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Airway surface liquid has innate antiviral activity that is reduced in cystic fibrosis

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Abstract

While chronic bacterial infections and inflammation are associated with progressive lung disease in cystic fibrosis (CF) patients, much less is known regarding the contributions of respiratory viral infections to this process. Clinical studies suggest that antiviral host defenses may be compromised in individuals with CF, and CF airway epithelia exhibit impaired antiviral responses *in vitro*. Here, we used the CF pig model to test the hypothesis that the antiviral activity of respiratory secretions is reduced in CF. We developed an *in vitro* assay to measure the innate antiviral activity present in airway surface liquid (ASL) from CF and non-CF pigs. We found that tracheal and nasal ASL from newborn non-CF pigs exhibited dose-dependent inhibitory activity against several enveloped and encapsidated viruses including Sendai virus (SeV), respiratory syncytial virus (RSV), influenza A, and adenovirus. Importantly, we found that the anti-SeV activity of nasal ASL from newborn CF pigs was significantly diminished relative to non-CF littermate controls. This diminution of extracellular antiviral defenses appears to be driven, at least in part, by the differences in pH between CF and non-CF ASL. These data highlight the novel antiviral properties of native airway secretions, and suggest the possibility that defects in extracellular antiviral defenses contribute to CF pathogenesis.

Key Words: cystic fibrosis, airway secretions, host defense, airway surface liquid, antiviral proteins

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disease among Caucasians, with patients exhibiting a median life expectancy in the early 40s (1). While CF is a multi-organ disease, most morbidity is caused by chronic lung disease characterized by acute and chronic bacterial infections and neutrophil-dominated inflammation (2). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (3, 4), a nucleotide-phosphorylation regulated anion channel that conducts Cl^-, HCO_3^- (5), and SCN^- (6) across epithelia. Much is still unknown about the link between CFTR dysfunction and lung disease progression.

Although CF newborns are commonly reported to have normal lungs, growing evidence indicates that airway host defenses are impaired as early as the first month after birth (7, 8). Studies using the CF pig model have provided insights into factors initiating CF lung disease. Newborn CF pigs lack airway inflammation, but within hours of birth their airways exhibit impaired antibacterial defenses compared to non-CF littermates (9). It was shown by several experimental approaches that this defect in antibacterial defenses can be attributed to a reduced bacterial killing activity in CF airway secretions (10), suggesting that abnormalities in the extracellular compartment contribute to this defect. These findings suggest that defective innate immunity contributes to early lung disease onset and progression.

The contribution of respiratory viral infections to CF lung disease onset and progression is not well understood. Although viral infection rates are similar between infants and children with CF

and their non-CF counterparts (11-13), disease severity from viral infections appears to be greater in children with CF. Compared with control subjects, children with CF exhibit a greater likelihood of lower respiratory tract involvement during viral infections (11, 13), higher rates of hospitalization from respiratory infections (11), and significant and persistent decreases in pulmonary function after viral infection (11, 14). Additionally, epidemiological studies point to an important association between respiratory viral infections and bacterial colonization of the lungs in early childhood for children with CF (15-17). Increased viral loads have been observed in the lungs of RSV-infected CF children relative to infected non-CF controls, despite similar infection prevalence between the two groups (18), suggesting that host defense mechanisms for restricting viral replication may be less effective in CF lungs. This concept is further supported by *in vitro* studies of cultured CF and non-CF airway cells, demonstrating that several aspects of antiviral host defense, including interferon signaling and apoptotic responses to viral infection, are perturbed in CF airway epithelia (19-22).

