Influenza Virus Infection Decreases Tracheal Mucociliary Velocity and Clearance of *Streptococcus pneumoniae*

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Influenza virus infections increase susceptibility to secondary bacterial infections, such as pneumococcal pneumonia, resulting in increased morbidity and mortality. Influenza-induced tissue damage is hypothesized to increase susceptibility to *Streptococcus pneumoniae* infection by increasing adherence to the respiratory epithelium. Using a mouse model of influenza infection followed by *S. pneumoniae* infection, we found that an influenza infection does not increase the number of pneumococci initially present within the trachea, but does inhibit pneumococcal clearance by 2 hours after infection. To determine whether influenza damage increases pneumococcal adherence, we developed a novel murine tracheal explant system to determine influenza-induced tissue damage and subsequent pneumococcal adherence. Murine tracheas were kept viable *ex vivo* as shown by microscopic examination of ciliary beating and cellular morphology using continuous media flow for up to 8 days. Tracheas were infected with influenza virus for 0.5–5 days *ex vivo*, and influenza-induced tissue damage and the early stages of repair to the epithelium were assessed histologically. A prior influenza infection did not increase pneumococcal adherence, even when the basement membrane was maximally denuded or during the repopulation of the basement membrane with undifferentiated epithelial cells. We measured mucociliary clearance *in vivo* and found it was decreased in influenza-infected mice. Together, our results indicate that exposure of the tracheal basement membrane contributes minimally to pneumococcal adherence. Instead, an influenza infection results in decreased tracheal mucociliary velocity and initial clearance of pneumococci, leading to an increased pneumococcal burden as early as 2 hours after pneumococcal infection.

Keywords: influenza virus; *Streptococcus pneumoniae*; mucociliary velocity; bacterial clearance and adherence; tracheal explants

CLINICAL RELEVANCE

Using a novel tracheal explant system, this research shows that influenza-induced damage to the tracheal epithelium does not increase pneumococcal adherence to the tracheal epithelium. It shows that an influenza infection increases the number of pneumococci within the trachea by inhibiting tracheal mucociliary velocity and clearance of pneumococci. This research will lead to a better understanding of how to prevent and/or treat secondary bacterial pneumonia after an influenza infection.

Secondary bacterial infections such as pneumonia caused by *Streptococcus pneumoniae* are associated with an increase in morbidity and mortality during influenza epidemics and pandemics (1–13). In 2002, influenza and pneumonia infections were the number one cause of infectious death in the United States (14). With the threat of an influenza pandemic in the near future, and rapidly increasing antibiotic resistance, it is critical to understand how a prior influenza infection increases susceptibility to *S. pneumoniae* infection. Several possible mechanisms may account for the increase in pneumococcal pneumonia associated with influenza infection, including influenza-induced damage to the tracheal epithelium and/or alterations in host cellular responses to bacterial pathogens (15–17). Although the exact mechanism(s) for increased susceptibility to secondary *S. pneumoniae* infections after influenza infection are not known, two competing hypotheses have been advanced to explain concurrent increased susceptibility. The first hypothesis is that influenza-induced phagocytic dysfunction (such as impaired neutrophil chemotaxis and bactericidal activities) increase susceptibility to *S. pneumoniae* infections (17–22). The other hypothesis states that influenza-induced damage to the respiratory epithelium alters the cellular characteristics of the epithelium by exposing surface molecules and cell receptors to which pneumococci more readily adhere (4, 23–25). However, another possibility is that epithelial damage could also result in defective mucociliary clearance mechanisms, leading to increased numbers of bacteria remaining in the respiratory tract. Nugent and Pesanti (15) found that influenza-infected mice cleared *Staphylococcus aureus* from their tracheas less efficiently than non–influenza-infected mice.

Previous studies have used *in vitro* cell culture assays to measure the adherence of *S. pneumoniae* to influenza-infected lung and bronchial epithelial cells (3, 26, 27). However, a limitation of this method is that undifferentiated or immortalized cells in monolayers may not express the same surface molecules as differentiated cells of the intact respiratory epithelium, and, therefore, may not represent cell types present during *in vivo* influenza infections. Several different cell types are exposed during the course of an influenza virus infection, including ciliated, pseudostratified, columnar epithelial cells, microvilli-covered cells, goblet cells, basal cells, undifferentiated epithelial cells, and differentiating epithelial cells (16, 24, 28). Influenza virus infection is cytolytic, resulting in denudation of the epithelium and exposure of the basement membrane (24). Consequently, pneumococci may adhere more readily to components of the basement membrane, such as fibronectin (29) and/or different cell types or proteins exposed or expressed during an influenza infection (23).

Tracheal explant systems, such as tracheal rings or whole-organ explants, from a variety of animal species, including hamsters, mice, rats, ferrets, pigs, guinea pigs, and chinchillas, have been widely used as an alternative to *in vitro* cell culture assays (30–39). Tracheal explants have been used to investigate the effects of toxins, such as mineral dusts, air pollution particles, and cigarette smoke, on tracheal function (32, 33, 39). In addition,

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the interactions of several bacterial and viral pathogens, such as *Mycoplasma* spp., *Bordetella* spp., influenza virus, cytomegalovirus, and *S. pneumoniae*, with the respiratory epithelium have been studied (30, 31, 34–38). In particular, *S. pneumoniae* adherence has been studied using chinchilla tracheal explants (30, 38). A distinct advantage of a tracheal explant system is that the differentiated tissue more accurately reproduces influenza and respiratory epithelial cell interactions with pneumococci than do *in vitro*–grown cellular monolayer assays of adherence.

In the present study, using an *in vivo* model of coinfection, we found that a prior influenza infection of 3 or 6 days did not affect the number of pneumococci in tracheas during the initial stages of pneumococcal infection (0–60 min). However, a prior influenza infection significantly increased the number of *S. pneumoniae* CFUs remaining within the trachea (either attached to the epithelium or within the lumen) after 2 hours of pneumococcal infection. These data suggest that an influenza infection either increases pneumococcal adherence to the tracheal epithelium and/or decreases pneumococcal clearance via the mucociliary escalator. Because influenza infection affects other host immune factors, such as neutrophil function, it is difficult to determine the extent of influenza-induced tissue damage as a contributing factor to the susceptibility to a *S. pneumoniae* infection *in vivo* (17–22). Therefore, we developed an *ex vivo* murine tracheal explant system to mimic influenza-induced tissue damage seen *in vivo* to determine how different stages of an influenza infection (including the initial presence of virus-infected cells, influenza-induced damage of the respiratory epithelium and resultant exposure of the basement membrane, and repair of the respiratory epithelium) affect *S. pneumoniae* adherence to the tracheal respiratory epithelium. Specifically, the tracheal explant model allows the effects of influenza-induced tissue damage on *S. pneumoniae* adherence to be determined independently of influenza-induced effects on the immune system, such as neutrophil dysfunction. In addition, this model allows us to simulate physiological shear conditions that may occur *in vivo* by adjusting the flow of media through the tracheas. Using our novel tracheal explant system, we found that an *ex vivo* influenza infection, resulting in exposure of the tracheal basement membrane, does not increase *S. pneumoniae* adherence to the tracheal epithelium. To determine whether influenza virus infection inhibits mucociliary clearance of pneumococci, we measured tracheal mucociliary velocity, and found that a prior influenza infection decreases tracheal mucociliary velocity, which is one mechanism that leads to decreased bacterial clearance. This decreased clearance would lead to increased pneumococcal numbers within the tracheal lumen and possible subsequent increased adherence to the tracheal epithelium. Together, our *in vivo* and *ex vivo* data indicate that the increased *S. pneumoniae* burden after an influenza infection *in vivo* most likely is due to influenza-induced defects in mucociliary clearance of pneumococci, and is not due to increased adherence of pneumococci to the damaged tracheal epithelium.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice, ages 10 to 16 weeks, from the National Cancer Institute (Frederick, MD) were used for all experiments. Animals were housed at the Montana State University Animal Research Center. All animal protocols were approved by the Montana State University Institute for Animal Care and Use Committee.

