Respiratory syncytial virus infection enhances *Pseudomonas aeruginosa* biofilm growth through dysregulation of nutritional immunity

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Clinical observations link respiratory virus infection and *Pseudomonas aeruginosa* colonization in chronic lung disease, including cystic fibrosis (CF) and chronic obstructive pulmonary disease. The development of *P. aeruginosa* into highly antibiotic-resistant biofilm communities promotes airway colonization and accounts for disease progression in patients. Although clinical studies show a strong correlation between CF patients' acquisition of chronic *P. aeruginosa* infections and respiratory virus infection, little is known about the mechanism by which chronic *P. aeruginosa* infections are initiated in the host. Using a coculture model to study the formation of bacterial biofilm formation associated with the airway epithelium, we show that respiratory viral infections and the induction of antiviral interferons promote robust secondary *P. aeruginosa* biofilm formation. We report that the induction of antiviral IFN signaling in response to respiratory syncytial virus (RSV) infection induces bacterial biofilm formation through a mechanism of dysregulated iron homeostasis of the airway epithelium. Moreover, increased apical release of the host iron-binding protein transferrin during RSV infection promotes *P. aeruginosa* biofilm development in vitro and in vivo. Thus, nutritional immunity pathways that are disrupted during respiratory viral infection create an environment that favors secondary bacterial infection and may provide previously unidentified targets to combat bacterial biofilm formation.

respiratory syncytial virus | nutritional immunity | cystic fibrosis | *Pseudomonas aeruginosa* | biofilm

Viral–bacterial interactions impact the development and evolution of chronic infections at many mucosal sites, including the airway (1–3). In the lung disease cystic fibrosis (CF), viral infections are linked to pulmonary function decline, antibiotic use, prolonged hospitalizations, and increased respiratory symptoms (4). Respiratory syncytial virus (RSV) is one of the most common viral copathogens in CF and is a culprit in disease progression, promoting early respiratory tract morbidity and reductions in lung function (5, 6). Moreover, beyond the morbidity associated with viral infections alone, RSV has been linked in clinical studies to the development of *Pseudomonas aeruginosa* coinfections and to the conversion to chronic *P. aeruginosa* colonization in CF patients (6–10). Although clinical associations between viral infection and the acquisition of colonizing *P. aeruginosa* are clear, the basic biology of this interaction is not understood.

The transition of acute bacterial infections to chronic infections often involves the development of bacterial aggregates, or biofilms. The combination of an up-regulation of antibiotic resistance genes and the production of a polymeric matrix surrounding the biofilm serves to protect bacteria from the hostile environment in the host (11). The development of biofilm in human disease has been studied intensely for its involvement in disease progression in CF. Biofilm development at a mucosal surface requires initial attachment of bacteria to a surface, followed by the formation and growth of microcolonies, resulting in the development of bacterial biofilms, which can undergo regulated dispersal and ultimately seed a new surface (12, 13). Our present understanding of bacterial biofilm development is largely limited to single-organism infections. Although we have long known of polymicrobial communities colonizing human tissues, there is a surprising gap in our understanding of how these communities develop, how they impact human disease, and how host defense mechanisms influence polymicrobial infections. Because our current antimicrobial approaches have limited success for chronic infections, elucidating the mechanism by which biofilms develop during polymicrobial infections may identify new therapeutic targets to combat biofilm persistence.

Many environmental cues have been described as contributing to the conversion of *P. aeruginosa* to a biofilm mode of growth; one such cue is iron. Nutrient iron is tightly regulated in the host through complex interactions among uptake, storage, and use in the cell. Nutritional immunity postulates that, because iron is required for microbial growth, respiration, and metabolism, the host employs many regulatory pathways to sequester free iron (14). In CF, elevated levels of iron in the airways of infected patients are correlated with frequency of exacerbation and have been proposed to play a role in airway colonization (15, 16). The sputum of CF patients contains elevated levels of ferrous iron, and these levels correlate with disease severity (17). Although increased iron in sputum is associated with CF lung disease severity, it still is unknown how iron homeostasis is altered in CF and how this alteration relates to airway infection.