Defensive responses to respiratory viruses are primarily mediated by the airway epithelium, a common site of virus replication and a first line of defense against inhaled microbes and other particulates. Serving as a vital barrier between the body and the external environment, the airway epithelium is home to cell-based mechanisms for recognizing and responding to viruses, including viral pattern recognition receptors and signaling pathways that initiate and amplify responses to viral pathogens. The airway epithelium is covered by an aqueous periciliary liquid bathing the cilia and mucus that traps microbes and allows for their removal from the airways, collectively termed airway surface liquid (ASL). Airway epithelia secrete a number of innate immune molecules into this ASL, including lysozyme, lactoferrin, defensins, cathelicidins, and

many others (23). Many of these are multifunctional molecules exhibiting a diverse array of defensive functions, which can include antibacterial, antiviral, and antifungal activities; biofilm prevention; opsonization; pro/anti-inflammatory effects; inhibition of host and pathogen proteases; and chemokine activity, among other functions (reviewed (23)). In addition, dendritic cells, neutrophils, and resident alveolar macrophages are present or may be recruited to the airways to participate in antiviral defenses and act as a bridge between the innate and adaptive immune responses to viruses.

While several studies have described defects in the cellular responses of CF airway epithelia to viral pathogens, relatively little is known regarding the impact that genotype-dependent differences in the extracellular environment have on antiviral defenses. Using an assay developed to measure the native antiviral activity present in airway secretions, we found that ASL from humans and pigs exhibits innate antiviral properties toward several relevant classes of respiratory viruses. Here we use this assay to test the hypothesis that extracellular antiviral host defenses are impaired in CF. We observed reduced antiviral activity in ASL from CF pigs relative to non-CF, raising the possibility that defects in extracellular antiviral host defense may play a role in the establishment of CF lung disease.

Materials and Methods

Collection and processing of airway surface liquid

Three-week old pigs were anesthetized and tracheal secretions were stimulated using methacholine as previously described (10). Airway surface liquid (ASL) was harvested by advancing a microsampling probe (model BC-401C; Olympus Optical Co, Tokyo, Japan)

through a pediatric bronchoscope inserted into the trachea. ASL was then collected by centrifuging at 10,000 x g for 10 minutes, irradiated at 80 Gy and stored at -80°C. Nasal ASL was collected from humans and newborn pigs by inserting a sterile polyester-tipped applicator (Puritan Medical Products Co, Guilford, ME, USA) into each nostril for 5 minutes. Nasal ASL was collected by microcentrifugation, irradiated at 8 krad, and stored at -80°C until use. Bronchoalveolar lavage (BAL) was collected from newborn CF and non-CF pigs according to previously described protocols (24).

Viruses

Viral inactivation assays were carried out using recombinant Sendai virus encoding an *eGFP* reporter gene (25, 26) as well as a recombinant respiratory syncytial virus encoding green fluorescent protein (RSV-GFP) upstream of the NS1 open reading frame (27). Influenza A virus (A/Puerto Rico/8/1934) encoding the *eGFP* gene in the NS segment (IAV-eGFP) (28) was kindly provided by Kevin Legge (University of Iowa, Iowa City, IA, USA). Recombinant, replication incompetent adenovirus encoding *eGFP* (Ad-eGFP) was prepared by the University of Iowa Viral Vector Core Facility.

Viral inactivation assay

To assess dose-dependent viral inactivation by airway secretions, increasing volumes of ASL were pre-incubated for 2 hours with 10^6 fluorescence forming units (ffu) of Sendai virus (SeV-eGFP), 4 x 10^5 plaque forming units (pfu) of respiratory syncytial virus (RSV-GFP), 2 x 10^5 pfu of adenovirus (Ad-eGFP), or 10^6 ffu of influenza A (IAV-eGFP). Virus:ASL mixtures were then brought up to a volume of 250 µL in 100 mM HEPES buffer (pH 7.4) and applied at a MOI of 5-

10 to appropriate cell lines: LLC-MK2 cells for SeV-eGFP, A549 cells for IAV-eGFP, and HEp-2 cells for RSV-GFP and Ad-eGFP. Cells were incubated with the virus:ASL mixtures for 1 hour at 37˚C, then mixtures were removed and replaced with cell culture medium. After overnight incubation at 37˚C and 5% CO_2, cells were harvested and GFP-positive cells in each condition quantified using a BD Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). "Relative infectivity" represents the number of GFP-positive cells arising from infection with ASL-treated virus, expressed as a percentage of the number of GFP-positive cells after infection with untreated virus.

Statistical analysis

All data are presented as means ± standard error about the mean. Statistical significance was determined using Student's *t*-tests or one-way ANOVA followed by Tukey's post-hoc tests, using Graphpad Prism (Version 6.02).