Infectious Agents

Influenza virus A/PR8/8/34 (PR8; H1N1) from the Trudeau Institute (Saranac Lake, NY) was used for all influenza infections. The virus was grown in the allantoic fluid of embryonated chick eggs and stored at −80°C. *S. pneumoniae* type 4 (ATCC 6304; American Type Culture Collection, Manassas, VA) was grown in Todd Hewitt broth (Becton Dickinson and Co., Sparks, MD) supplemented with 0.5% yeast extract (THY; Fisher Scientific, Fair Lawn, NJ) at 37°C with 5% CO_2 until mid–log phase of growth. Stock cultures in THY with 10% glycerol (Sigma, St. Louis, MO) were snap frozen in liquid nitrogen and stored at −80°C until needed. Before use, stock cultures were washed twice at 16,000 × *g* with Dulbecco's PBS (D-PBS; Gibco, Grand Island, NY) and resuspended to the appropriate concentration. *S. pneumoniae* CFUs were approximated using the optical density at 450 nm. CFUs were confirmed by plating serial dilutions on neomycin blood agar plates (30 μg/ml neomycin; Becton Dickinson Microbiology Systems, Cockeysville, MD) using the drop plate method, as previously described (40).

In Vivo Infection Model: Pneumococcal Adherence and Clearance

Mice were lightly anesthetized with isoflurane and infected intranasally with 600 plaque-forming units (PFUs) PR8 influenza virus in 100 μl of sterile Hanks' balanced salt solution (HBSS; 50 μl/nare). *S. pneumoniae*–infected–only mice were mock influenza–infected intranasally with 100 μl HBSS for 6 days. At either Day 3 or 6 after influenza infection, mice were challenged intranasally with 10^7 CFUs of *S. pneumoniae* in 100 μl of sterile D-PBS (50 μl/nare) for 0 to 2 hours. At various time points after *S. pneumoniae* infection (0, 30, 60, or 120 min), mice were killed by deep pentobarbital anesthesia followed by exsanguination, and the tracheas were removed, placed in 2 ml HBSS, snap frozen in liquid nitrogen, and stored at −80°C. *S. pneumoniae* CFUs and influenza PFUs were enumerated as described subsequently here.

Tracheal Explant Media

Tracheal explants were maintained with continuous media flow through the tracheal lumen of RPMI 1640 (Gibco) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO), Hepes (20 mM; Gibco), neomycin (30 μg/ml; Cellgro, Herndon, VA), and gentamicin (5 μg/ml; Gibco) (cRPMI) at a flow rate of 0.1 ml/minute. Before influenza infection, tracheal explants were incubated for 24 hours with continuous flow of cRPMI supplemented with penicillin (200 units/ml) and streptomycin (200 μg/ml) (cRPMI-pen/strep; Gibco) at a flow rate of 0.1 ml/minute to remove normal bacterial flora from the explants.

Tracheal Explant System

Mice were anesthetized intraperitoneally with sodium pentabarbitol and exanguinated. For each tracheal explant, the trachea was separated from the esophagus and cut below the larynx. The trachea, lungs, and heart were removed *en bloc* and placed in a sterile Petri dish, where the heart was removed. The lungs were transected below the bronchial bifurcation, leaving part of the lungs attached to the trachea for added weight. The excised trachea was then placed in cRPMI. A blunted 18-gauge needle (Becton Dickinson and Co.) was inserted through an opening in the filter cap of a sterile 0.2-μm vented cap 162 cm^2 cell culture flask (Corning Inc., Corning, NY), and the cap was removed from the flask. The needle was then inserted into the trachea, and the cap and attached trachea were placed back onto the flask. The portion of lung still attached to the trachea caused the trachea to hang straight down from the point of attachment to the needle.

To sterilely maintain medium influent, two holes were burned into the lid of a sterile 1,000-ml filter unit receiver (Nalge Nunc International, Rochester, NY) with a 45-mm neck using a heated 1/8" slotted screwdriver. Two 100- to 200-μl Drummond Microdispenser replacement tubes (Drummond Scientific Co., Broomall, PA) were placed into each of the holes and sealed using a silicone adhesive sealant (Henkel Corporation, Rocky Hill, CT). To allow filtered air into the system to aerate the medium, 5.5 cm of 1.85-mm internal diameter (ID) silicone tubing (Cole-Parmer Instrument Co., Vernon Hills, IL) was attached to the exterior end of one of the glass tubes, and a 0.2-μm, 10-mm membrane filter (Whatman Inc., Clifton, NJ) was attached to the tubing using a 1/16" male luer fitting (Cole-Parmer). To the interior end of the other glass tube, 22 cm of 2.29-mm ID silicone tubing (Cole-Parmer) was attached and placed in the medium to allow for media flow. The exterior end of the tube was attached to 83 cm of 1.52-mm ID

silicone tubing (Cole-Parmer), which was attached to the yellow end of two-stop tubing (1.52-mm ID yellow/blue; Cole-Parmer) with a straight 1/16" connector (Cole-Parmer). The blue end of the two-stop tubing was attached to 107 cm of 1.52-mm ID silicone tubing with a straight 1/16" connector. This tubing was connected to a 5" microbore extension set with a "T" connector and prepierced injection site (needle port) (Abbott Laboratories, North Chicago, IL) using a male 1/16" luer fitting. The microbore extension kit was then inserted into the 18-gauge needle in the flask with the trachea, as described above. The two-stop tubing was connected to an Ismatec IPC High Precision Multichannel Dispenser pump (Cole-Parmer), and the flow rate was calibrated to 0.1 ml/minute. Before use, the medium reservoir lid and all attached glass tubes and silicone tubing were autoclaved for sterility.

Tracheal Explant Infection Model

Tracheal explants were incubated at 37°C and 5% CO_2 for 24 hours with cRPMI-pen/strep at a continuous flow rate of 0.1 ml/minute to remove normal bacterial flora. After 24 hours, the medium was changed to cRPMI without pen/strep. To assess viability of tracheal explants, uninfected tracheas were incubated for up to 8 days. Trypan blue (MP Biomedicals, LLC, Aurora, OH) was used to measure the volume needed to fill both the needle and the tracheal lumen. For influenza-infected tracheas, medium flow was stopped, and approximately 5×10^5 PFUs of PR8 in 100 μl HBSS were added by injecting a 23-gauge needle (Becton Dickinson and Co.) into the needle port. To allow for viral attachment, tracheal explants were incubated with PR8 for 1 hour before resuming flow with medium. Tracheal explants were then incubated for up to 5 days with or without PR8 at 37°C and 5% CO_2.

S. pneumoniae was added to the tracheal explants at designated time points after influenza infection. Frozen stocks of *S. pneumoniae*, as described previously here, were used for all experiments. The inoculum was prepared by washing the *S. pneumoniae* twice in D-PBS and resuspending the bacteria with D-PBS to the desired concentration. To confirm actual CFUs of the inoculum, serial dilutions of the inoculum were plated.

For *S. pneumoniae* adherence assays, medium flow was stopped and 1 ml of inoculum with approximately 5×10^7 CFUs *S. pneumoniae* (resulting in $\sim 10^6$ CFUs within tracheal lumen) was injected into the needle port with a 23-gauge needle. Tracheal explants were then incubated for 1 hour with *S. pneumoniae* to allow for *S. pneumoniae* adherence. Medium flow was then resumed for 30 minutes to remove nonadherent *S. pneumoniae*. To measure adherence based on initial inoculum size of *S. pneumoniae*, tracheal explants were incubated with *S. pneumoniae* as described above, except with the following concentrations of *S. pneumoniae*: 5×10^3, 5×10^5, 5×10^6, 5×10^7, or 5×10^8 CFUs/ml/trachea. The approximate numbers of *S. pneumoniae* CFUs within the tracheal lumen were 10^2, 10^4, 10^5, 10^6, and 10^7, respectively.

Quantification of PFUs and CFUs

At designated time points, the remaining lung tissue from the explants was removed, and the tracheas were homogenized through a sterile wire screen. Samples were serially diluted and plated using the drop plate method (40) to enumerate *S. pneumoniae* CFUs. Plates were incubated overnight at 37°C and 5% CO_2. For PFU enumeration, the remainder of each sample was snap frozen in liquid nitrogen and stored at −80°C. Samples were serially diluted and incubated with Madin-Darby canine kidney cells using the plaque assay method, as previously described (41).

