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In Vivo Evaluation of Adeno-Associated Virus Gene Transfer in Airways of Mice with Acute or Chronic Respiratory Infection

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Abstract

Patients with cystic fibrosis (CF) often suffer chronic lung infection with concomitant inflammation, a setting that may reduce the efficacy of gene transfer. While gene therapy development for CF often involves viral-based vectors, little is known about gene transfer in the context of an infected airway. In this study, three mouse models were established to evaluate adeno-associated virus (AAV) gene transfer in such an environment. *Bordetella bronchiseptica* RB50 was used in a chronic, nonlethal respiratory infection in C57BL/6 mice. An inoculum of $\sim 10^5$ CFU allowed *B. bronchiseptica* RB50 to persist in the upper and lower respiratory tracts for at least 21 days. In this infection model, administration of an AAV vector on day 2 resulted in 2.8-fold reduction of reporter gene expression compared with that observed in uninfected controls. Postponement of AAV administration to day 14 resulted in an even greater (eightfold) reduction of reporter gene expression, when compared with uninfected controls. In another infection model, *Pseudomonas aeruginosa* PAO1 was used to infect surfactant protein D (SP-D) or surfactant protein A (SP-A) knockout (KO) mice. With an inoculum of $\sim 10^5$ CFU, infection persisted for 2 days in the nasal cavity of either mouse model. Reporter gene expression was approximately $\sim$2.5-fold lower compared with uninfected mice. In the SP-D KO model, postponement of AAV administration to day 9 postinfection resulted in only a two fold reduction in reporter gene expression, when compared with expression seen in uninfected controls. These results confirm that respiratory infections, both ongoing and recently resolved, decrease the efficacy of AAV-mediated gene transfer.

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disorder among Caucasians, occurring at a rate of approximately 1 in 2000 people (Bobadilla *et al.*, 2002). As a disease caused by a single defective gene, CF airway disease is ideal for treatment with gene therapy (Lee *et al.*, 2005). However, over 20 years have elapsed since the discovery of the CF transmembrane conductance regulator and the associated *CFTR* gene, and still, no viable gene therapy option exists for curing or alleviating airway disease (Birault *et al.*, 2013). A number of obstacles to gene therapy for CF exist (Ferrari *et al.*, 2002; Oakland *et al.*, 2012), but one major obstacle has yet to be extensively studied: how an active respiratory infection affects gene transfer in the airway.

Patients with CF are characterized by chronic respiratory infections by opportunistic bacteria (Chattoraj *et al.*, 2010). In particular, *Pseudomonas aeruginosa* colonizes the lungs of most CF patients indefinitely (Govan and Deretic, 1996). The initial presentation of infection is not fatal, but as CF patients cannot clear the bacteria from their lungs, a persistent inflammatory state arises that causes continuous infection and eventually leads to permanent pulmonary damage (Sinn *et al.*, 2011). Little research has been done to investigate the effects of microbial infection on airway gene transfer.

Deterioration of the lung in CF patients is caused by a cycle of infection by opportunistic pathogens and neutrophilic inflammation (Makam *et al.*, 2009). By the age of 17, nearly 70% of CF patients have *P. aeruginosa* in their sputum at levels of 10^6–10^8 CFU/gr sputum (Davis *et al.*, 1996), and approximately 80% of adult CF patients are chronically infected with *P. aeruginosa* that cannot be eliminated by antibiotic therapy (Cystic Fibrosis Foundation, 2012). In a phase II gene therapy trial with adeno-associated virus (AAV)-CFTR, nearly 70% of CF patients receiving the AAV2 vector

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were *P. aeruginosa* colonized (Moss *et al.*, 2004). The presence of infection was an endpoint of measurement in the trial and was not a basis for exclusion from the trial. Separately, a phase IIB trial with AAV2-CFTR that included 3 doses of 10^{13} particles administered 30 days apart failed to demonstrate efficacy (Moss *et al.*, 2007). In this clinical trial of 102 CF subjects, the number of days of antibiotic use was considered a marker for trial monitoring. Infection in CF airways is expected during the progress of the gene therapy regimen, and about 15% of CF subjects were on antibiotics while they received aerosolized AAV2 for this study.

In a clinical trial of interferon gamma-1b with 66 CF patients, sputum bacterial density was 7.1 Log_{10} CFU/gr, with 80–90% of enrolled subjects having *P. aeruginosa* infection (Moss *et al.*, 2005). The sickest CF patients have chronic lung infection, but the presence of *P. aeruginosa* in sputum has not been a criterion for exclusion in airway-directed gene therapy clinical trials. The failure of CF AAV gene therapy trials may be due in part to the difficulty of gene delivery to lungs that sustain and harbor a chronic disease state because of infection.

The paucity of research in this area may be related to the lack of a relevant animal model necessary to study such effects. *CFTR* knockout (KO) mice do not develop the pulmonary abnormalities typically associated with the lung disease in humans (Pezzulo *et al.*, 2012). Additionally, *P. aeruginosa* is not a natural murine pathogen and cannot naturally establish a lasting infection in wild-type (WT) mice, unless administered in an artificial biofilm (i.e., embedded in beads composed of agar, agarose, or seaweed alginate) (Hoffmann *et al.*, 2005). Administration of free bacteria results in either rapid clearance of the organism or acute sepsis and death (van Heeckeren and Schluchter, 2002).

We present here three alternatives for studying gene transfer in an infected airway. The first involves a natural murine pathogen, *Bordetella bronchiseptica* (*Bb*) RB50, to establish a chronic infection in C57BL/6 mice. *Bb* infects a number of members of the mammalian family, and in rodents, rabbits, cats, dogs, and pigs, it typically establishes an asymptomatic infection in the nasal cavity that persists indefinitely (Gueirard *et al.*, 2003; Irie and Yuk, 2007). In the lower respiratory tract, the infection is enduring, but transient, with the bacterial load reducing to almost baseline within 45 days (Gueirard *et al.*, 2003). The second and third scenarios utilize *P. aeruginosa* PAO1 to establish an acute infection in surfactant protein D (SP-D) and surfactant protein A (SP-A) KO mice, respectively. SP-D and SP-A are pulmonary collectins that are important in innate immunity against various bacterial and viral pathogens (Cheng and Palaniyar, 2013). Decreased or lack of SP-D and SP-A has been implicated in the pathogenesis of CF airway disease (Postle *et al.*, 1999). When KO mice were engineered to lack either SP-D or SP-A, they were found to have decreased ability to effectively clear *P. aeruginosa* (Giannoni *et al.*, 2006). The resulting infection was still transient, lasting only a few days, but the resulting inflammatory response was exaggerated when compared with WT mice. As hyperinflammatory responses are also typical of CF patients, the SP-D and SP-A KO mice emulate key inflammatory aspects of the disease when compared with WT mice.