Significance

***Pseudomonas aeruginosa* is the major respiratory pathogen that promotes disease progression in chronic lung diseases such as cystic fibrosis (CF) and resides in antibiotic-resistant biofilm communities in the lungs of patients. Little is known about host factors that contribute to the development of bacterial biofilms in the lung. We have observed that respiratory virus coinfection and the antiviral immune response aid in the transition of *P. aeruginosa* to a biofilm mode of growth through inappropriate release of the nutrient iron. Defining molecular mechanisms by which *P. aeruginosa* biofilms develop in the lung affords a better opportunity to target therapies to eliminate life-threatening infections in CF and in other chronic lung diseases.**

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Using CF lung disease as a model to understand viral–bacterial interactions at a mucosal surface, we use a coculture system for bacterial biofilm development in association with the airway epithelium. Using RSV, we demonstrate that virus coinfection and the subsequent antiviral IFN response dramatically enhance the growth of *P. aeruginosa* biofilm in association with the airway epithelium. In addition, we show that virus infection impairs nutritional immunity, allowing the apical release of transferrin and thus increasing bioavailability of iron to promote the growth of *P. aeruginosa* biofilm. These findings offer new insight into the complex interaction among two pathogens and the host during polymicrobial infections and suggest a mechanism by which nutritional immunity plays a critical role in regulating pathogen persistence in the airway.

Results

Respiratory Virus Infections Promote *P. aeruginosa* Biofilm Growth on Airway Epithelial Cells. To determine if respiratory virus infections promote the growth of *P. aeruginosa* biofilm on airway epithelial cells, human ΔF508/ΔF508 cystic fibrosis bronchial epithelial cells (CFBE41o-, hereafter called "AECs") were grown as a polarized monolayer and were infected with RSV, followed by infection with GFP-tagged *P. aeruginosa* (strain PAO1) in a flow chamber. Biofilm growth was analyzed by live-cell microscopy, as described previously (Fig. 1 *A* and *B*) (18, 19). Surprisingly, RSV coinfection significantly enhanced the growth of *P. aeruginosa* biofilm on the surface of AECs in a time-dependent manner, peaking at 72 h after RSV infection, as measured in a static coculture biofilm model (Fig. 1*C*) (19). We also found that RSV infection induced biofilm growth of three CF clinical isolate strains of *P. aeruginosa* (Fig. S1*A*) and on primary CF and non-CF human bronchial epithelial (HBE) cells (Fig. 1*D* and Fig. S1*A*).

Using an abiotic biofilm growth assay in a 96-well microtiter dish (20), we found no differences in biofilm formation in *P. aeruginosa* biofilms grown in the presence or absence of RSV (Fig. S2*A*). In addition, although bacterial attachment was increased when cells were infected simultaneously with RSV and *P. aeruginosa* (Fig. S2*B*), as has been shown previously (21), *P. aeruginosa* attachment was not increased on cells infected with RSV before bacterial infection, whereas biofilm formation was greatly increased (Fig. S2*C*). Moreover, biofilm growth on the apical surface of AECs infected with RFP-tagged RSV (RSV-RFP) showed a random distribution (Pearson correlation coefficient of 0.072 ± 0.021) (Fig. S2*D*), demonstrating that biofilms did not form specifically on virus-infected cells.

Stimulation of Bacterial Biofilm Formation Is Induced by Infection of AECs by Disparate Viruses. To examine whether virus-enhanced *P. aeruginosa* biofilm formation was specific for RSV coinfection, we infected AECs with human rhinovirus-14 (hRV14) or adenovirus-5 (Ad5), two additional respiratory viral pathogens commonly found in CF patients (4), before bacterial infection. Coinfection with hRV14 or Ad5 also increased the growth of *P. aeruginosa* biofilm on AECs (Fig. 1*E*). Importantly, cytotoxicity was not detected for RSV, hRV, or adenovirus infections, as measured by lactate dehydrogenase release and transepithelial electrical resistance, a measure of epithelial barrier integrity (Fig. S3). Taken together, these results indicate that several respiratory viruses relevant to CF lung disease can stimulate the growth of *P. aeruginosa* biofilm on AECs.

Antiviral IFN Signaling Increases the Growth of *P. aeruginosa* Biofilm. Host cells commonly respond to viral infections through the induction of antiviral IFNs. Because we observed an enhancement of bacterial biofilm growth by diverse respiratory viruses, we focused