Histologic Examination

Ciliary beating was visualized microscopically using a Zeiss LSM 510 Meta Confocal Microscope (Carl Zeiss Microimaging, Thornwood, NY) and an Olympus CK2 inverted microscope (Olympus America, Inc., Melville, NY). Tracheas were instilled with 3% agarose and cut into 750-μm rings using a Leica VT1000S vibratome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). The agarose plugs were removed from the rings using forceps, and the rings were placed in cRPMI for microscopy.

For morphological examination of the respiratory epithelium, tracheal explants were fixed at various time points after incubation with or without influenza infection for at least 24 hours in 10% phosphate-buffered formalin (Fisher Scientific), embedded in paraffin, and cut into 5-μM sections. Sections were then stained using hemotoxylin and eosin (H&E), and morphology of the respiratory epithelium was examined using a Nikon Eclipse E800 microscope (Nikon, Inc., Melville, NY). For visualization of β-tubulin and Clara cell secretory protein (CCSP), sections were blocked with avidin solution (Vector Laboratories Inc., Burlingame, CA) in D-PBS/0.2% BSA (Serologicals Corp., Norcross, GA) (D-PBS/BSA) for 15 minutes, rinsed with D-PBS/BSA, blocked with biotin solution (Vector Laboratories) in D-PBS/BSA for 15 minutes, and rinsed with D-PBS/BSA. For β-tubulin detection, slides were incubated with 50 μg/ml mouse anti–β-tubulin IV antibody (BioGenex, San Ramon, CA) using the Vector M.O.M. Fluorescein kit (Vector Laboratories). Control slides were incubated with 50 μg/ml mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). For CCSP staining, slides blocked with avidin solution in 5% normal rabbit serum (Jackson Immunoresearch Laboratories) and 5% normal donkey serum (Chemicon International, Temecula, CA) (NR/NDS) for 15 minutes, rinsed with D-PBS/BSA, blocked with biotin solution in NR/NDS for 15 minutes, and rinsed with D-PBS/BSA. Slides were incubated with 4 μg/ml goat anti-Clara cell 10 protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For CCSP staining, all antibodies were diluted in 2.5% NR/NDS and all incubations were 30 minutes at room temperature, followed by rinses with D-PBS/BSA. Control slides were incubated with 4 μg/ml goat IgG (Jackson ImmunoResearch Laboratories). All slides were incubated with 4 μg/ml Alexa Fluor 546 rabbit anti-goat IgG antibody (Molecular Probes, Invitrogen Corporation, Carlsbad, CA), followed by three rinses with pure D-PBS. Slides were coverslipped with Immu-Mount mounting medium (Thermo Shandon, Pittsburgh, PA). Slides were viewed using the Nikon Eclipse E800 microscope and imaged using the Nikon Digital Camera DXM1200 and MetaVue software (Molecular Devices Corp., Downingtown, PA).

For visualization of influenza virus and β-tubulin, tracheal explants were instilled with Tissue-Tek OCT (Sakura Finetek, Torrance, GA) and snap frozen in liquid nitrogen. Frozen tracheal explants were cut into serial 5-μm sections and air-dried. For visualization of tissue structure in adjacent sections, tissue sections were stained using hematoxylin and eosin. For immunofluorescence, tissue sections were fixed in acetone/alcohol (75/25) at room temperature for 5 minutes, then washed three times for 3–5 minutes each in D-PBS. Sections were blocked with avidin solution (Vector Laboratories) in (D-PBS/BSA) for 15 minutes, rinsed with D-PBS/BSA, blocked with biotin solution (Vector Laboratories) in D-PBS/BSA for 15 minutes, and rinsed with D-PBS/BSA. For β-tubulin detection, slides were stained as described above. For influenza detection, slides were blocked with avidin solution in 5% normal goat serum (ICN Biomedicals, Inc., Aurora, OH) and 5% normal donkey serum (Chemicon International) (NG/NDS) for 15 minutes, rinsed with D-PBS/BSA, blocked with biotin solution in NG/NDS for 15 minutes, and rinsed with D-PBS/BSA. Slides were incubated with 4 μg/ml biotin conjugated goat anti-influenza virus A H1N1 (Accurate Chemical and Scientific Corporation, Westbury, NY). For influenza staining, all antibodies were diluted in 2.5% NG/NDS and all incubations were 30 minutes at room temperature followed by rinses with D-PBS/BSA. Control slides were incubated with 4 μg/ml biotinylated goat IgG (Jackson ImmunoResearch Laboratories). All slides were incubated with 10 μg/ml streptavidin Alexa Fluor 568 conjugate (Molecular Probes, Invitrogen Corporation) followed by three rinses with pure D-PBS. Slides were coverslipped with Immu-Mount mounting medium (Thermo Shandon). Slides were viewed using the Nikon Eclipse E800 microscope and imaged using the Nikon Digital Camera DXM1200 and MetaVue software (Molecular Devices Corp.).

Mucociliary Velocity Measurements

Mucociliary velocities of uninfected and influenza-infected tracheas were calculated by measuring the movement of latex beads using previously described protocols, with slight modifications (42, 43). Tracheas of anesthetized mice were exposed, and a small anterior window was cut. Tissue surrounding the trachea was held to the side using two 26-gauge needles. Mice were placed on a 30° inclined platform inside a 37°C humidified chamber, with their heads elevated above their bodies. Water was constantly pumped through the chamber

to maintain a consistent temperature. Deep blue 0.8-μm latex beads (Sigma) were diluted in an equal volume of PBS, and 2 μl were placed on the posterior mucosal surface of the inferior end of the trachea. Movement of the beads was measured using an ocular micrometer on a stereomicroscope. Mucociliary velocity (mm/min) was calculated by determining the length of time required for movement of beads over a defined distance (0.58 mm). If no bead movement was detected after 1 minute, the mucociliary velocity was recorded as 0 mm/min. Velocity measurements were repeated five times for each mouse to determine the mean mucociliary velocity for each mouse. To verify influenza virus infections in mice used for mucociliary velocity measurements, lungs were snap-frozen, homogenized, and influenza virus PFUs were enumerated as described previously (41).

Statistical Analysis

Data were analyzed using one-way ANOVA followed by the Bonferroni post test (GraphPad Prism Software, San Diego, CA). Significant differences were reported for P values of less than 0.05.

RESULTS

In Vivo S. pneumoniae Adherence and Clearance

We recently showed that a prior influenza infection of 6 days, but not 3 days, significantly increased the severity of a secondary pneumococcal pneumonia (17). To determine whether a prior *in vivo* infection increases initial adherence of *S. pneumoniae* to the tracheal epithelium *in vivo*, thereby increasing the likelihood of progression to pneumonia, we compared *S. pneumoniae* adherence to tracheas from uninfected mice to that of tracheas from mice infected with influenza for 3 or 6 days. Tracheas from mice infected with influenza for 3 or 6 days had similar influenza PFUs, whereas those from uninfected mice had none (Figure 1A). A prior influenza infection of either 3 or 6 days did not increase the number of pneumococci initially adherent to the tracheas (0 min) (Figure 1B). At 30 and 60 minutes after infection, pneumococcal numbers did not significantly differ between any of the groups examined. Together, these data indicate that a prior influenza infection of 3 or 6 days does not affect the number of pneumococci present within the trachea during the initial stages of a pneumococcal infection (0–60 min).

To determine whether a prior influenza infection affects tracheal bacterial clearance, we compared the number of pneumococci present after 120 minutes of infection to the number initially present in non–influenza-infected mice and in mice infected with influenza virus for 3 or 6 days. By 120 minutes after pneumococcal infection, non–influenza-infected mice had cleared 98.9% of the initial pneumococci from their tracheas, resulting in significantly fewer pneumococci remaining in their tracheas at 120 minutes than at 0 minutes ($P < 0.001$; Figure 1B). Mice infected with influenza for 3 days did not clear pneumococci from their tracheas during the 120-minute pneumococcal infection ($P > 0.05$; Figure 1B). Mice infected with influenza for 6 days had 528.6% more pneumococci present in their tracheas after 120 minutes than they initially had at 0 minutes ($P < 0.001$; Figure 1B).