In this report, we employed these mouse models to study AAV-mediated gene transfer in infected airways. Figure 1 depicts the experimental design used. The presence of an on-going or even recently resolved infection decreased AAV-mediated gene transfer efficiency in the airway, validating the use of these models to optimize gene transfer for relevant airway diseases like CF.

Materials and Methods

Bacterial strains and growth conditions

Bb strain RB50 was a kind gift from Dr. Yasuhiko Irie, University of Washington, Seattle, WA. *Bb* was cultured in Stainer–Scholte broth or on Bendet Gengou (BG) blood agar (BD Diagnostic Systems) at 37°C. *P. aeruginosa* PAO1 was a kind gift from Dr. Robert Bucki, University of Pennsylvania, Philadelphia, PA. The bacterium was cultured at 37°C in Miller's Lysogeny Broth (Mediatech) or on Pseudosel Agar (cetrimide agar) from BD Diagnostic Systems.

AAV preparation

The AAV9 vectors flanked with AAV2 inverted terminal repeats contained a firefly luciferase (*ffluc*) reporter gene fused to a nucleus localization sequence at the N-terminus under the transcriptional control of the cytomegalovirus-enhanced chicken-β-actin promoter. Vectors were produced by the Penn Vector core as previously described (Bell *et al.*, 2011).

Mice

C57BL/6 male mice were purchased from Charles River Laboratories, while SP-D and SP-A KO mice (Botas *et al.*, 1998) were maintained in-house at the University of Pennsylvania. Mice were age-matched and used between 7 and 14 weeks of age. For all experiments, a group size of at least three mice was used for each experimental cohort. All animals were maintained at the Animal Facility of the Translation Research Laboratories at the University of Pennsylvania under protocols reviewed and approved by the University of Pennsylvania's Institutional Animal Care and Use Committee. Before all intranasal administrations, mice were anesthetized by an intraperitoneal injection of ketamine/xylazine (70/7 mg/kg) and were subsequently suspended from their dorsal incisors (hind quarters supported) for dosing.

Preparation of inoculum

Bacteria were grown overnight in their appropriate liquid media at 37°C with shaking at 250 rpm. Approximately 16–18 hr later, the bacteria were diluted into fresh broth to an optical density ($\lambda = 600$ nm) reading of 0.1 and were allowed to grow until midlogarithmic phase (optical density at $\lambda = 600$ nm of ~ 0.5 for *Bb* RB50 and ~ 0.4 for *P. aeruginosa*). At that point, the bacteria were harvested and washed once in phosphate-buffered saline (PBS) before being diluted into fresh PBS at the desired concentration (controlled by evaluation of optical density at 600 nm).

Mouse infection model for evaluation of airway transduction

Before challenge, mice were anesthetized intraperitoneally and suspended from their dorsal incisors, as described earlier. Mice were then intranasally (i.n.) challenged with a 30 μl bolus (delivered as two 15 μl aliquots, one into each

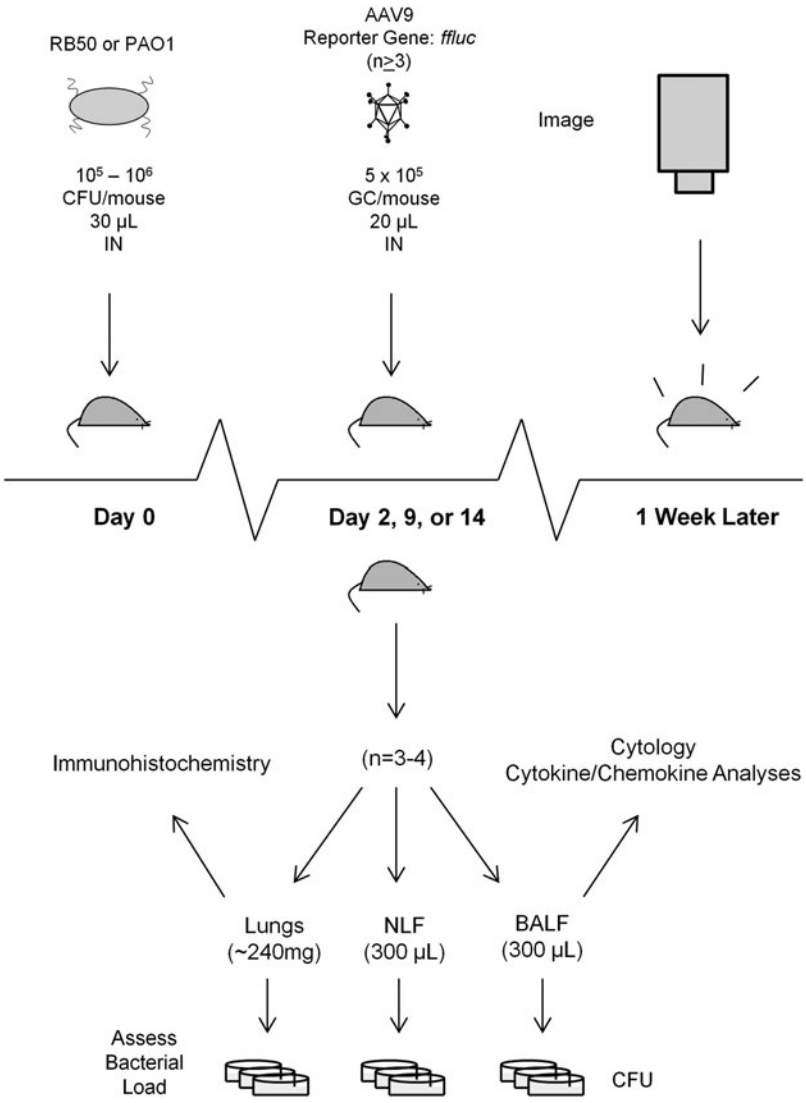

FIG. 1. Schematic of experimental design. Mice are i.n. infected with freshly cultured bacteria on day 0. On day 2, 9, or 14, an AAV9 vector containing a *ffluc* reporter gene is introduced to the recently infected airway via intranasal instillation. Three to four randomly selected mice are also taken on the day of AAV administration for evaluation of bacterial load and characterization of immune response at the time of vector administration ($n=3$ for uninfected controls, $n=4$ for infected groups). Approximately every week thereafter, the remaining mice are dosed with luciferin and subjected to live imaging to evaluate *ffluc* expression. AAV, adeno-associated virus; BALF, bronchoalveolar lavage fluid; *ffluc*, firefly luciferase; i.n., intranasally; NLF, nasal lavage fluid.

nostril) of *Bb* RB50 or *P. aeruginosa* PAO1 to achieve approximately 10^5 CFU/mouse. At specified time points later (i.e., 2, 9, or 14 days after infection), mice were i.n. administered 5×10^{10} genome copies of AAV vector in 20 μl (10 μl into each nostril).