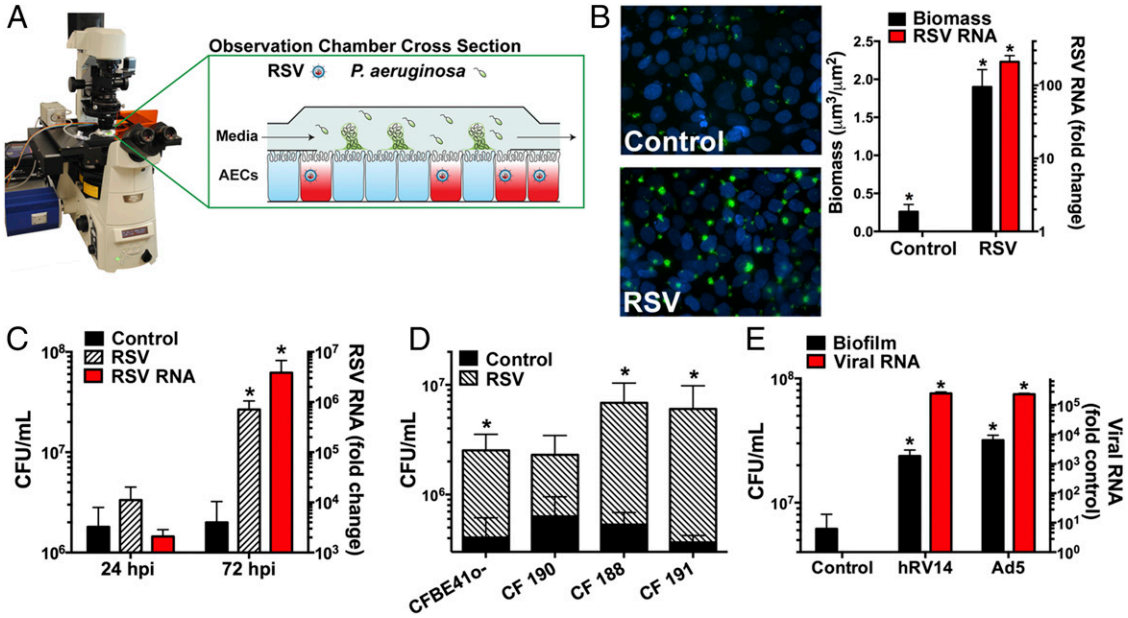

Fig. 1. Respiratory viral infection promotes the growth of *P. aeruginosa* biofilm on AECs. (*A*) The setup for live-cell biotic biofilm imaging and a cross-sectional view of the micro-observation chamber. (*B*, *Right*) *P. aeruginosa* (GFP) biofilms imaged by live-cell microscopy after 6 h of growth. AEC cell nuclei are shown with Hoechst (blue) staining. (*Left*) Biofilm biomass was quantified using COMSTAT (black bars, left *y* axis). RSV RNA was measured by quantitative RT-PCR (qRT-PCR) to assess RSV infection (red bar, right *y* axis). (*C*) RSV-enhanced biofilm growth is time dependent. In a static coculture biofilm assay, AECs were infected with RSV (striped bars) or were mock-infected [Eagle's minimal essential media (MEM) control; black bars] for the indicated times followed by *P. aeruginosa* infection. *P. aeruginosa* biofilm was assessed by cfu enumeration (Left *y* axis). RSV RNA was measured by qRT-PCR to assess RSV infection (red bars, right *y* axis). (*D*) RSV stimulates the growth of *P. aeruginosa* biofilm on well-differentiated CF HBEs. *P. aeruginosa* biofilms were grown on primary CF HBEs using the static coculture biofilm assay and were quantified by cfu enumeration. (*E*) Other respiratory viruses enhance the growth of *P. aeruginosa* biofilm on AECs. *P. aeruginosa* biofilms were grown in a static coculture biofilm assay on AECs infected with hRV or AdV or were mock-infected (MEM control). Biofilms were quantified by cfu enumeration (black bars, left *y* axis). Viral RNA was measured by qRT-PCR to assess virus infection (red bars, right *y* axis). RSV, RSV-infected AECs. For all experiments $n \geq 3$. Data are presented as mean ± SD; $^*P < 0.05$ versus control.

on common pathways that might be induced in infected cells in response to virus infection. To do so, we first measured IFN-λ (IL-29/28B) production during the course of RSV infection and observed that IFN-λ levels in the apical airway surface liquid peaked 72 h post-RSV infection (see Fig. 3*A*). Consistent with previous studies (22), type I IFN production (IFN-β) also was produced by AECs during RSV infection, but to a lesser extent than IFN-λ (Fig. S4 *A* and *B*). Treatment of AECs with purified IFN-λ1 (IL-29, 100 ng/mL) (Fig. 2 *B* and *C*) enhanced the growth of *P. aeruginosa* biofilm on the surfaces of AECs. To confirm that signaling through the IFN-λ receptor was required for IFN-stimulated biofilm growth, we targeted both components of the heterodimeric IFN-λ receptor, IL-28Rα and IL-10Rβ. RNAi-mediated knockdown of IL-28Rα (Fig. S5*A*) reduced IFN-λ–stimulated biofilm growth on AECs (Fig. 2*D*). In addition, using a neutralizing antibody against IL-10Rβ during RSV infection, we observed reduced *P. aeruginosa* biofilm growth (Fig. S5*B*). IFN-β signaling through the IFN α/β receptor (IFNAR) also induced biofilm growth (1,000 U/mL) (Fig. S4 *C*–*F*). Taken together, these data support the conclusion that IFN signaling in response to respiratory viral infection is necessary and sufficient to enhance the growth of *P. aeruginosa* biofilm.