Importantly, a prior influenza infection of 3 or 6 days significantly decreased pneumococcal clearance by 120 minutes after pneumococcal infection compared with uninfected mice ($P < 0.001$; Figure 1B). Mice infected with influenza for 3 days had 100-fold more pneumococci remaining in their tracheas than uninfected mice ($P < 0.001$; Figure 1B). In tracheas from mice infected with influenza for 6 days, there was a 220-fold increase in the number of pneumococci remaining compared with pneumococci in tracheas from uninfected mice ($P < 0.001$; Figure 1B). In addition, tracheas from mice infected with influenza for 6 days had 2.1-fold more pneumococci remaining than those from mice infected with influenza for 3 days before

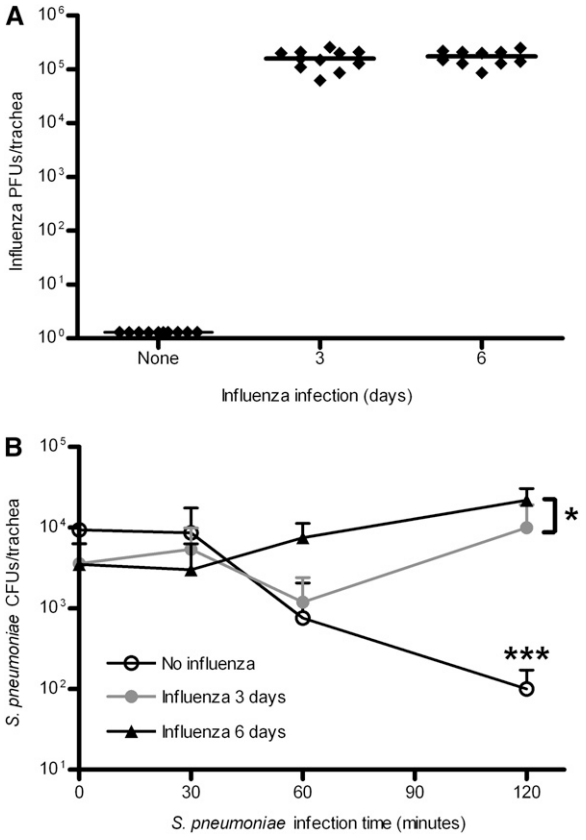

Figure 1. Effects of influenza infection on pneumococcal clearance. (*A*) Influenza plaque-forming units (PFUs) in tracheas from uninfected mice or mice infected with influenza virus for 3 or 6 days. *Line* represents median value for each infection. (*B*) Initial adherence and clearance of *Streptococcus pneumoniae* in tracheas from uninfected mice or mice infected with influenza for 3 or 6 days. ***$P < 0.001$, significant differences between mice infected with *S. pneumoniae* only and mice coinfected with influenza and *S. pneumoniae*; *$P < 0.05$ significant differences between mice coinfected with influenza for 3 or 6 days followed by *S. pneumoniae*. Data expressed as means (±SD). Data analyzed using one-way ANOVA followed by the Bonferroni post test. Results represent 5–6 mice for 0- and 30-minute time points, and 8–11 mice for 60- and 120-minute time points.

the *S. pneumoniae* infection ($P = 0.0184$; Figure 1B). These data indicate that a prior influenza infection of 3 or 6 days significantly decreases *S. pneumoniae* clearance from the trachea, with greater deficiencies in clearance occurring at 6 days after influenza infection.

Together, our data indicate that a prior influenza infection does not affect the number of pneumococci in the trachea (either in the lumen or attached to the epithelium) during the first hour of a pneumococcal infection, but does result in an increase in the number of pneumococci within the trachea by 2 hours after a pneumococcal infection compared with tracheas from mice without a prior influenza infection.

Tracheal Explant System

We found that a prior influenza infection *in vivo* decreases clearance of pneumococci as early as 2 hours after infection. We also found that a prior influenza infection *in vivo* does not increase the initial numbers of pneumococci deposited within the trachea. However, with this model of *in vivo* influenza infection, we were not able to determine directly how influenza-induced damage to the respiratory epithelium influences pneumococcal

adherence. We therefore developed a murine whole-tracheal explant system to examine *S. pneumoniae* adherence independently of other changes in the host immune response.

Tracheas were removed and attached to needles of the tracheal explant system (Figure 2). A portion of the lung was left attached to the trachea to add weight to the trachea, allowing for complete exposure of the respiratory epithelium (Figure 2). Fresh medium (cRPMI) was pumped through the tracheal explants at a constant flow rate of 0.1 ml/min to keep the tissue evenly hydrated and supplied with nutrients. Infectious agents were injected through the needle port of the microbore extension set. After incubation with influenza virus or *S. pneumoniae*, flow was resumed to remove unattached virus or bacteria. Spent medium (waste) was collected in the tissue culture flask and discarded (Figure 2).

Viability of Uninfected Tracheal Explants

To determine how *ex vivo* culture affected the viability and morphology of tracheal explants, we used both confocal and light microscopy. Ciliary beating was determined using both an inverted microscope and a confocal microscope as a measure of viability. The cellular morphology of tracheal explants was examined using H&E-stained sections at various time points of *ex vivo* culture (0–8 d). Cilia were observed using both H&E staining as well as staining for β-tubulin (Figure 3). We found that tracheal explants remained viable for the 8-day period studied, as shown by cellular morphology and ciliary beating. The cilia and respiratory epithelium appeared normal, with a ciliated, pseudostratified, columnar epithelium present in uninfected tracheas (Figure 3). In addition, ciliary beating could be observed during the entire 8 days of culture (data not shown). Together, these data indicate that our explant system can be used to monitor trachea function for at least 8 days *ex vivo*.

Establishment of an Influenza Infection in Tracheal Explants

We injected trypan blue into tracheal explants through the needle port to determine the minimum volume needed to fill the tracheal lumen. We found that a volume of 40 μl filled the lumen, and therefore used volumes of at least 40 μl for influenza virus and *S. pneumoniae* infections to ensure complete coverage of the tracheal epithelium by the inoculum (data not shown). Tracheal explants were infected with PR8 influenza virus for 0.5 to 5 days, and viral PFUs were enumerated at each time point after infection. Influenza PFUs were detected as early as 12 hours after influenza infection ($3.5 \times 10^1 \pm 1.6 \times 10^1$ PFUs/trachea), and could be detected for up to 5 days (Figure 4). Influenza PFUs increased to a peak of 2.0×10^3 ($\pm 4.5 \times 10^3$) PFUs/trachea at 3 days in culture, and gradually decreased to 1.9×10^2 ($\pm 4.1 \times 10^2$) PFUs/trachea by 5 days of culture (Figure 4). These results demonstrate that an influenza infection can be initiated and maintained with infectious influenza virus particles present in our tracheal explant system for up to 5 days.