To evaluate *ffluc* expression, mice ($\sim$20 g) were anesthetized and suspended before 20 μl of D-luciferin (15 mg/ml) was i.n. administered as two 10 μl aliquots, one into each nostril. After 5 min, mice were imaged for 60 sec with the IVIS Xenogen imaging system. Quantification of signal was calculated with Living Image 2.5.1 software.

Evaluation of bacterial load

To determine bacterial load, three samples were taken from each randomly selected mouse: (a) lungs, (b) bronchoalveolar lavage fluid (BALF), and (c) nasal lavage fluid (NLF). Before the lungs were harvested, BALF was collected by instilling 500 μl of sterile PBS through a cannula into the trachea. Fluid was collected and re-instilled into the lungs for a total of three times before being collected into a 1.5 ml tube. The lungs ($\sim$110–150 mg per lung) were then excised. One lung was inflated with a 1:1 mixture of Tissue Tek OCT and PBS, embedded in OCT in a storage cassette and flash-frozen with cooled 2-methylbutane. Frozen lungs were kept at -80°C until further histological processing. The other lung was homogenized in 1.5 ml of sterile PBS for evaluation of bacterial load. For the NLF samples, mice were decapitated and a cannula attached to a 1 ml syringe containing 300 μl of sterile PBS was placed into the tracheal remnant. PBS was then flushed through the nasal passages and collected through the nares into a 1.5 ml tube. This recovered fluid was used to flush the nasal cavity another two times, for a total of three flushes. All samples were kept on ice until further processing.

To determine the CFU counts in each sample, solutions were diluted 10-, 100-, and 1000-fold. Ten-microliter aliquots of each dilution were spotted in duplicate on the appropriate solid selection agar for each bacterial strain (BG blood agar for *Bb* and Pseudosel agar for *P. aeruginosa*). Plates were incubated at 37°C for 48 hr for *Bb* RB50 and 16 hr for *P. aeruginosa*. The number of colonies at each dilution was then counted, and the concentration of CFU of each sample was back calculated from the dilution factor.

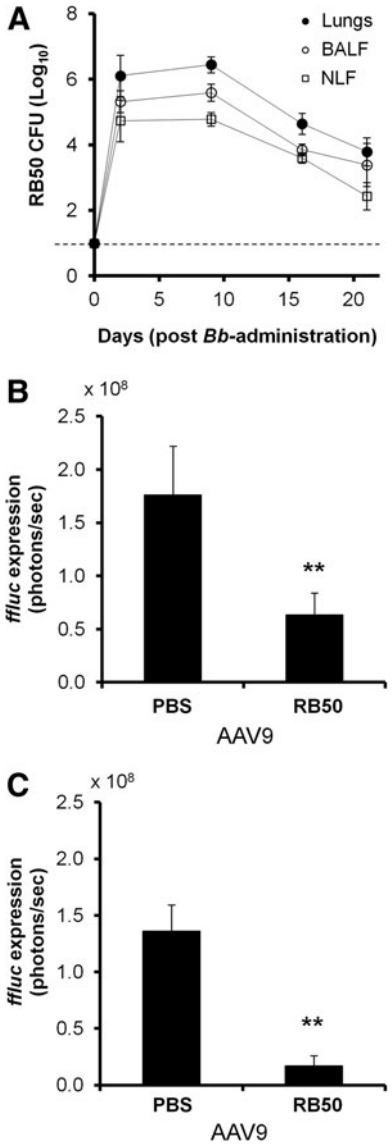

FIG. 2. Effects of a sustained *Bb* RB50 airway infection on AAV9-mediated transduction in C57BL/6 mice. **(A)** RB50 colonization of C57BL/6 mouse airway. Approximately 10^5 CFU of bacteria were delivered i.n. in 30 μl. Bacterial colonization was analyzed through harvest of lungs, BALF, and NLF from 3 to 4 randomly selected mice at specified time points over the course of 21 days. The plots show means ± standard deviations for $n = 4$–11 mice tested at each time point in 4 different experiments. Samples taken from uninfected mice were clear of RB50 at all time points. Dashed line denotes detection limit. **(B** and **C)** AAV9-mediated transduction in wild-type C57BL/6 mice recently infected with RB50. Two or 14 days after receiving intranasal inoculations of either sterile PBS or $\sim 10^5$ CFU of *Bb*, mice were i.n. dosed with AAV9.*ffluc*. Mice were assessed for gene expression 1 week later: **(B)** mice that received AAV on day 2 of infection and **(C)** mice that received AAV on day 14 of infection. Plots show quantification of luminescence from mouse nasal cavity. Results are presented as means, and error bars represent the standard deviation for the six mice imaged at each time point. Significantly lower signal was observed from the nasal cavities of infected mice (**$p < 0.01$, Student's *t*-test, $n = 6$). *Bb*, *Bordetella bronchiseptica.*

BALF cell analysis

Cytospin slides were prepared using 50 μl of freshly isolated BALF diluted into 100 μl of fresh PBS. After centrifugation (Shandon Cytospin 3; Thermo Fisher Scientific) for 5 min at 1,000 rpm, cells were allowed to air-dry for 10 min. Cells were then fixed with 10% neutral buffered formalin, washed in PBS, stained with Nuclear Fast Red (Sigma Aldrich), dehydrated with a series of ethanols, and cleared with xylene according to standard protocols. Cells were studied and characterized using light microscopy (IX81; Olympus America).

Lung immunohistochemistry

Immunofluorescence staining was performed on frozen lung sections. Sections were fixed in acetone at −20°C for 7 min, air-dried, blocked in 1% donkey serum in PBS for 20 min, and incubated with primary rat antibodies diluted in blocking buffer against CD8 (clone 53–6.7; BD Biosciences; 1:20), CD4 (clone RM4-5; BD Biosciences; 1:20), and Mac-2 (clone M3/38; Cedarlane; 1:200) for 45 min. After washing in PBS, sections were stained with secondary donkey antibodies labeled with FITC or TRITC (Jackson Immunoresearch Laboratories) for 30 min, washed in PBS, and mounted in Vectashield containing DAPI (Vector Laboratories). For detection of neutrophils, cryosections were fixed in 4% paraformaldehyde in PBS for 10 min. Sections were then permeabilized and blocked in 0.2% Triton containing 1% donkey serum for 30 min and stained with a rabbit antibody against myeloperoxidase (Abcam) followed by secondary antibodies and washing steps as described above.

