgG1, IgG2a and IgA were detected using biotin-labeled goat anti-mouse IgG1, IgG2a (Southern Biotech, 1070-08, 1080-08) or IgA (Gibco, M3115) followed by streptavidin-alkaline phosphatase (AP) (Jackson ImmunoResearch Laboratories Inc., 016-050-084), and developed with p-nitrophenyl phosphate (Sigma-Aldrich, N3254) substrate.

Enzyme-linked immunospot (ELISPOT) assays: ELISPOT assays were performed on splenocytes and LNs cells as previously described {Garlapati, 2012 #650}. Briefly, cells were stimulated with Î” F protein (2 µg/ml) and spots were developed using biotinylated IFN-Î³- or IL-5- (BD Biosciences, 554410, 554397) or goat anti-mouse IgA specific antibody, AP-conjugated streptavidin and BCIP/NBT (Sigma-Aldrich, B5655) as the substrate.

Analysis of CD8 + T cells from lungs by flow cytometry: Lung single-cell suspensions were generated as previously described {Garg, 2014 #942}. To analysis of RSV F 85-93- specific CD8 + T cells, lung mononuclear cells were surface stained with H-2Kd-F 85-93 MHC Class I pentamer (ProImmune, F149) together with labeled antibodies specific for CD8 + T cells (BD Pharmingen, 553031). For intracellular detection of IFN-Î³ in CD8 + T cells, lung mononuclear cells were stimulated with 1 Î¼M of F 85-93 peptide (KYKNAVTEL) and cell surface staining was performed, followed by intracellular cytokine staining using Cytofix/Cytoperm (BD Pharmingen, 554715) and APC- conjugated anti-IFN-Î³ antibody (BD Biosciences, 554413). After staining, cells were acquired by flow cytometry (BD Biosciences), and data were analyzed using Kaluza Software (Version 1. 2). Cells were gated for live cells, singlets and lymphocytes and then analyzed for indicated markers.

Virus titration and virus neutralization assay: Virus titrations were performed with individual lungs at day 4 post- challenge as described previously {Garg, 2014 #942}. Results are expressed as PFU/g of lung tissue. RSV-specific neutralization titers were determined by plaque reduction assays. Serum samples were mixed with 500 PFU/well of RSV strain A2 for 1 h at 37°C. The sample-virus mixtures were transferred to HEp-2 cell monolayers and incubated for 4 days at 37 °C, and the cells were fixed and stained with 0. 5% crystal violet.

Statistical analysis: All data were analyzed using GraphPad PRISM version 6 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Differences among all groups were examined using Student t-tests, one-way ANOVA, followed by a Newman-Keuls post test. Differences were considered significant if P < 0. 05.

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### Figure legends

Fig. 1. RSV Î” F-specific systemic humoral immune responses in mice. (A) Serum IgG1 and IgG2a titers before challenge (B/C), (B) Serum IgG1 and IgG2a titers after challenge (A/C) with RSV, (C) Serum VN antibody titers determined before (B/C) and after (A/C) RSV challenge, and (D) Numbers of IFN-Î³ and IL-5 secreting splenocytes determined in response to in vitro restimulation with Î” F protein. BALB/c mice were immunized once IN with Î” F formulated with TriAdj and challenged three weeks later with RSV. Control groups were immunized with PBS and challenged with RSV (Placebo) or mock-challenged (Placebo/mock). ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Virus neutralization titers are expressed as the highest dilution of serum that resulted in <50% of cells displaying cytopathic effects. Cytokine secreting cell numbers are expressed as the difference in the number of spots between Î” F-stimulated wells and medium-control wells. Bars indicate median values with interquartile ranges. \*\*P < 0. 01; \*\*\*P < 0. 001.

Fig. 2. Mucosal immune responses to RSV Î” F protein in mice after challenge with RSV. The IgA titer (A), Virus titer (B), Percentages of Î” F-specific CD8 + T cells (C), and Î” F-specific IFN-Î³ secreting CD8 + T cells (D) were measured in the lung after RSV challenge. Mice were immunized and challenged as described in the legend for Fig. 1. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Virus replication in the lungs is expressed as pfu per gram of lung tissue. Bars represent median values with interquartile ranges. \*P < 0. 05; \*\*P < 0. 01; \*\*\*P < 0. 001.

Fig 3. Long-term systemic immune responses to RSV Î” F protein. IgG (A) and VN (B) titers were measured at different times after vaccination. BALB/c mice were immunized once IN with Î” F formulated with TriAdj and challenged with RSV on day 150. Control groups were immunized with PBS and challenged with RSV (Placebo) or mock-challenged (Placebo/mock). ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Virus neutralization titers are expressed as the highest dilution of serum that resulted in <50% of cells displaying cytopathic effects. Symbols represent median values with interquartile ranges.

Fig 4. Systemic immune responses to RSV Î” F protein. (A) Serum IgG1 and IgG2a titers before challenge (B/C), (B) Serum IgG1 and IgG2a titers after challenge (A/C) with RSV, (C) Serum VN antibody titers determined after (A/C) RSV challenge, and (D) Numbers of IFN-Î³ and IL-5 secreting splenocytes determined in response to in vitro restimulation with Î” F protein. Mice were immunized and challenged with RSV as described in the legend for Fig. 3. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. Virus neutralization titers are expressed as the highest dilution of serum that resulted in <50% of cells displaying cytopathic effects. Cytokine secreting cell numbers are expressed as the difference in the number of spots between Î” F-stimulated wells and medium-control wells. Bars indicate median values with interquartile ranges. \*\*P < 0. 01; \*\*\*P < 0. 001.

Fig 5. Long term mucosal immune responses to RSV Î” F protein. IgA titers (A), numbers of IgA secreting LNs cells (B), Virus titers (C) were determined after (A/C) RSV challenge. Mice were immunized and challenged with RSV as described in the legend for Fig. 3. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum. IgA secreting cell numbers are expressed as the difference in the number of spots between Î” F-stimulated wells and medium-control wells. Virus replication in the lungs is expressed as pfu per gram of lung tissue. Bars represent median values with interquartile ranges. \*\*P < 0. 01; \*\*\*P < 0. 001.