Effects of Influenza Virus Infection on Respiratory Epithelium of Tracheal Explants

Influenza-induced tissue damage and influenza-infected cells were visualized using influenza-infected tracheal explants after 1 to 5 days of *ex vivo* culture and compared with uninfected tracheal explants (Figure 5). Influenza virus particles were visualized in influenza-infected tissues using immunofluorescence. Influenza-induced tissue damage was shown using H&E-stained sections, and uninfected tracheal explants were stained for comparison (Figures 3 and 5). Influenza-infected tracheal explants were also stained with a negative-control antibody (biotinylated goat IgG; data not shown). At 1 day after influenza infection, influenza-induced damage to the epithelium was evident and influenza-infected cells were present, as shown by immunofluorescence (Figure 5). Staining for influenza virus and β-tubulin showed influenza virus–infected epithelial cells (Figure 6). At 2 days after influenza infection, most of the epithelium was denuded, exposing the basement membrane and undifferentiated basal cells (Figure 5). Sloughed-off influenza-infected epithelial cells were also seen 1 day and 2 days after influenza infection using immunofluorescence (Figure 5). At 3 days after infection, basal cells were seen migrating over the exposed basement membrane, as evidenced by the intense violet H&E staining of the tracheal epithelium, and some influenza-infected cells were visible by immunofluorescence assay (Figure 5 and data not shown). At 4 and 5 days after infection, the epithelium appeared almost normal, with some influenza-antigen–positive cells present at 4 days, but few to

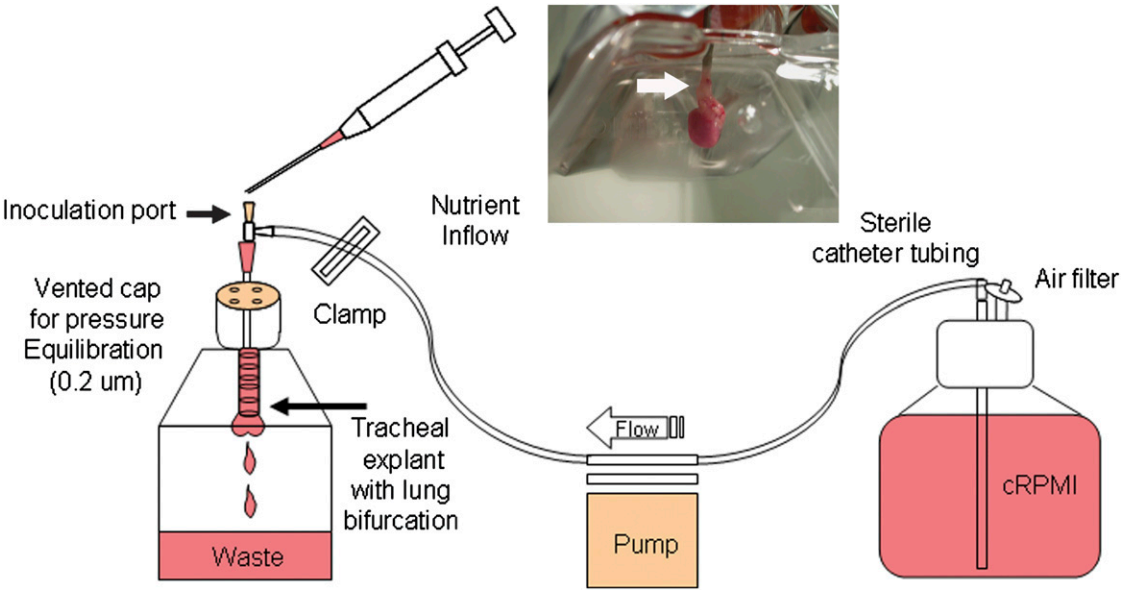

Figure 2. Diagram of tracheal explant system showing media reservoir connected to pump and flask with tracheal explant. Tracheas were suspended from blunt-ended 18-gauge needles, which were connected to silicone tubing through a pump with a constant media flow rate of 0.1 ml/min. Media (cRPMI) and explants were incubated at 37°C in 5% CO_2. *Inset*: picture of tracheal explant and portion of lung connected to needle inside flask. The *arrow* indicates trachea attached to needle.

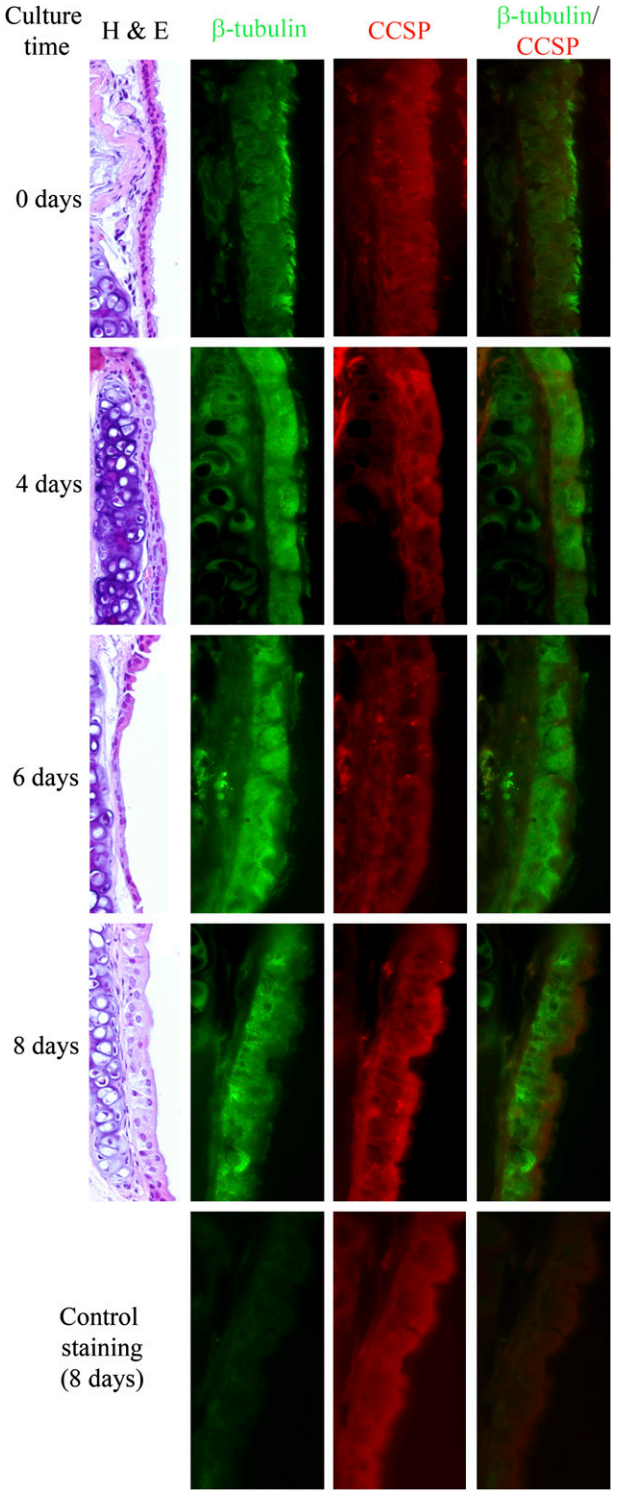

Figure 3. Uninfected tracheal explants at 0, 4, 6, and 8 days after *ex vivo* culture stained with hemotoxylin and eosin (H&E) or for β-tubulin and Clara cell secretory protein (CCSP). Intact cilia are present at all time points. Tracheal respiratory epithelium is on *right-hand side* of images. Magnification, 400× for H&E staining, 600× for β-tubulin and CCSP staining.

none present at 5 days (Figure 5). Thus, the tracheal explant system enabled examination of both influenza-infected cells and influenza-induced tissue damage, as well as resolution of viral infection and tissue repair.

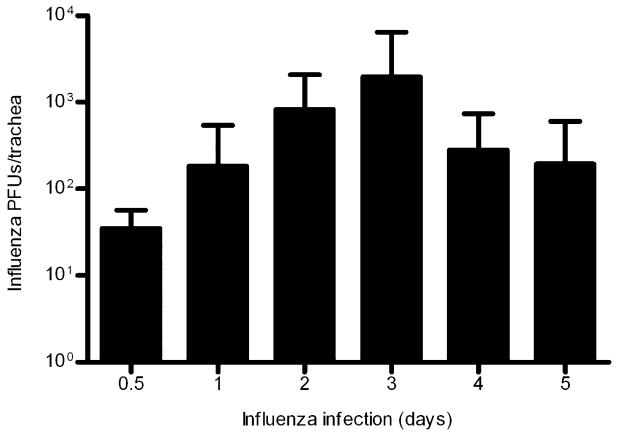

Figure 4. Influenza PR8 virus plaque-forming units (PFUs) in tracheal explants at various time points after *ex vivo* influenza infection. Data are expressed as means (±SD). Results represent 12–15 tracheas per day of influenza infection.