Results

Airway secretions exhibit antiviral activity

Tracheal (29) and nasal airway secretions (30, 31) have previously been shown to have broad spectrum antibacterial activity. However, the possible antiviral properties of airway secretions are less well established and, to our knowledge, have not been studied. To probe for such activity, we developed an assay to measure the innate antiviral activity of airway secretions, using nasal and tracheal ASL collected from 3-week old methacholine-stimulated pigs as a model. Porcine nasal and tracheal ASL were incubated with Sendai virus (SeV), a member of the *Paramyxoviridae* family of negative sense single stranded enveloped RNA viruses, for 2 hours at

37°C, 5% CO_2. For these studies, we used a recombinant SeV expressing an enhanced green fluorescent protein (eGFP) reporter (SeV-eGFP), to assess viral infection. After the 2-hour incubation period, the virus:ASL mixtures were applied to LLC-MK2 cells, a rhesus monkey kidney epithelial cell line that is permissive for SeV. After ~18 hours incubation at 37°C, we quantified eGFP-positive cells by flow cytometry. As shown in **Figure 1A**, pre-incubation with porcine nasal or tracheal ASL reduced infection by SeV-eGFP in a dose-dependent manner. In time course studies with SeV-eGFP, we observed that the majority of antiviral activity occurred within the first 30 minutes of virus:ASL pre-incubation, with maximal viral inhibition consistently occurring after 2 hours of incubation (**Supplemental Figure E1**). For this reason, we selected a 2 hour incubation time for subsequent experiments.

Using this experimental approach, we then asked whether ASL inhibited the infectivity of other respiratory viruses. We found that porcine nasal and tracheal ASL also have inhibitory activity against the related pneumovirus, respiratory syncytial virus (RSV-GFP) (**Figure 1B**). Similar to the SeV-eGFP results, RSV-GFP infection was significantly reduced in the presence of nasal ASL; tracheal ASL displayed a more modest effect against this virus, with an approximate 25% reduction in RSV-GFP infection levels. We also found that, in addition to their effects against SeV and RSV, porcine nasal and tracheal ASL dose-dependently reduced infectivity of the orthomyxovirus, influenza A virus (IAV-eGFP) (**Figure 1C)** and serotype 5 adenovirus (Ad-eGFP) (**Figure 1D**). Unlike SeV, RSV, and IAV, adenovirus is an encapsidated non-enveloped DNA virus. These results suggest that ASL possesses inhibitory activity against a range of respiratory viral pathogens.

Of interest, we noted that similar antiviral activity could be detected in airway secretions from humans. We found that human nasal ASL reduced the infectivity of SeV-eGFP (**Figure 2A**), RSV-GFP (**Figure 2B**), IAV-eGFP (**Figure 2C**), and Ad-eGFP (**Figure 2D**) in a dose-dependent manner similar to that of porcine nasal and tracheal ASL, suggesting that this property is a conserved feature of ASL. For the remainder of our studies, we selected SeV as a model virus because of its close antigenic and genetic homology to human parainfluenza virus 1 (HPIV-1). HPIV-1, along with HPIV-3, causes one-third of the lower respiratory tract infections in children under the age of 5 in the United States (32). Of note, human parainfluenza viruses are also implicated in pulmonary exacerbations in children with CF (7, 33, 34).

To begin to characterize the antiviral activity we observed in porcine and human ASL, we tested whether the activity was heat labile. Porcine tracheal ASL was heated for 30 minutes at 56˚C, 65˚C, or 95˚C, or left on ice for 30 minutes. Heat treatment at 56˚C is sufficient to inactivate components of the complement system, while most proteins are denatured at 95˚C; the 65˚C treatment condition was chosen as an intermediate temperature. Heat-treated ASL samples were then incubated with SeV-eGFP for 2 hours before being used to infect LLC-MK2 cells as described above. ASL incubated at 56˚C exhibited a modest, non-significant reduction in anti-SeV activity compared to ASL incubated on ice (**Figure 3A**). ASL anti-SeV activity was significantly reduced when heated to 65˚C or 95˚C, with 95˚C treatment causing the greatest reduction in ASL antiviral activity. The heat-lability of ASL antiviral activity suggests that proteins are responsible for the activity. In addition, the partial reduction in ASL antiviral activity after incubation at 56˚C and 65˚C implies that multiple components contribute to the antiviral activity.