Iron Released from RSV-Infected AECs Stimulates Biofilm Growth. Because IFNs are secreted factors, we next determined whether the apical airway surface liquid [hereafter termed "conditioned medium" (CM)] from RSV-infected cells was capable of enhancing biofilm growth in the absence of AECs. We found that the CM from RSV-infected cells increased biofilm growth on abiotic surfaces, as assessed by microscopy (Fig. 3*A*) and a 96-well microtiter biofilm assay (Fig. 3*B*). Importantly, treatment of *P. aeruginosa* under cell-free conditions with purified IFN-λ1 (100 ng/mL) had no effect on biofilm growth (Fig. S6). Because we observed that CM from RSV-infected cells stimulated biofilm growth, we hypothesized that RSV infection and subsequent IFN signaling induced the secretion of a biofilm stimulatory factor. Iron is an essential nutrient for many bacteria and is required for the formation of *P. aeruginosa* biofilm on both abiotic and biotic surfaces (18, 23, 24). To determine if iron homeostasis is altered during RSV infection, we measured total iron levels in CM from mock- or RSV-infected cells. RSV infection resulted in a time-dependent and dose-dependent increase in extracellular iron in the CM from CF AECs and primary CF HBEs (Fig. 3*C* and Fig. S7 *A* and *B*) as well as in the CM from primary non-CF HBEs (Fig. S7*C*). Importantly, the presence of iron in CM was required for the enhancement of biofilm growth in response to RSV infection, because an iron-chelating agent (Chelex-100) dramatically decreased CM-induced biofilm growth (Fig. 3*D*). When exogenous iron ($FeCl_3$) was added to CM from RSV-infected cells after treatment with Chelex-100, biofilm growth was restored (Fig. 3*D*). Collectively, these results indicate that RSV infection enhances iron release by AECs, thus increasing iron availability and biofilm formation by *P. aeruginosa*.

Transferrin Is Apically Released in Response to RSV Infection. To examine the mechanism by which RSV infection promotes apical iron release, we investigated whether the abundance of iron transporters and iron-binding proteins in AECs was altered by virus infection. We did not observe a significant difference in the abundance of the iron transporters ferroportin or divalent metal-ion transporter 1 (DMT1) during RSV infection (Fig. S8*A*). Similarly, the abundance of iron-binding proteins, including transferrin, ferritin, and lactoferrin, was not changed by RSV infection (Fig. S8*A*).

Because we observed increased iron levels in CM from RSV-infected cells (Fig. 3*C*), with no corresponding change in the expression of iron transporters, we next examined whether RSV infection alters the release of iron-binding proteins into the CM. Indeed, we found increased abundance of transferrin in the CM from RSV-infected AECs (Fig. 4*A*). Transferrin is a host iron-binding protein that can be used as a source of iron and can support the growth of multiple bacterial species, including *P. aeruginosa* (25–27). Notably, transferrin promotes biofilm growth by *P. aeruginosa*