S. pneumoniae Adherence to Uninfected and Influenza-Infected Tracheal Explants

To determine the effect of *S. pneumoniae* inoculum size on initial adherence to tracheal explants with and without influenza infections, tracheas were either mock-influenza–infected or infected with influenza virus for 1 day, followed by *S. pneumoniae* infection with 5×10^3, 5×10^5, 5×10^6, 5×10^7, or 5×10^8 *S. pneumoniae* CFUs/ml in PBS. In both influenza-infected and non–influenza-infected tracheas, there were no detectable viable pneumococci adherent to the tracheal explants with an inoculum of 5×10^3 CFUs/ml (Figure 7). Pneumococci adhered to the tracheal explants in a dose-dependent manner, with maximal adherence occurring with an inoculum of 5×10^8 *S. pneumoniae* CFUs/ml ($3.1 \times 10^4 \pm 2.5 \times 10^4$ CFUs/trachea) (Figure 7). However, *S. pneumoniae* adherence to influenza-infected tracheas was not significantly increased compared with adherence to non–influenza-infected tracheas at inoculum sizes of 5×10^5, 5×10^6, and 5×10^7 *S. pneumoniae* CFUs/ml ($P > 0.05$; Figure 7). Unless otherwise noted, the inoculum for the remainder of tracheal explants was 5×10^7 *S. pneumoniae* CFUs/ml. These data indicate that *S. pneumoniae* adherence to both uninfected and influenza-infected tracheal explants occurs in a dose-dependent manner.

We measured adherence of *S. pneumoniae* to uninfected tracheas and tracheas infected with influenza for up to 5 days in *ex vivo* culture to determine whether influenza virus affected initial adherence of *S. pneumoniae* in the tracheal explants. A prior influenza infection did not increase pneumococcal adherence to tracheal explants after 1 hour of incubation at any of the times after influenza infection that we examined (Figure 8). These data indicate that a prior influenza infection does not increase pneumococcal adherence to the tracheal epithelium of explants at any of the stages of influenza infection examined (from early during an *ex vivo* influenza infection when influenza-infected cells are present and influenza-induced damage to the respiratory epithelium has begun, to the later stages when the epithelium is denuded and the basement membrane is exposed, or during the initial stages of epithelial repair).

Mucociliary Velocity

To determine whether an influenza virus infection alters tracheal mucociliary velocity, and thereby decreases pneumococcal clearance, we measured the movement of latex beads in

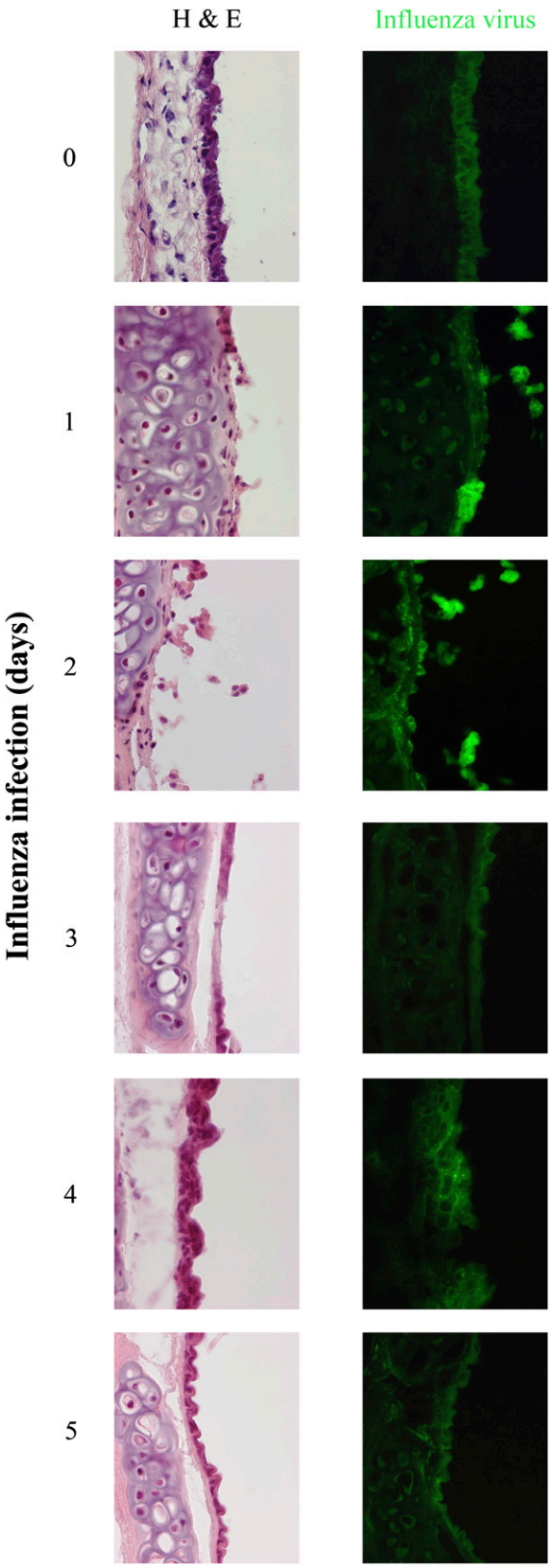

Figure 5. Uninfected tracheal explants and tracheal explants infected *ex vivo* with influenza virus for 1–5 days. Hemotoxylin and eosin (H&E) staining and corresponding influenza immunofluorescence staining of serial sections from tracheal explants at various time points after *ex vivo* influenza infection. Magnification, 600×. Tracheal respiratory epithelium is on *right-hand side* of images.

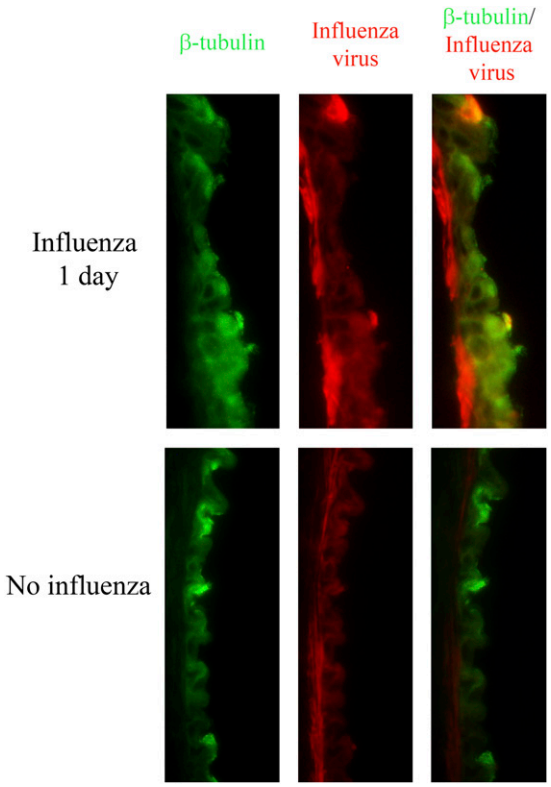

Figure 6. Influenza-infected epithelium of tracheal explant. Colocalization of immunofluorescence staining for influenza virus (*red*) and β-tubulin (*green*) in tracheal explant infected with influenza virus for 1 day. Uninfected tracheal explant shows β-tubulin staining only. Magnification, 600×. Tracheal respiratory epithelium is on *right-hand side* of images.

tracheas of uninfected mice and mice infected with influenza for 3 or 6 days. The mean mucociliary velocity of uninfected tracheas was 6.3 (±1.9) mm/min (Figure 9A). An influenza infection of 3 days decreased tracheal mucociliary velocities to an average of 1.5 (±2.9) mm/min, significantly slower than uninfected tracheas ($P < 0.001$; Figure 9A). Only 4 of the 10 mice examined had detectable mucociliary velocities. Of these 4 mice, only 2 velocity measurements were at levels similar to those seen with uninfected mice. More drastic effects were seen in mice infected with influenza for 6 days. None of these mice had any detectable movement of latex beads, resulting in a mean tracheal mucociliary velocity of 0.0 (±0.0) mm/min ($P < 0.001$ compared with uninfected mice; Figure 9A). No significant differences in tracheal mucociliary velocities were seen between mice infected with influenza virus for 3 or 6 days ($P > 0.05$; Figure 9A). Enumeration of influenza virus PFUs in lungs of these mice confirmed that all influenza-infected mice were infected with influenza virus (Figure 9B). Uninfected mice had no detectable influenza PFUs (Figure 9B). Mice infected with influenza virus for 3 days had significantly more influenza PFUs than mice infected with influenza virus for 6 days ($P < 0.001$; Figure 9B). Together, these data indicate that an influenza infection of 3 or 6 days dramatically decreases tracheal mucociliary velocities.