Proteins from serum can reach the airways through transudation or, in the case of IgA, specific transcellular transport pathways. For this reason, many host defense proteins present in blood may be found in ASL or bronchoalveolar lavage (BAL) fluid. We therefore considered that proteins such as complement or immunoglobulins might contribute to the antiviral activity of ASL. To test this hypothesis, we assessed antiviral activity in serial dilutions of serum from newborn pigs using the SeV-eGFP infectivity assay. For this study, the serum was either left untreated or heat treated for 30 minutes at 56˚C to inactivate complement. Untreated serum had potent, dose-dependent antiviral activity against SeV-eGFP (**Figure 3B**). However, in contrast to tracheal ASL, serum completely lost its anti-SeV activity after heating to 56˚C. Together with the ASL heat lability studies, these results suggest that the ASL antiviral components are not solely derived from serum (although we cannot exclude the possibility that serum-derived proteins make some contribution to the overall antiviral activity in ASL).

ASL from CF pigs has reduced antiviral activity

Earlier studies indicated that CF pigs have impaired antimicrobial defenses at birth, and that a relatively acidic ASL pH leads to reduced antibacterial properties of their airway secretions (29). We hypothesized that CF pig ASL would similarly have reduced antiviral activity compared to non-CF ASL. To test this, we collected nasal ASL from newborn $CFTR^{-/-}$ pigs (CF pigs) and non-CF littermates ($CFTR^{+/+}$ and $CFTR^{+/-}$). As shown in **Figure 4**, CF pig nasal ASL exhibited significantly reduced antiviral activity against SeV-eGFP, relative to that of non-CF pig nasal ASL. This reduced antiviral activity was seen consistently throughout a range of tested volumes of ASL (**Figure 4**). We confirmed that newborn CF pig nasal ASL has a reduced pH compared

to non-CF littermates (**Figure 5A**), in agreement with previous reports for newborn CF pig tracheal ASL (29), newborn human nasal fluid (35), airway breath condensate (36), human primary airway epithelial cells (37), and human submucosal gland secretions (38).

To test the hypothesis that the reduced ASL pH in CF impairs antiviral activity, we predicted that increasing the pH of CF nasal ASL would shift antiviral activity toward non-CF levels. We tested this by measuring the antiviral activity of CF and non-CF nasal ASL at 3 defined pHs (pH 6.8, 7.4, and 8.0). In contrast to the experiments with unmodified ASL shown in **Figures 1-4**, here the pH was clamped in each condition using buffer. As shown in **Figure 5B**, while the CF ASL continued to display a slight (non-significant) trend toward reduced antiviral activity relative to the non-CF ASL at each pH, the genotype-dependent differences in ASL antiviral activity largely disappear when pH is clamped in this way.

We also addressed this hypothesis by asking whether altered pH might impact the antiviral functions of individual antimicrobial proteins and peptides (AMPs) with known antiviral activity in the airways. (Of note, the overall protein abundance did not differ significantly between CF and non-CF nasal ASL, **Supplemental Figure E2**). The human and porcine cathelicidins LL-37 and protegrin-1, human β-defensin 3 (HBD-3), and lysozyme all displayed antiviral activity against SeV-eGFP in our infectivity assay (**Figure 6**). Each of these peptides, except lysozyme, had activity that was increased at pH 6.8, though statistically significant differences between different pH conditions were only observed for HBD-3. Human β-defensin 2 (HBD-2) and lactoferrin did not exhibit significant antiviral activity against SeV-eGFP in this assay.