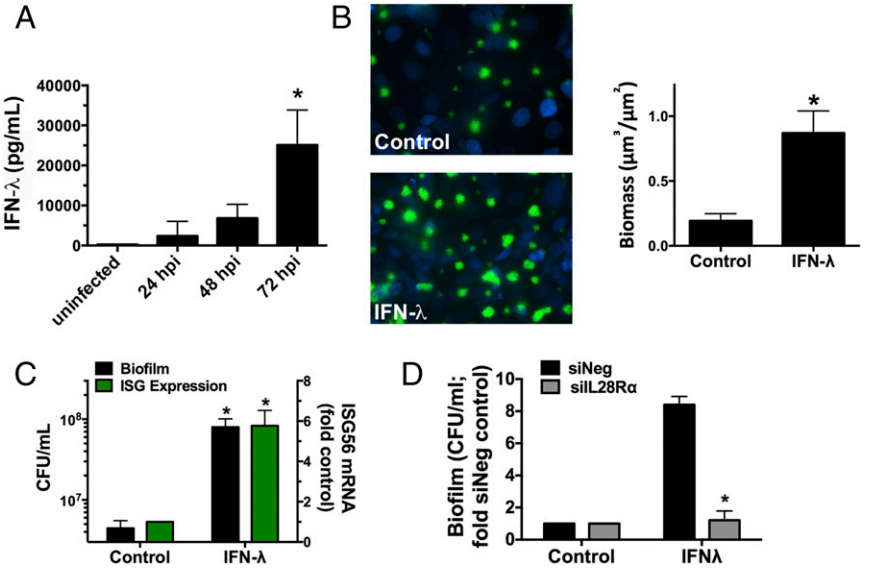

Fig. 2. Type III IFN (IFN-λ) signaling stimulates the growth of *P. aeruginosa* biofilm. (*A*) RSV infection induces IFN-λ secretion from AECs. Cells were infected with RSV for the indicated number of hours (hpi), and IFN-λ1/3 (IL-29/28B) release was measured by ELISA. (*B* and *C*) IFN-λ treatment stimulates the growth of *P. aeruginosa* biofilm on AECs. *P. aeruginosa* biofilm growth increased on AECs treated for 12 h with IFN-λ1 (100 ng/mL), as assessed by live-cell microscopy (*B*) or a static coculture biofilm assay (*C*). Epithelial cell nuclei are shown with Hoechst (blue) staining. The *P. aeruginosa* biofilm (GFP, green) biomass was calculated for each condition using COMSTAT (*B*). *P. aeruginosa* biofilm was assessed by cfu enumeration (black bars, left *y* axis) (*C*). IFN-λ1 signaling was confirmed with ISG56 induction by qRT-PCR (green bars, right *y* axis) (*C*). (*D*) Signaling via IL-28Rα is required for biofilm growth during IFN-λ treatment. AECs were transfected with scrambled siRNA (siNeg) or siRNA targeting IL-28Rα (siIL28Rα) and were treated with IFN-λ1 (100 ng/mL) for 12 h, and *P. aeruginosa* biofilms were grown in a static coculture biofilm assay. *P. aeruginosa* biofilm growth was quantified by cfu enumeration and displayed as fold change compared with siNeg-transfected cells. IFN-λ, IFN-λ–treated AECs. For all experiments $n \geq 3$. Data are presented as mean ± SD; $^*P < 0.05$.