BALF cytokine analysis

A multiplex bead assay based on the Luminex technology was used to measure cytokine/chemokine levels in BALF samples that were collected from mice as described earlier. A 25-panel multianalyte cytokine/chemokine kit (Millipore) was used according to the manufacturer's instructions. Briefly, 25 μl of samples were incubated overnight at 4°C with capture beads against G-CSF, GM-CSF, IFN-γ, TNF-α, RANTES, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, and MIP-2 on a rocking platform. The following day, beads were washed using a hand-held magnetic block (Millipore) and stained with biotin antibodies at room temperature for 1 hr. The beads were further stained for 30 minutes using streptavidin detection antibodies before a series of final washes. Beads were resuspended in sheath fluid and samples were read on a Luminex 200 instrument (Luminex), and the levels of each cytokine were determined by regressing against a seven-point standard curve (Bioplex Manager, Biorad).

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2007. The Student's *t*-test was used to determine significance of differences between two groups.

Results

To evaluate the effects of a chronic bacterial infection on gene transfer in the airway, C57BL/6 mice were i.n.

challenged with approximately 10^5 CFU of *Bb* RB50 or sterile PBS. Mice were tested for evidence of infection through day 21 postinfection, and in all RB50-infected mice, bacteria were detected at significant levels (10^4–10^7 CFU) in both the nasal cavity and lung throughout the duration of the study (Fig. 2A). All samples from mice receiving only sterile PBS showed no evidence of RB50 at all time points tested (data not shown). On day 2 or day 14 of infection, mice were given an intranasal dose of AAV9.*ffluc*. One week after AAV administration, mice were subjected to live whole-animal luminescent imaging. At both time points, the luminescent signal in the nasal cavity of the infected mice was significantly lower ($p < 0.01$, Student's *t*-test) than that observed in the nasal cavity of the uninfected mice (Fig. 2B and C). It is worthwhile to note that the loss in expression was greater (eightfold; Fig. 2C) when AAV administration was delayed to day 14 of infection. The significant difference in *ffluc* expression in the nasal cavity between uninfected mice and infected mice receiving AAV on day 14 was sustained for 50 days after AAV delivery (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hum).

To determine whether a transient acute infection would have a similar effect, *P. aeruginosa* PAO1 was used to infect SP-D KO mice. Mice were i.n. challenged with approximately 10^5 CFU of PAO1 and control mice given sterile PBS. On day 2 or day 9 postinfection, a representative sampling of mice (uninfected $n = 3$, infected $n = 4$) was taken to determine the status of the infection in the lungs and nose at the time of AAV administration (Fig. 3A and B). Samples from uninfected mice showed no evidence of PAO1 at both time points tested (data not shown). On day 2, PAO1 was only found in the nasal cavity at relatively low levels (~ 80 CFU). Lung and BALF samples isolated from the lower respiratory tract showed no evidence of infection. By day 9, the majority of mice had cleared the bacteria from their airways. However, despite the limited presence of bacteria in the nasal cavity, *ffluc* expression was still