Discussion

Here, we report that ASL from the airways of pigs and humans exhibits antiviral properties against a range of clinically relevant respiratory viruses, including RSV, influenza, and adenovirus. While it has long been recognized that airway epithelia mediate defensive responses to viral pathogens through cell-based signaling pathways, such as interferon production and inflammasome activation (39), there is less appreciation of the role that secreted proteins and peptides in the extracellular environment play in antiviral defenses. Our findings in pigs and humans suggest that - similar to what is known regarding antibacterial defenses - secretion of a suite of antiviral effector molecules into the ASL is likely a conserved mechanism forming an "antiviral shield" to protect the airways from inhaled viruses.

Based on our heat lability studies, we propose that the antiviral activity of porcine ASL is largely protein-mediated and represents the combined activities of multiple secreted factors. Antiviral properties have been described for a number of individual proteins and peptides found in human ASL, including LL-37 (40), HBD-2 (41) and HBD-3 (42), lactoferrin (43), lysozyme (44), SLPI (45), and the collectins surfactant protein A (SP-A) (46) and surfactant protein D (SP-D) (47). Several mechanisms are responsible for the antiviral actions of these proteins and peptides, including direct binding to and aggregation of viruses through interactions with lipid bilayers and/or viral glycoproteins, and in some cases disruption of viral membranes (40, 41). In contrast, lactoferrin inhibits parainfluenza virus 2 (PIV2) infection by binding to cellular proteins rather than through direct interaction with the virus (43). Additionally, airway mucins are thought to contribute to antiviral defenses by interacting with viruses in ways that impede their movement and/or prevent their uptake (48-50). Proteomic analysis indicates that many of the antimicrobial

factors found in human airways can also be detected in porcine ASL, such as lactoferrin, lysozyme, SP-A, SP-D, SLPI, and numerous mucins including MUC1, -2, -4, -5B, -5AC, -13, -16, and -19 (51). While LL-37 is unique to humans, porcine ASL contains a related molecule, the cathelicidin protegrin-1 (51), which displays antiviral activity against several different viruses (52, 53). We expect that these proteins, and likely others, contribute to the broad-spectrum antiviral activity of porcine ASL.

Our studies demonstrate that this innate antiviral property of ASL is diminished in CF. This is consistent with a growing body of literature supporting the idea that antiviral defenses are impaired in CF airways. Compared with non-CF cells, CF airway epithelia have been shown to support increased replication of parainfluenza virus 3 (PIV3) (22), influenza (21), and rhinovirus (19, 20), and viral loads are increased in the lungs of CF mice after experimental infection with RSV (54). To date, these studies have focused on dysregulated interferon signaling, possibly as a consequence of chronic inflammation in the airway tissues that cells were derived from, as a central mechanism for this defect. In support of this, Xu and colleagues reported delayed production of interferon-β in CF cells upon infection with influenza virus, along with altered expression of a number of interferon-inducible and antiviral response genes (21). Another report described blunted expression of the intracellular molecules 2',5'-oligoadenylate synthetase 1 (OAS1) and nitric oxide synthase 2 (NOS2) in CF airway epithelia, both at baseline and after infection with PIV3 (22).

Our results suggest that, in addition to this defect in the cellular interferon response, compromised antiviral activity in the extracellular compartment is also a likely contributor to

impaired antiviral defenses in CF. Importantly, our experiments were performed using ASL from newborn CF pigs. CF pig airways lack inflammation at birth (24), and would have had minimal exposure to infectious or other immunostimulatory agents at the time of sample collection. Therefore, the observation of decreased antiviral activity in newborn airway secretions argues against the idea that this phenotype is a secondary consequence of inflammation, and instead suggests that diminished antiviral capacity is an inherent property of CF ASL.

The finding that CF pig ASL is intrinsically less antiviral than non-CF ASL echoes the observation that bacterial killing is impaired in the airway surface liquid of CF pigs (10). In the case of this bacterial killing defect, antibacterial activity can be restored by adjusting the pH of CF ASL to non-CF levels (10), indicating that loss of antibacterial activity in these secretions is primarily due to altered function of secreted antimicrobial proteins and peptides (AMPs) in the lower pH of CF ASL. In the current study, the finding that CF and non-CF nasal ASL exhibited similar antiviral activity when tested at equivalent pHs suggests that the disease-related reduction in pH is likely contributing to the reduced antiviral activity of CF ASL.