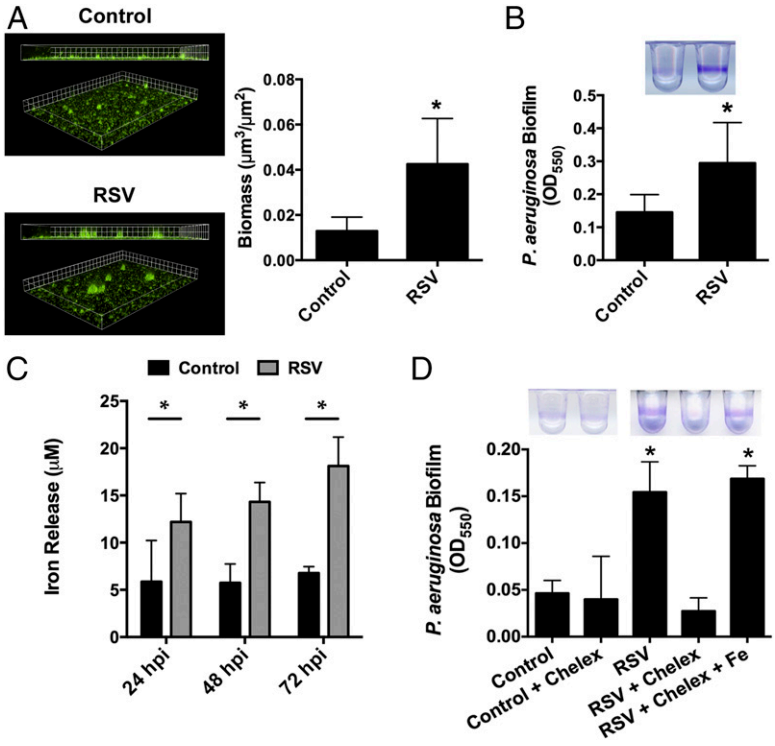

Fig. 3. RSV infection enhances iron release from AECs to stimulate biofilm growth. (*A* and *B*) RSV infection stimulates the release of a biofilm-stimulatory factor that promotes *P. aeruginosa* formation. AECs were infected with RSV or were mock-infected (MEM control) for 72 h, and the apical CM was collected. (*A*) *P. aeruginosa* (GFP) was grown in the presence of CM in static abiotic biofilm assays. Epifluorescence microscopy was used to measure the growth of *P. aeruginosa* biofilm (GFP, green), and biomass was quantified using Nikon Elements (grid unit = 9 μm). (*B*) *P. aeruginosa* biofilms were grown in CM for 24 h in a 96-well microtiter biofilm assay. (*C*) Total iron was increased in apical CM collected from AECs infected with RSV or mock-infected (MEM control) for the indicated number of hours postinfection. (*D*) Iron in RSV CM is required for the growth of *P. aeruginosa* biofilm. Ninety-six–well microtiter biofilm assays were performed to measure the growth of *P. aeruginosa* in CM. Divalent metal cations were chelated with Chelex-100 (labeled "Chelex" in the figure), and iron was added back with $FeCl_3$ (8 μM) after Chelex-100 treatment. RSV, RSV-infected AECs. For all experiments $n \geq 3$. Data are presented as mean ± SD; $^*P < 0.05$.

in a dose-dependent manner (18). To determine whether transferrin is necessary for the stimulation of biofilm growth by CM from RSV-infected cells, we grew *P. aeruginosa* in transferrin-depleted CM (Fig. S8*B*) and found that a significant reduction in biofilm growth when CM from RSV-infected cells was depleted of transferrin (Fig. 4*B*). Collectively, these data suggest that RSV infection stimulates the apical release of transferrin from AECs to promote the formation of *P. aeruginosa* biofilms.

RSV Infection Increases the Availability of Airway Iron in Vivo. To extend our in vitro findings to what might occur in vivo, we used a neonatal mouse model of RSV infection (28) and measured iron levels in the bronchoalveolar lavage fluid (BALF) up to 1 wk postinfection. RSV infection increased iron concentrations in the airways significantly, compared with mock-infected controls (Fig. 4*C* and Fig. S9*A*). In agreement with the increased abundance of iron, BALF from RSV-infected mice was able to support in vitro *P. aeruginosa* biofilm growth (Fig. S9*B*). Although RSV infection resulted in increased inflammation, there was no evidence of severe bronchiolar epithelial damage or of sloughing or rupture of the epithelial barrier, which could account for the increase of iron in the airways (Fig. S9 *C* and *D*). In addition, consistent with our finding in polarized human AECs, transferrin abundance was increased in the BALF of RSV-infected mice (Fig. 4*D*), further supporting a role for iron release as a key mediator of biofilm formation in response to RSV infection of AECs.

Discussion

Respiratory viral infections trigger exacerbations in chronic lung disease such as CF and predispose patients to bacterial colonization, but identifying the molecular mechanism(s) underlying viral–bacterial interactions has been elusive (7, 10, 29). Because of the paucity of animal models to study the development of chronic bacterial infections, we used a unique coculture model in which the early stages of *P. aeruginosa* biofilm development in association with polarized human AECs can be observed by high-resolution, live-cell imaging. We demonstrate that respiratory virus infection of CF and non-CF AECs and the subsequent induction of IFN signaling pathways results in the increased formation of bacterial biofilm. Moreover, we show that biofilm growth is increased through a dysregulation of host nutritional immunity mechanisms, resulting in the increased release of iron-bound transferrin during virus infection. This release of iron-bound transferrin promotes the transition of *P. aeruginosa* to a biofilm mode of growth (Fig. S10). Importantly, our findings propose a molecular mechanism underlying the common clinical observation that respiratory virus infection in patients with chronic lung disease promotes chronic bacterial colonization and disease progression (7, 10, 29). Our studies have important implications, beyond CF pathogenesis, for understanding how complex microbial communities interact during disease in the lung.

Few studies have investigated the interaction between viruses and *P. aeruginosa* in the airways. It has been shown that *P. aeruginosa* may modulate the antiviral response, although the consequences of these interactions on the progression of *P. aeruginosa* infection remain unclear (30, 31). Previous studies have shown that RSV coinfection promotes bacterial adherence to nonpolarized AECs (21, 32). In our model using well-differentiated AECs, we show that preceding RSV infection decreased *P. aeruginosa* adherence to AECs while greatly enhancing the development of bacterial biofilm. We interpret these results, coupled with abiotic biofilm assays that are not altered with virus exposure, as indicating that a direct interaction between virus and bacteria is not responsible for enhancement of chronic *P. aeruginosa* infections during respiratory viral infection. Instead, using a coculture model of biofilm development in association with the airway epithelium, our data suggest a mechanism by which the host innate immune response to the viral pathogen creates a local environment at the mucosal surface that promotes chronic bacterial infection.