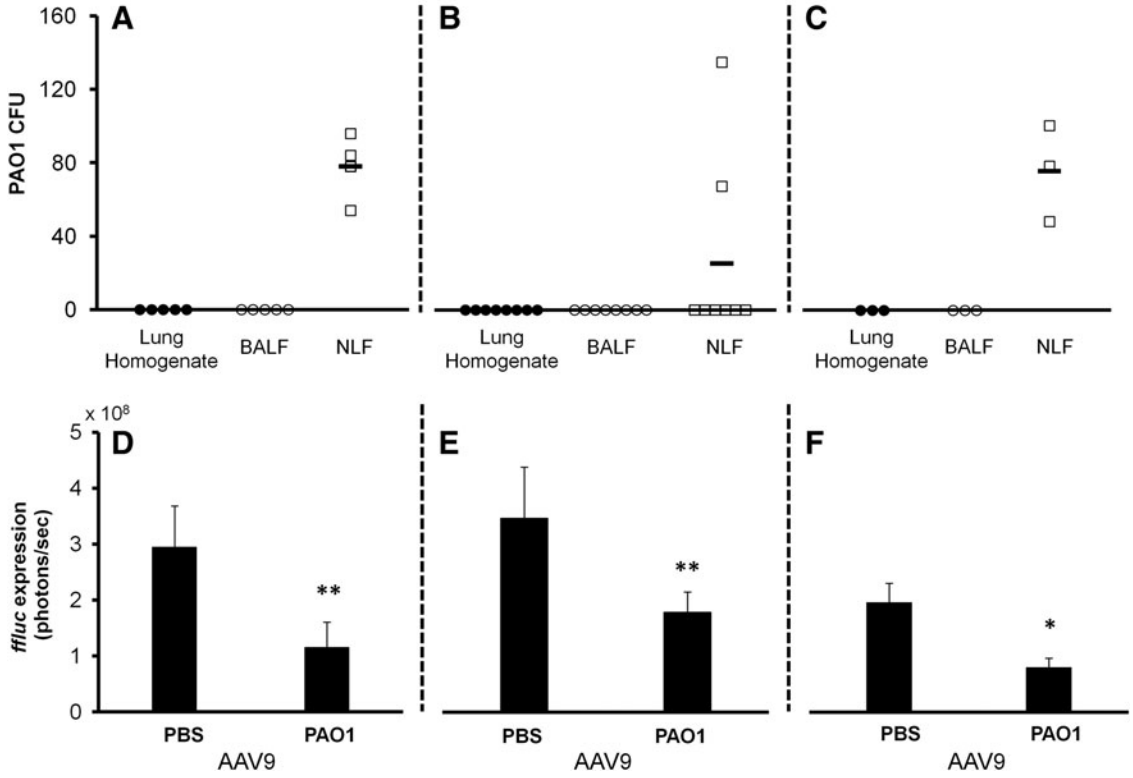

FIG. 3. Effects of a transient *Pseudomonas aeruginosa* PAO1 airway infection on AAV9-mediated transduction in SP-D or SP-A KO mice. **(A–C)** PAO1 colonization of SP-D or SP-A KO mouse airway. Approximately 10^5 CFU of bacteria were delivered i.n. in 30 μl. Bacterial colonization was analyzed through harvest of lungs, BALF, and NLF from three or four randomly selected mice at specified time points: **(A)** SP-D KO mice at day 2 postinfection, **(B)** SP-D KO mice at day 9 postinfection, and **(C)** SP-A KO mice at day 2 after infection. Plots show means ± standard deviations for $n = 5$–7 mice tested at each time point in 3 different experiments for SP-D KO mice and $n = 3$ mice from a single experiment for SP-A KO mice. Samples taken concurrently from uninfected mice were clear of PAO1 at all time points tested. **(D–F)** AAV9-mediated transduction in SP-D or SP-A KO mice recently infected with PAO1. Two or 9 days after being challenged with sterile PBS or approximately 10^5 CFU of *P. aeruginosa* PAO1, mice were i.n. dosed with AAV9.*ffluc*. One week later, mice were imaged for gene expression: **(D)** SP-D KO mice that received AAV on day 2 of infection, **(E)** SP-D KO mice that received AAV on day 9 of infection, and **(F)** SP-A KO mice that received AAV on day 2 of infection. Plots show quantification of luminescence from mouse nasal cavity. Results are presented as means, and error bars represent the standard deviation for the mice imaged at each time point. Significantly lower signal was observed from the nasal cavities of infected mice (**$p < 0.01$, *$p < 0.05$, Student's *t*-test, uninfected $n = 6$, infected SP-D KO mice receiving AAV on day 2 $n = 11$, infected mice SP-D KO receiving AAV on day 9 $n = 8$, $n = 7$ for both SP-A KO mice groups). KO, knockout; SP-A, surfactant protein A; SP-D, surfactant protein D.

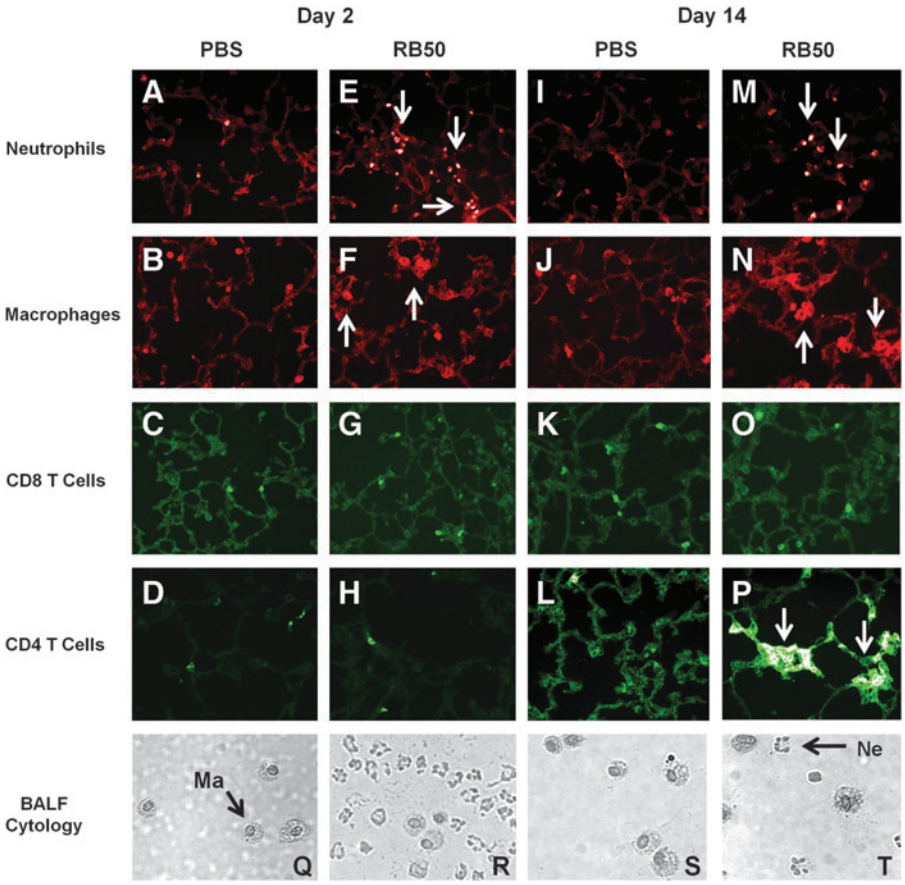

FIG. 4. Characterization of immune response at time of AAV administration in *Bb* chronic infection model. **(A–P)** Lung histology of C57BL/6 mice transduced at specified time points during *Bb* airway infection. Lungs were harvested on the day of AAV administration and sectioned and stained with antibodies against antigens for the specified cell type. Representative images are shown here: sections from **(A–D)** uninfected mice on day 2, **(E–H)** infected mice on day 2, **(I–L)** uninfected mice on day 14, and **(M–P)** infected mice on day 14. Notable neutrophil and macrophage infiltration was observed in samples from infected lungs. Day 9 showed an elevated CD4 T cell population as well. Arrows indicate evidence of elevated cell populations. Magnification, ×20. **(Q–T)** Cytospin preparations of cells recovered from fresh BALF samples taken on the day of AAV administration: **(Q** and **S)** samples taken from uninfected mice on day 2 and day 14, respectively; **(R** and **T)** samples taken from infected mice on day 2 and day 14, respectively. Representative macrophages (Ma) and neutrophils (Ne) are indicated in the figure. Samples from uninfected mice showed only macrophages, while samples from infected mice showed a prominent neutrophil presence in addition to the macrophage cell population at both time points. Color images available online at www.liebertpub.com/hum

significantly lower ($p < 0.01$, Student's *t*-test) in the noses of infected mice for both cases (Fig. 3D and E). Consistent observations were seen at subsequent time points through to day 14 posttransduction (Supplementary Figs. S2–S3).

To determine whether infection would have similar effects on gene expression in mice lacking SP-A, the SP-A KO mice were i.n. challenged with $\sim 10^5$ CFU of PAO1 or sterile PBS. On day 2 postinfection, mice (uninfected $n = 3$, infected $n = 4$) were peeled to determine the status of infection in the SP-A nasal and lung airways at the time of AAV administration (Fig. 2C). As observed in the SP-D KO mice, bacteria were found only in the nasal cavity at relatively low levels (~80 CFU) on day 2 postinfection, and samples from uninfected mice showed no evidence of PAO1 (data not shown). Additionally, similar trends on reporter gene expression were also observed; *ffluc* expression was significantly lower ($p < 0.05$, Student's *t*-test) in the nasal cavity of infected mice (Fig. 2F). Consistent observations were seen at subsequent time points through day 21 posttransduction (Supplementary Fig. SF4).

The immune response at the time of AAV administration was then characterized to provide insights into the observed mechanisms. Cytology on fresh BALF samples indicated that actively infected mice were characterized by substantial neutrophil infiltration on day 2 of infection (Figs. 4R, 5R, and 6J). Samples from uninfected mice only showed evidence of alveolar macrophages (Figs. 4Q, 5Q, and 6I). Interestingly, uninfected SP-D KO mice had a small number of neutrophils in their BALF samples in addition to macrophages that were characterized by a foamy and/or multinucleated appearance (Fig. 5Q). By day 9 post-PAO1 infection, many of the neutrophils had band, rather than segmented, nuclei (Fig. 5T), indicative of a shift toward immature precursors.