DISCUSSION

The trachea is thought to play a role in the progression of *S. pneumoniae* from the nasal mucosa, where it asymptomatically

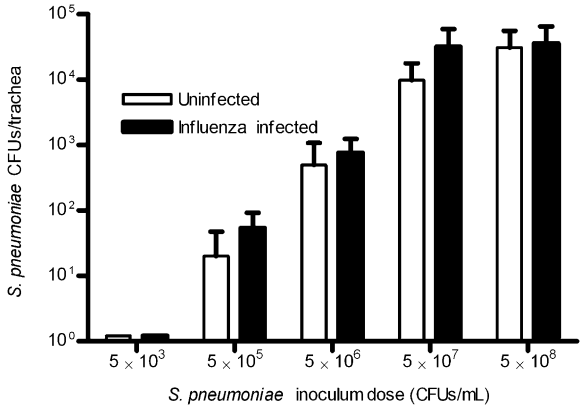

Figure 7. Effect of *Streptococcus pneumoniae* inoculum dose on *S. pneumoniae* adherence to uninfected and influenza-infected (1 d) tracheal explants. Tracheas were inoculated with 5×10^3, 5×10^5, 5×10^6, 5×10^7, or 5×10^8 *S. pneumoniae* CFUs/ml for 1 hour to allow for adherence. Data are expressed as means (±SD). Data were analyzed using one-way ANOVA followed by the Bonferroni post test. Results represent four to five tracheas per dose, except eight tracheas were used for the 5×10^7 dose.

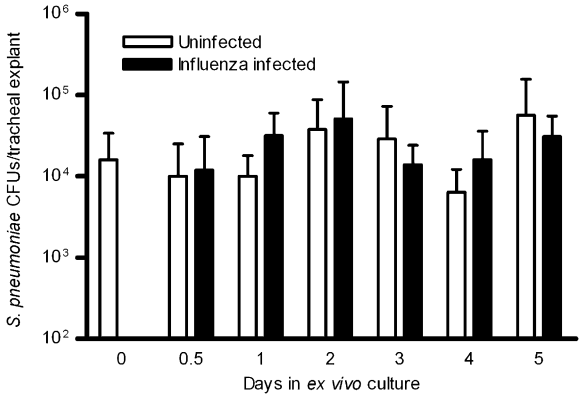

Figure 8. Effect of influenza infection on *Streptococcus pneumoniae* adherence to tracheal explants at various times after influenza infection (0.5–5 d). Data are expressed as means (±SD). Data were analyzed using one-way ANOVA followed by the Bonferroni post test. Results represent 8–10 tracheas per time point, except 0 and 0.5 days with and without influenza, which had 14 and 15 tracheas each, respectively.

colonizes the nasal epithelium, to the lungs, resulting in pneumonia (15, 44). Changes to the tracheal epithelium induced by influenza virus may increase susceptibility to a secondary *S. pneumoniae* infection by increasing pneumococcal adherence to the tracheal epithelium and/or decreasing the clearance of *S. pneumoniae* via the mucociliary escalator of the trachea, because many of the ciliated cells are destroyed (15, 24). This impairment of clearance and enhanced progression of *S. pneumoniae* due to an influenza infection would allow more *S. pneumoniae* to reach the lungs (15). In other studies examining susceptibility of mice to *S. pneumoniae* after an influenza infection in the lungs, such an increase in susceptibility to *S. pneumoniae* was seen around 7 days after influenza infection, when influenza-induced tissue damage of the tracheal respiratory epithelium is greatest (4, 9, 17, 24, 28, 45).

Our *in vivo* coinfection results indicate that a prior influenza infection does not increase the initial number of pneumococci found within the trachea during the first hour of infection, but it does decrease mucociliary velocity, and thereby reduces pneumococcal clearance during the first 2 hours after pneumococcal infection at both 3 and 6 days after an influenza infection. The defects in pneumococcal clearance were greatest at 6 days after influenza infection. Nugent and Pesanti (15) previously reported that a prior influenza infection of 7 days reduced clearance of *S. aureus* at both 3 and 6 hours after *S. aureus* infection. By measuring the number of pneumococci present in tracheas immediately after inoculation, we were able to directly measure both the number of pneumococci initially present within the trachea, and the clearance of these bacteria over time. Our study is the first to examine how a prior influenza infection affects both initial adherence and pneumococcal clearance over 2 hours. Our results at 120 minutes confirm those of Plotkowski and colleagues (24), who showed that tracheas from mice infected *in vivo* with influenza and infected with *S. pneumoniae in situ* for 90 minutes had more *S. pneumoniae* adherent than non–influenza-infected tracheas. This increase in adherence was greatest at 6 days after influenza infection, when tissue damage to the tracheal respiratory epithelium was the greatest (24). Although they concluded that this increase in pneumococci was due to increased adherence, our results indicate that this increase may be due to decreased bacterial clearance rather than increased adherence. In a normal, healthy trachea with an intact respiratory epithelium, pneumococci are removed by ciliary beating and the resulting movement of mucus. Other studies have shown that pneumococci adhere primarily to the mucus layer, and not to the respiratory epithelium (46, 47). Therefore, pneumococci adherent to the mucus layer would normally be removed from the trachea via the mucociliary escalator. However, during an *in vivo* influenza infection, ciliated epithelial cells are destroyed, and this clearance process is disrupted (15), which may allow *S. pneumoniae* to adhere to the damaged epithelium more readily due to increased dwell time, as seen with our studies and those of Plotkowski and colleagues (24). Decreased clearance would facilitate pneumococcus-laden mucus to be inhaled into the lower airways, where the small airway epithelium may be more susceptible to pneumococcal adherence and invasion.

In support of this hypothesis, we found that mucociliary velocity, as measured by the movement of fluorescent latex beads, was significantly decreased in tracheas of influenza-infected mice. Importantly, all mice infected with influenza for 6 days, and most mice infected with influenza for 3 days, had no detectable mucociliary movement. This study is the first, to our knowledge, to directly measure the effects of influenza virus infection on tracheal mucociliary velocity. Mucociliary clearance is a critical component of the airway defense mechanism, and therefore is crucial for preventing the spread of bacteria from the trachea to the lungs (48). Influenza-induced decreases in mucociliary velocity resulting in decreased pneumococcal clearance would allow more pneumococci to remain inside the tracheal lumen, and would increase the likelihood of pneumococci entering the lungs to cause pneumonia.

With our *in vivo* model of coinfection, we were not able to directly measure how influenza-induced tissue damage affects pneumococcal adherence to the tracheal epithelium, because other aspects of the immune system, such as neutrophil function, may have been affected by the influenza infection (17–22). Other influenza-induced changes could affect pneumococcal numbers within the tracheas, such as changes in the rate of bacterial division, bacterial killing by neutrophils, and removal of epithelial cells with adherent pneumococci by mucociliary clearance. We consequently developed a novel murine tracheal explant method to study how an influenza infection directly affects the tracheal respiratory epithelium and subsequent