Although the antiviral effects of IFNs are well recognized (33), studies now suggest that an appropriate antiviral IFN response to respiratory viral infection has unfavorable effects on secondary bacterial infections. For example, in acute models of influenza–bacterial coinfection, the increased bacterial load in the airways of mice after influenza challenge has been attributed to the antiviral IFN response to the virus (34–36). Little is known about how IFN

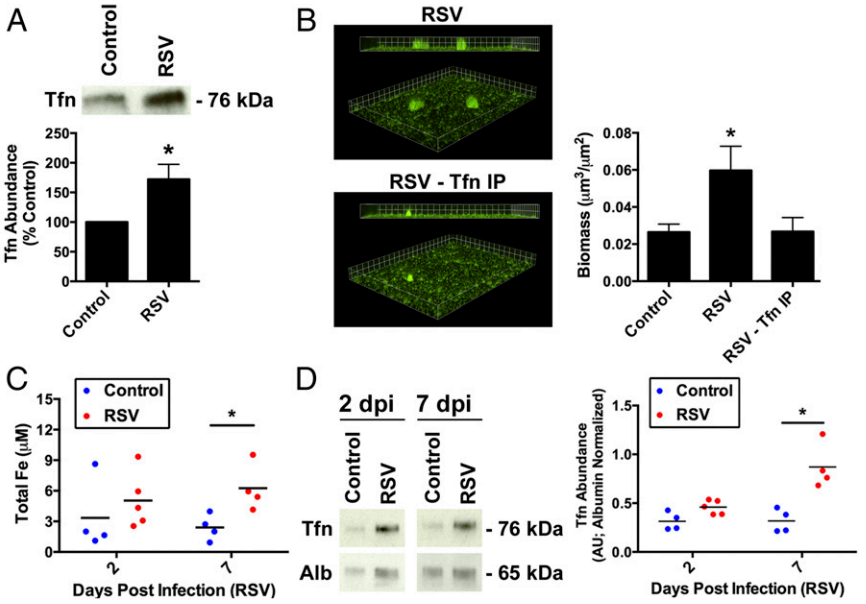

Fig. 4. Transferrin release increases in response to virus infection in vitro and in vivo. (*A* and *B*) AECs were infected with RSV or were mock-infected (MEM control) for 72 h and then apical CM was collected. (*A*) RSV infection increases transferrin abundance in apical CM, as measured by Western blot analysis. (*B*) Transferrin depletion reduces the growth of *P. aeruginosa* biofilm in RSV CM. Apical CM was collected from RSV-infected AECs depleted of transferrin by immunoprecipitation (RSV-Tfn IP). *P. aeruginosa* (GFP) biofilms were grown in transferrin-replete and -depleted RSV CM in static abiotic biofilm assays. Biofilm biomass was quantified using Nikon Elements (grid unit = 8.5 μm). (*C* and *D*) Total iron (*C*) and transferrin abundance (*D*) were increased in BALF recovered from neonatal mice infected with RSV or mock-infected (PBS control) for the indicated number of days postinfection (dpi), as measured by iron assay or Western blot analysis, respectively. Horizontal lines indicate mean values. Alb, albumin; RSV, RSV infection; Tfn, transferrin. All experiments were repeated, with at least four mice per group; $*P < 0.05$.

induction or signaling pathways influence chronic bacterial infection. In acute models of *P. aeruginosa* infection, the ability to clear *P. aeruginosa* infection is improved significantly in mice lacking IL-28Rα, indicating that IFN-λ signaling may contribute adversely to pulmonary *P. aeruginosa* infections in vivo (37). Our data extend previous findings to suggest that IFN signaling also promotes the growth of *P. aeruginosa* biofilm and chronic infections.

Our study suggests the dysregulation of nutritional immunity, specifically iron homeostasis, as a primary mechanism by which RSV promotes the transition of *P. aeruginosa* to a biofilm mode of growth. Iron plays an essential role in the development of biofilms both on abiotic surfaces and in association with AECs for *P. aeruginosa* and other bacterial species (18, 23, 24, 38). A strong positive correlation has been reported among increased iron levels, *P. aeruginosa* load, and disease severity in the CF lung, but the effect of respiratory viral infection on iron levels has not been investigated (15, 17, 39). In our studies we found that, during the course of infection, RSV increased apical iron release from the airway epithelium and that iron chelation dramatically reduced biofilm growth, implying that iron chelation is effective in counteracting virus-mediated iron release and biofilm growth. Although *P. aeruginosa* biofilms grown on AECs display significantly higher antibiotic resistance than biofilms formed on abiotic surfaces, the use of iron chelation compounds significantly increases antibiotic-mediated disruption of biofilms on cells (40). Moreover, it has been demonstrated recently that *P. aeruginosa* dispersed from biofilms by chemical induction is highly sensitive to iron stress (41). This high sensitivity to iron limitation suggests that iron chelation compounds may play a role in preventing the spread of bacteria to new infection sites. A novel approach to inhibiting *P. aeruginosa* growth and biofilm formation uses the transition metal gallium (Ga^{3+}) to disrupt *P. aeruginosa* iron metabolism (42, 43). Therefore, the coadministration of iron chelators that can prevent bacterial iron acquisition and compounds that disrupt bacterial iron metabolism may be an important therapeutic strategy during respiratory virus seasons to prevent the development and spread of chronic bacterial infections in CF patients.