Histology on frozen lung sections showed similar trends (Figs. 4–6). Day 2 of infection was characterized by significant neutrophil and macrophage infiltration for the *Bb* chronic infection and the PAO1 acute infection in the SP-A KO model (Figs. 4 and 6E–H). Day 2 of infection was also characterized by an elevated CD8 T cell population in the

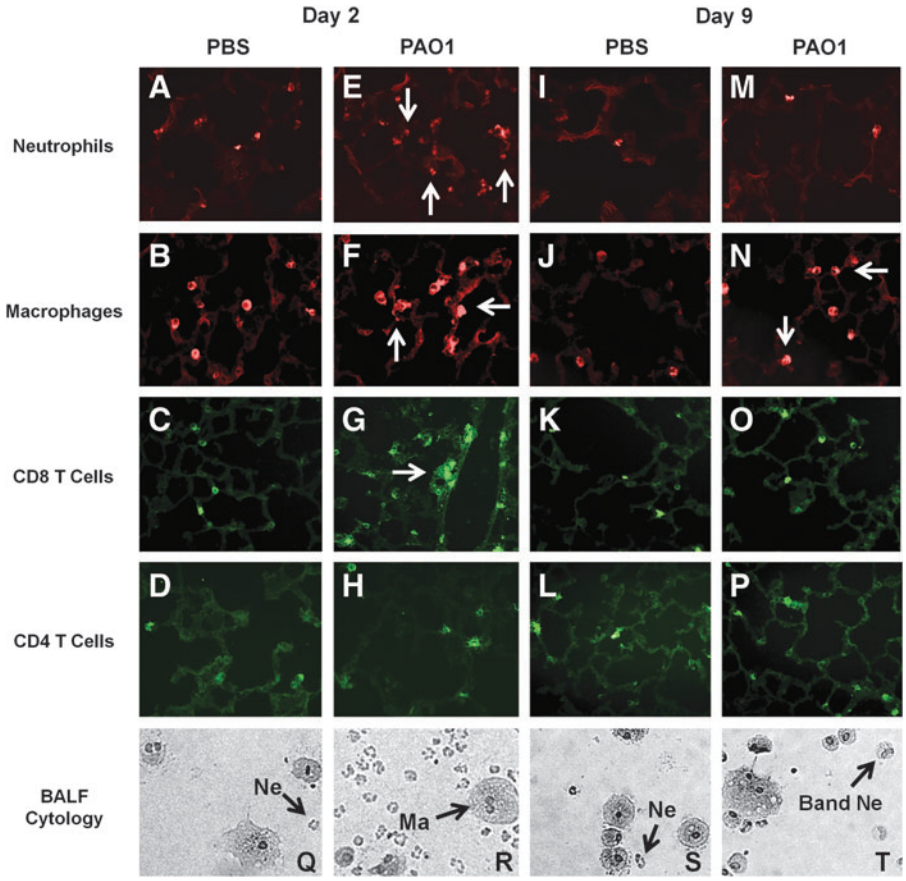

FIG. 5. Characterization of immune response at the time of AAV administration in SP-D KO/PAO1 acute infection model. **(A–P)** Lung histology of SP-D KO mice transduced at specified time points during PAO1 airway infection. Lungs were harvested on the day of AAV administration and sectioned and stained with antibodies against antigens for the specified cell type. Representative images are shown here: sections from **(A–D)** uninfected mice on day 2, **(E–H)** infected mice on day 2, **(I–L)** uninfected mice on day 9, and **(M–P)** infected mice on day 9. Day 2 samples from uninfected mice displayed evidence of neutrophils, macrophages, and CD8 T cells. Infected samples at this time point showed slightly elevated levels of the same cell types, as indicated by the arrows. By day 9, only macrophages remain slightly elevated. Magnification, ×20. **(Q–T)** Cytospin preparations of cells recovered from fresh BALF samples taken on day of AAV administration: **(Q** and **S)** samples taken from uninfected mice on day 2 and day 9, respectively; **(R** and **T)** samples taken from infected mice on day 2 and day 9, respectively. Representative macrophages (Ma), neutrophils (Ne), and band neutrophils (band Ne) are indicated in the figure. Day 2 samples from uninfected mice showed macrophages and a small neutrophilic presence. Infected samples at this same time point displayed a much larger neutrophils population. By day 9, both macrophages and neutrophils were still seen. However, neutrophils on day 9 had band (instead of segmented) nuclei, indicative a shift toward immature precursors. Color images available online at www.liebertpub.com/hum

SP-A KO/PAO1 model (Fig. 6G). Interestingly, in the SP-D KO/PAO1 acute infection model, neutrophils and macrophages were present in the lungs of uninfected mice (Fig. 5A and B). We also observed small numbers of CD8 T cells in the uninfected lung and these increased slightly with infection. By day 9 postinfection with PAO1, lung samples from the SP-D KO mice showed little to no differences between uninfected and infected groups for all cell types detected (Fig. 5I–P). Day 14 of *Bb* infection in C57BL/6 mice, on the other hand, was characterized by elevated neutrophil and macrophage populations (Fig. 4M and N). Additionally, day 14 samples from infected lungs also showed a larger presence of CD4 T cells but not CD8 T cells (Fig. 4O and P).

Analysis of cytokine/chemokine levels in BALF samples isolated from a representative sampling of mice (typically uninfected $n=3$, infected $n=4$) at the time of AAV administration demonstrated the most notable differences on day 2 of infection for all infection models (Fig. 7). Of the 25 analytes measured, 9 of them (GM-CSF, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12p40, IL-13, and IL-15) showed no differences between uninfected and infected groups in any of infection models at any time points used in this study (data not shown). The remaining 16 (G-CSF, IFN-γ, IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17, IP-10, KC, MCP-1, MIP-1γ, MIP-1β, MIP-2, TNF-α, and RANTES) displayed substantial differences between uninfected and infected groups on day 2 postinfection in at least one of the infection models tested (Fig. 7). Determination of statistical significance for differences observed in the SP-A KO/PAO1 model was limited by the number of samples available for analysis ($n=2$ for each group). At the later time points tested, that is, at day 14 after *Bb* infection and at day 9 after PAO1 infection, many of the elevated analytes (i.e., G-CSF, IFN-γ,