However, our experiments also suggest a somewhat more complicated picture with respect to the effect of pH on the antiviral activity of native secretions. In the airways, ASL pH is regulated by secretion of HCO_3^- and H^+ (55). Contrary to our hypothesis, reducing the pH of non-CF nasal ASL to a lower, more “CF-like” level had only a very modest effect on overall antiviral activity. Further, the effect of pH on the activities of individual antimicrobial molecules was variable, with some molecules (LL-37, protegrin-1, HBD-3) exhibiting increased antiviral activity at lower pH, while others (lysozyme) were unaffected by pH changes. We speculate that this lack

of consistent pH-dependence reflects the fact that the secreted antimicrobial factors in ASL inactivate bacteria and viruses by distinct mechanisms.

We note that while adjusting the pH in the SeV infectivity assay significantly diminished the magnitude of the CF/non-CF difference in antiviral activity, CF ASL still showed a slight impairment in activity (not statistically significant) with respect to non-CF ASL at all pHs tested (**Figure 5B**). This observation suggests that pH differences may provide only a partial explanation for the defect in CF secretions. It is possible that differences in the composition of CF and non-CF ASL may additionally influence antiviral activity. For example, while the overall protein abundance between CF and non-CF nasal ASL is not significantly different (**Supplemental Figure E2**), it is possible that CF ASL is lacking in a specific factor (or several factors) which accounts for some proportion of the antiviral activity in airway secretions. In an earlier study, no significant differences were noted in the abundances of selected AMPs (lysozyme, lactoferrin, PLUNC, SP-A) in tracheal ASL from newborn CF and non-CF pigs (10). However, it is currently unknown whether levels of other antimicrobial agents may be altered in CF secretions. Additionally, it has been reported that protease/antiprotease balance is altered in CF ASL, which could potentially impact innate immunity by causing aberrant cleavage and inactivation of secreted antiviral factors (56-58). Further study will be needed to better understand whether these additional mechanisms contribute to the reduced antiviral activity in CF ASL.

In conclusion, we found that human and porcine ASL is innately antiviral, with broad spectrum activity against representative enveloped and encapsidated respiratory viruses. Further, the

potency of this antiviral activity is reduced in ASL from CF pigs relative to non-CF controls. These findings implicate extracellular antiviral mechanisms in defending the airways from inhaled viral pathogens, and also suggest that altered extracellular defenses may provide an advantage to invading viruses in CF airways. The activities of secreted antiviral molecules in the extracellular milieu are part of a larger, multilayered antiviral defense system, and further studies are needed to determine the overall impact of this defect in CF. It is possible that this reduction in the antiviral properties of CF airway surface liquid leads to a weakening of antiviral host defense in CF airways, which is then further impaired by chronic inflammation associated with the progression of CF lung disease. This reveals a previously underappreciated aspect of airway innate immunity and provides insight into how defects in this system may contribute to dysfunctional host defenses in CF.

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Figure Legends

Figure 1. Porcine ASL has innate antiviral activity against respiratory viruses. (A) SeV-eGFP, (B) RSV-GFP, (C) IAV-eGFP, or (D) Ad-eGFP, were incubated with increasing amounts of porcine tracheal or nasal ASL for 2 hours at 37˚C and 5% CO_2. Virus:ASL mixtures were then brought up to 250 μL volume in 100 mM HEPES buffer (pH 7.4) and used to infect appropriate permissive cell lines. Viral infection was quantified 24-hours post infection via flow cytometry analysis of eGFP expression. Infectivity is expressed as the number of GFP-positive cells observed in a given condition, as a percentage of the number of GFP-positive cells seen for the untreated (virus alone) condition. Results are displayed as mean ± SE (n = 3-4 individual pig donors).