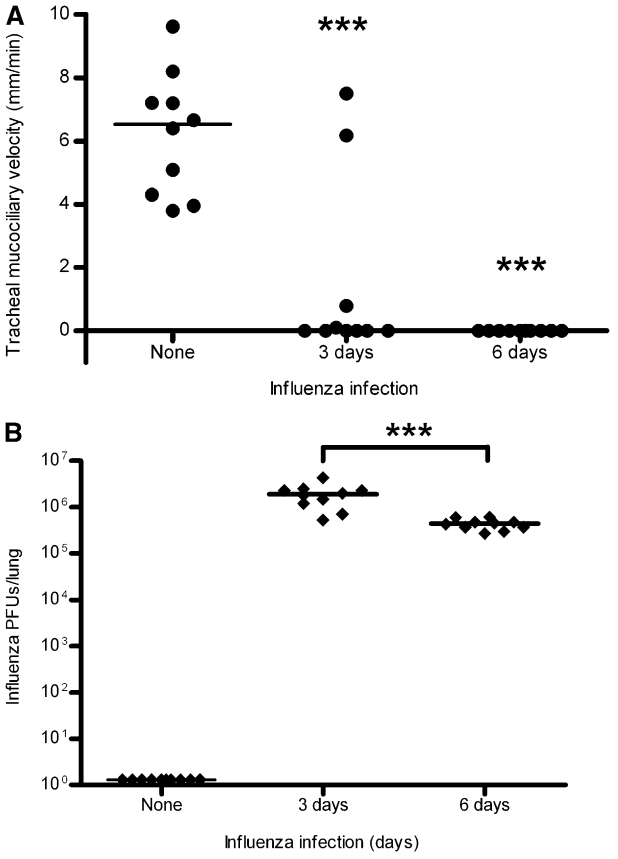

Figure 9. Effects of influenza infection on tracheal mucociliary velocity. (*A*) Tracheal mucociliary velocities of uninfected mice or mice infected with influenza virus for 3 or 6 days. *Line* represents median value for each infection. ***$P < 0.001$, significant differences between uninfected mice and influenza-infected mice. Data were analyzed using one-way ANOVA followed by the Bonferroni post test. (*B*) Lung influenza plaque-forming units (PFUs) from mice used for tracheal mucociliary velocity measurements. *Line* represents median value for each infection. ***$P < 0.001$, significant differences between mice infected with influenza for 3 or 6 days. Data were analyzed using one-way ANOVA followed by the Bonferroni post test.

S. pneumoniae adherence independently of neutrophil function. A murine system was developed because most *in vivo* coinfection studies are done in mice (4, 9, 17, 24).

The tracheal explant model that we developed has several advantages over other methods, such as cell culture or the tracheal ring model, for studying interactions between bacteria and host cells. One advantage is that this model uses an intact tissue, complete with a fully differentiated respiratory epithelium and basement membrane, whereas cell culture methods rely on only one individual cell type, making it difficult to reproduce the actual respiratory tissue. Another advantage is that our tracheal explant system allows for controlled, synchronous infection and tissue damage throughout the trachea. In addition, the infection process is expedited compared with an *in vivo* infection, as the inoculum is concentrated in the trachea rather than distributed throughout the entire respiratory tract. With an *in vivo* infection, the infection is difficult to synchronize, because it spreads throughout the entire respiratory tract, resulting in different stages of infection in different areas of the respiratory tract (49).

A third advantage of our tracheal explant model is that only specific adhesion to the tracheal epithelium is measured, because there is no sectioning of tissue involved, and it is a closed system. This method eliminates nonspecific adherence to cut areas and the trachea adventitia, which can occur with the tracheal ring model. By using a closed system, we can also control the amount of medium, influenza virus, and/or bacteria that each trachea receives, and flow can be adjusted to simulate shear forces found *in vivo*. In addition, nonadherent influenza virus particles and *S. pneumoniae* are efficiently removed from the system by resuming the flow of medium. With our tracheal explant system, shed viral particles and influenza-infected cells produced during the infection are removed from the trachea by the media flow, resulting in lower influenza PFUs than seen with *in vivo* infections. In addition, any differences in mucociliary clearance are eliminated due to both the removal of mucus by the media, and the direction and velocity of the media flow. Both of these features of the explant system result in elimination of mucociliary clearance, allowing for examination of adherence independently of mucociliary clearance. Finally, because influenza virus affects neutrophil function (17–22), an important advantage of the explant model is that systemic and confounding effects of influenza infection that are present *in vivo*, such as changes in neutrophil function, are eliminated, allowing for the direct examination of influenza's effects on bacterial adherence due specifically to direct tissue damage to the epithelium.

Although our tracheal explant system has several advantages for examining the effects of influenza infection on subsequent pneumococcal infection and adherence to the epithelium, it also has some limitations. First, unlike tracheal epithelial cells found *in vivo*, the epithelial cells in the explants do not have a mucus layer or aqueous layer on their apical surfaces. In addition, the cells are placed in a submerged environment with a continuous flow of media moving over them that is not normally encountered *in vivo*. Finally, over 8 days of *ex vivo* culture, there are changes in cellular morphology as well as gene expression and protein localization (β-tubulin expression is decreased and is located primarily in the cytoplasm rather than the apical surface). Despite these limitations, we believe that our tracheal explant system offers a more advanced and relevant way to examine influenza-induced tissue damage and subsequent pneumococcal adherence than previously established methods.

Our study is the first study to examine how an *ex vivo* influenza infection affects *S. pneumoniae* adherence using murine tracheal explants. Previous studies using explant models to study *S. pneumoniae* adherence have looked only at adherence to normal tracheal epithelium (30, 50). The explant model that we developed allowed us to examine *S. pneumoniae* adherence to uninfected respiratory epithelium, as well as influenza-infected respiratory epithelium during all stages of influenza infection, from initial infection causing possible changes in cellular receptors (0.5 d), to epithelial damage and exposure of the basement membrane (1–2 d), to initial repair and regeneration of the epithelium, when basal cells and undifferentiated cells are present (3–5 d). Previous studies of *in vivo* influenza infections have shown a similar time frame for damage and repair of the tracheal epithelium after an influenza infection (24, 28, 45). Ramphal and colleagues (28) showed evidence of desquamation as early as 24 hours, and complete desquamation by 3 days after influenza infection, with repair starting at Day 5 and completion by Day 14 after influenza infection. Azoulay-Dupuis and colleagues (45) used a guinea pig model of influenza infection, and found that tracheal epithelial damage occurred as early as 48 hours after infection, and lasted up to 7 days, with repair taking 2 weeks. Our *in vivo* pneumococcal adherence data were further corroborated by *ex vivo* results showing that influenza infection did not affect adherence of *S. pneumoniae* to tracheal explants at any of the

times after influenza infection that we examined. Because the pneumococci inoculated into the tracheal explants were subjected to the same flow velocity regardless of influenza infection, this result is consistent with a mucociliary clearance defect, rather than pneumococcal adherence, being a major contributor to the bacterial load in the lumen of the tracheas. Our dose–response curve further supports this conclusion, as increasing the number of pneumococci in the inoculum increased pneumococcal adherence, but the presence of a prior influenza infection did not increase this adherence further. If an influenza virus infection increased pneumococcal adherence to explants, these differences should have been apparent with our dose–response curve.

In summary, we found that an *in vivo* influenza infection of 3 or 6 days did not affect the number of pneumococci within the trachea during the initial stages of infection (0–60 min). However, by 2 hours after pneumococcal infection, a prior influenza infection of 3 or 6 days significantly increased the number of pneumococci remaining in the tracheas, indicating that either adherence was increased, or mucociliary clearance was decreased. To further examine whether influenza-induced changes to the epithelium affect pneumococcal adherence, we developed a novel murine tracheal explant system that can be used to study a variety of cellular processes, such as viral and/or bacterial infections and mechanisms of epithelial repair. We used this model to study the effects of influenza virus on the respiratory epithelium of the trachea and subsequent *S. pneumoniae* adherence. We showed that tracheal explants remained viable for at least 8 days, and that influenza infections can be maintained for at least 5 days. However, our results show that a prior influenza infection did not increase *S. pneumoniae* adherence to tracheal explants at any of the time points after influenza infection that we examined, including when the basement membrane was maximally exposed. Using influenza-infected mice, we found that tracheal mucociliary velocities were significantly decreased, or even stopped completely, in mice infected with influenza virus for 3 or 6 days. Together, our *in vivo* and *ex vivo* data indicate that influenza-induced tissue damage to the tracheal epithelium results in increased pneumococci within the trachea and decreased mucociliary velocity, which is one mechanism that accounts for decreased pneumococcal clearance from the trachea. Decreased pneumococcal clearance increases the likelihood of pneumococci to remain in the trachea (possibly attached to mucus), and to eventually spread to the lungs to cause pneumonia.

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