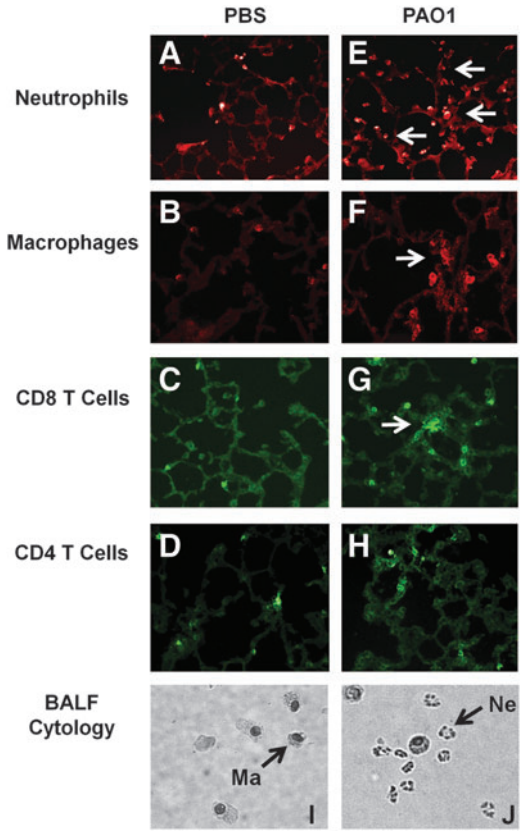

FIG. 6. Characterization of immune response at time of AAV administration in SP-A KO/PAO1 acute infection model. **(A–H)** Lung histology of SP-A KO mice transduced on day 2 during PAO1 airway infection. Lungs were harvested on the day of AAV administration and sectioned and stained with antibodies against antigens for the specified cell type. Representative images are shown here: sections from **(A–D)** uninfected mice and **(E–H)** infected mice. On day 2, elevated levels of neutrophils, macrophages, and CD8 T cells were evident in samples from infected lungs (see arrows). Magnification, ×20. **(I–J)** Cytospin preparations of cells recovered from fresh BALF samples taken on day of AAV administration: **(I)** samples taken from uninfected mice and **(J)** samples taken from infected mice. Representative macrophages (Ma) and neutrophils (Ne) are indicated in the figure. Samples from uninfected mice showed only macrophages, while infected samples showed obvious neutrophil infiltration in addition to the macrophage cell population. Color images available online at www.liebertpub.com/hum

IL-1β, IL-6, IL-10, IL-12p70, IP-10, MCP-1, and MIP-1γ) returned to baseline concentrations observed in samples from uninfected mice. Those that did not return to baseline included IL-1α, IL-17, KC, MIP-1β, MIP-2, TNF-α, and RANTES in samples obtained from *Bb*-infected mice. In the SP-D KO/PAO1 model, levels of all cytokines and chemokines had returned to baseline by day 9 postinfection.

Discussion

CF airway disease remains an ideal candidate for treatment by gene therapy, despite its limited success seen so far. Individuals with CF are characterized by chronic respiratory infections that are primarily responsible for the high morbidity and early mortality rates associated with the disease. However, limited research has been done to determine the effects of active airway infections on pulmonary gene transfer. The lack of research on this topic is not surprising as no convenient animal model exists to study such effects. Three different mouse models were established in this study to remedy this issue. The first involved creating a chronic infection in C57BL/6 mice with a natural murine pathogen, *Bb* RB50. The second and third models utilized PAO1 to establish an acute, but persistent, infection in SP-D and, separately, SP-A KO mice, respectively.

When an AAV vector was administered to the airways of infected mice on day 2 postinfection, transduction efficiency was similarly reduced in all mouse models, despite remarkable differences in the duration and intensity of all infection models. The approximate threefold reduction in gene expression was seen regardless of several additional factors, including the bacterium used, the strain of mouse used, or the bacterial load at the time of AAV administration. This suggests that immune response at the time of vector administration was the main cause for the observed reduction in gene transfer efficiency.

Characterization of these immune responses at day 2 of infection showed that the responses in each infection model were generally similar to each other. Analyses showed that day 2 inflammatory responses were dominated by macrophage and neutrophil infiltration, the latter of which is characteristic of the infected CF lung (Makam *et al.*, 2009). However, in the SP-D KO/PAO1 acute infection model, elevated levels of macrophages and neutrophils were present even in uninfected lung samples, suggesting that their presence alone is insufficient to reduce gene transfer.

While immune responses at day 2 were generally similar between all infection models studied, one striking difference was observed between the chronic and transient acute infection models. In the SP-D and SP-A KO/PAO1 infection models, CD8 T cell activation/recruitment was observed in the infected lungs. In the lungs of *Bb*-infected mice, the numbers of CD8 T cells were not elevated compared with uninfected mice. Despite this apparent difference, we observed a similar level of reduction in gene expression in all three infection models, suggesting that CD8 T cells are not directly involved in the reduction of gene transfer.

When AAV administration was delayed to a later time point postinfection, different effects on AAV-mediated gene expression were seen between the *Bb* chronic infection and the SP-D KO/PAO1 acute infection models. In the *Bb* chronic infection model, postponement of AAV administration to day 14 postinfection resulted in a significant (8-fold, $p < 0.01$, Student's *t*-test) reduction in gene expression, when compared with the expression seen in uninfected controls. In the SP-D KO/PAO1 acute infection model, however, delay of AAV administration to day 9 postinfection resulted in a $\sim$2-fold reduction. The notable differences observed in the immune response at these time points include (1) elevated levels of macrophages, segmented neutrophils, and CD4 T cells in *Bb*-infected mice but not in PAO1-infected mice and (2) elevated levels of IL-1α, IL-17, KC, MIP-1β, MIP-2, TNF-α, and RANTES in *Bb*-infected mice but not PAO1-infected mice. While PAO1-infected SP-D mice did display elevated levels of neutrophils in their BALF samples, many of these cells showed a band, rather than a segmented, nucleus, indicative of immature