Iron is a critical nutrient for many cellular processes in humans, but the quantity and location of iron must be regulated tightly to prevent infection (14). Even in the absence of infection, extracellular iron is bound to high-affinity iron-binding proteins, such as transferrin, to maintain extremely low levels of free iron. We found that RSV infection promoted the apical release of transferrin from AECs and that transferrin contributed significantly to the formation of *P. aeruginosa* biofilms. The mechanism(s) by which *P. aeruginosa* responds to the environmental conditions imposed by RSV infection, including the presence of transferrin, are important to consider. One mechanism by which *P. aeruginosa* overcomes iron sequestration is through the siderophore pyoverdine, which facilitates iron acquisition from transferrin and is important for bacterial growth and biofilm formation (24, 44). The presence of pyoverdine in CF sputum suggests the importance of pyoverdine-mediated iron uptake by *P. aeruginosa* in the CF lung (45). Taken together, these studies suggest that pyoverdine-mediated acquisition of iron from transferrin may be an important mechanism by which chronic *P. aeruginosa* infections initially develop in the CF lung and that respiratory viral infection may instigate such a microenvironment. In support of this hypothesis, longitudinal analyses of CF clinical isolates suggest that *P. aeruginosa* pyoverdine production is high early in infection (46, 47).

In summary, many clinical studies in patients with chronic lung disease report viral–bacterial interactions that result in poor bacterial clearance and disease progression. In the current study, we demonstrate that respiratory viral infections dysregulate host iron homeostasis mechanisms, promoting harmful secondary bacterial infections. By improving our mechanistic understanding of viral–bacterial coinfections, these studies aid in the development of new treatments to target complex infectious diseases. Moreover, because mounting clinical evidence suggests that many infectious diseases are polymicrobial in nature, our studies likely have implications for studying complex microbial communities during other disease processes.

Materials and Methods

Cell Lines, Virus Infection, and Bacterial Strains. The immortalized human CF bronchial epithelial cell line CFBE41o- (referred to herein as "AECs") was isolated from a ΔF508/ΔF508 patient, cultured on Transwell filters, and grown at the air–liquid interface for 7–10 d. Primary HBE cells were obtained (with informed consent under the protocol approved by the Institutional Review Board at the University of Pittsburgh, as described in ref. 19) from explanted lungs of CF patients and were cultured on Transwell inserts under air–liquid conditions Cells were infected with the purified human A2 strain of RSV [multiplicity of infection (MOI) of seeded cells = 1], hrV14 (MOI = 1), or human Ad5 (MOI = 1) for 72 h. The *P. aeruginosa* strain PAO1 constitutively expressing GFP was used as previously described (19, 40).

Coculture Biofilm Experiments. Live-cell imaging was used to image bacterial biofilms grown on epithelial cells in the presence or absence of virus infection, as described in refs. 18, 19, and 40). *P. aeruginosa* static coculture biofilm experiments were performed as described previously (19, 40).

Iron Analysis. CM was collected from AECs, cell debris was removed by centrifugation at 1,400 × *g*, and the CM was stored at 4 °C. Total iron in CM was measured using with the QuantiChrom Iron Assay Kit (BioAssay Systems).

In Vivo RSV Infection Studies. In vivo RSV infection studies were carried out in strict accordance with the recommendations in the NIH *Guide for the Care and Use of Laboratory Animals* (48) and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) (protocol number 14023340). Mice were handled according to IACUC guidelines, and all efforts were made to minimize animal suffering. Mice were housed at the University of Pittsburgh Division of Laboratory Animal Resources. Neonatal mice (pups) from BALB/cJ mice were intranasally inoculated with 5×10^5 pfu/g body weight of RSV line 19 or PBS, as previously described (28). BALF was harvested for analysis by iron assay, and transferrin abundance was assessed by Western blot.

Statistical Analysis. GraphPad Prism version 6.0 (GraphPad) was used for statistical analysis. Means were compared using Student's *t* test or, for multiple comparisons, ANOVA with Tukey's post hoc test. $P < 0.05$ was considered significant.

Additional methods can be found in *SI Materials and Methods*.

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