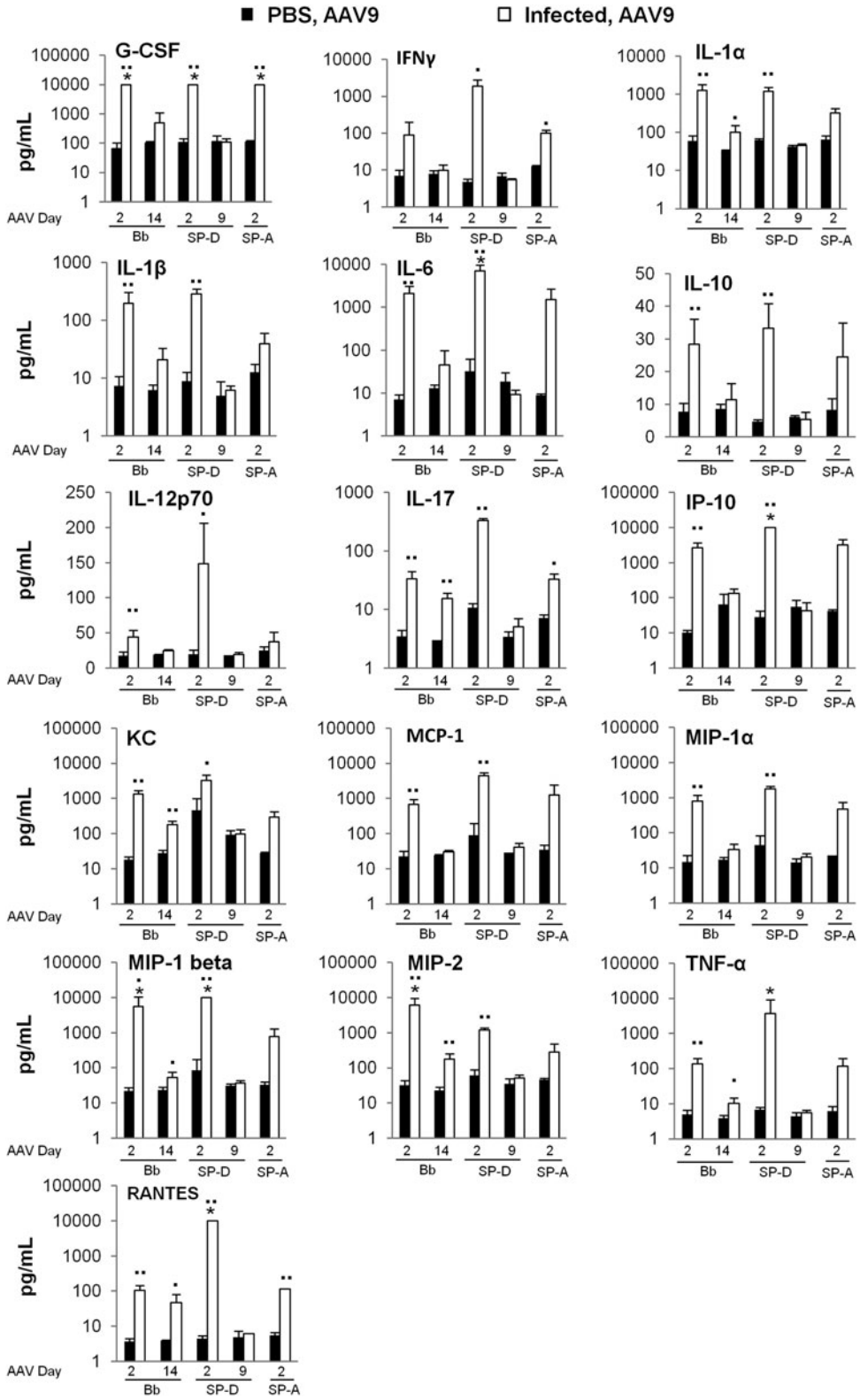

FIG. 7. BALF cytokine/chemokine levels at time of AAV administration. Cytokine/chemokine levels were determined using a Milliplex 25-plex premixed magnetic mouse cytokine/chemokine array and Luminex bead reader according to the manufacturer's instructions. Single asterisk (*) indicates samples that exceeded the upper limit of detection of the assay, approximately 10,000 pg/ml. Results are presented as means, and error bars represent the standard deviation (■■ $p < 0.01$, ■ $p < 0.05$, Student's *t*-test, uninfected $n = 2$–5, infected $n = 3$–7 for all groups except SP-A KO infected mice, where $n = 2$ for both uninfected and infected mice).

neutrophilic precursors. Taken together, these results indicate that activated, mature neutrophils and CD4 T cells may be responsible for causing significant reduction in gene expression observed in the *Bb*-infected mice. In particular, the presence of the CD4 T cells may be playing the more significant role, as that cell type was not present at the earlier AAV administration time point (day 2 postinfection), which reduced gene expression by only ~3-fold.

Additionally, it is worthwhile to note that one of the elevated cytokines on day 14 of the chronic infection, IL-17, is produced by CD4 T cells and has been implicated in the stimulation of airway mucin gene expression (Chen *et al.*,

2003). This could suggest the development of an enhanced mucus barrier by day 14 of the chronic infection. Such a phenomenon could explain the significant loss in gene transfer efficiency at this later time point for AAV administration. The enhanced mucus layer would prevent transduction by hindering normal transport of vector to the target cells.

Regardless, it is likely that in all our models, the inflammatory microenvironment and cytokine release profile following infection resulted in the breaking of immune tolerance to AAV particles and/or their gene products. In several studies, AAV vector administrations targeting liver have proven to be safe and rarely elicit an immune response, even when challenged with an adenovirus expressing a similar transgene. However, co-administration of TLR ligands, including CpG-containing oligodeoxynucleotides (ODNS), resulted in loss in liver transgene expression (Breous *et al.*, 2011; Martino *et al.*, 2009). Additionally, CpG-containing ODNs have also been proven to be potent adjuvants for adenovirus-mediated vaccinations against certain tumor antigens (Rieger and Kipps, 2003; Salucci *et al.*, 2006). In these studies, adenovirus vectors expressing the targeted antigens are coadministered with CpG ODNs in order to trigger a sufficient immune response that establishes immunity against those antigens. With this in mind, it would be unsurprising if the bacterial presence in our infection models is serving as a form of adjuvant, triggering sufficient immunity to the AAV vector and/or to the ffluc product to result in a diminished expression or visualization thereof.

In each of our models, free bacteria were administered to the nares of healthy mice to facilitate a prolonged infection of the respiratory tract. The administration of free bacteria contrasts with the established practice of artificially embedding bacteria in agar beads before depositing them into the airway through a tracheal incision. The methods presented here are thus simpler and less invasive. Additionally, our methods do not limit the infection to the conducting airways, which is not the case with methods involving embedded beads. Because of their size, the beads create a mechanical block to the bronchi, resulting not only in limited bacterial access to the respiratory airways but also in increased morbidity, unwanted lung damage, and potentially even collapse or closure of the lung (Wu *et al.*, 2000).

To the best of our knowledge, this the first study to examine the effects of an active, naturally occurring respiratory infection on AAV airway gene transfer. Our findings clearly show that the efficiency of AAV-mediated gene transfer in the airway is negatively impacted by the presence of an on-going or recently resolved bacterial infection. These results underscore the importance of considering such circumstances when developing potential genetic treatments for relevant diseases like CF and potentially other airway diseases such as asthma. The methods validated here provide simple and minimally invasive techniques that will allow this type of infected environment to be more easily accounted for.

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Author Disclosure Statement

No competing financial interests exist.

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