Figure 2. Human nasal ASL has innate antiviral activity against respiratory viruses. (A) SeV-eGFP, (B) RSV-GFP, (C) IAV-eGFP, or (D) Ad-eGFP, were incubated with increasing amounts of human nasal ASL for 2 hours at 37˚C and 5% CO_2. Virus:ASL mixtures were then brought up to 250 μL volume in 100 mM HEPES buffer (pH 7.4) and used to infect appropriate permissive cell lines. Viral infection was quantified 24-hours post infection via flow cytometry analysis of eGFP expression. Infectivity is expressed as the number of GFP-positive cells observed in a given condition, as a percentage of the number of GFP-positive cells seen for the untreated (virus alone) condition. Results are displayed as mean ± SE (n = 3 adult donors).

Figure 3. The antiviral activity in porcine ASL is heat labile and does not represent transudated serum components. (A) Porcine tracheal ASL was heated at 56˚C, 65˚C, 95˚C, or incubated on ice, for 30 minutes. After cooling to room temperature, ASL samples (2.5 μL per condition) were incubated with 10^6 ffu SeV-eGFP for 2 hours at 37˚C, 5% CO_2. The virus:ASL mixtures were brought up to a volume of 250 μL in 100 mM HEPES buffer (pH 7.4) before

infecting LLC-MK2 cells. Viral infection was quantified 24-hours post infection via flow cytometry analysis of eGFP expression. Infectivity is expressed as the number of GFP-positive cells observed in a given condition, as a percentage of the number of GFP-positive cells seen for the untreated (virus alone) condition. Results are presented as mean ± SE. (n = 3 replicate experiments) *$p < 0.05$; ***$p < 0.001$ as determined by one-way ANOVA followed by Tukey's multiple comparisons test. Only statistically significant differences are noted. (B) Porcine serum was either heat-treated at 56˚C or left on ice for 30 minutes. The serum was then serially diluted and incubated with SeV-eGFP (10^6 ffu) for 1 hour at 37˚C, 5% CO_2. The samples were brought up to a volume of 250 μL in Opti-MEM medium and anti-SeV activity was assessed as described in (A). Results are presented as mean ± SE (n = 3 replicate experiments).

Figure 4. The innate antiviral activity of ASL from newborn CF pigs is reduced compared to non-CF pig secretions. SeV-eGFP was incubated with increasing amounts of nasal ASL from newborn CF and non-CF pigs for 2 hours at 37˚C, 5% CO_2. The virus:ASL mixtures were then brought up to a volume of 250 μL in 100 mM HEPES buffer (pH 7.4) and used to infect LLC-MK2 cells. Viral infection was quantified 24-hours post infection via flow cytometry analysis of eGFP expression. Infectivity is expressed as the number of GFP-positive cells observed in a given condition, as a percentage of the number of GFP-positive cells seen for the untreated (virus alone) condition. Results are presented as mean ± SE. *$p < 0.05$ for area under the curve as determined by Student's t-test. (n = 6 non-CF, 8 CF)

Figure 5. pH dependence of antiviral activity in CF and non-CF nasal ASL. (A) The pH of newborn CF and non-CF pig nasal ASL was measured *ex vivo* with a Restech Dx-pH probe immediately prior to antiviral activity measurements. (n = 6 non-CF, 8 CF) Results are presented as mean ± SE. *$p < 0.05$ as determined by Student's *t*-test. (B) The pH of newborn CF and non-

CF nasal ASL was adjusted by mixing 1 μL of each sample with 25 μL 100 mM HEPES buffer at pH 6.8, 7.4, or 8.0. The viral inactivation assay was then carried out as described in Methods (n = 16 non-CF, 13 CF). Results are presented as mean ± SE.

Figure 6. Antiviral activity of individual host defense molecules at varying pHs. SeV-eGFP was incubated with increasing amounts of **(A)** human LL-37, **(B)** porcine protegrin-1, **(C)** human β-defensin 3 (HBD-3), **(D)** human lysozyme, **(E)** human β-defensin 2 (HBD-2), or **(F)** human lactoferrin for 2 hours at 37˚C and 5% CO_2. Host defense proteins were suspended in 100 mM HEPES buffers at pH 6.8, 7.4, or 8.0. After the 2 hour incubation, SeV-eGFP infectivity was determined as described in Methods. Results are presented as mean ± SE (n = 3 replicate experiments). $^{**}p < 0.01$; $^{***}p < 0.001$ for area under the curve as determined by one-way ANOVA followed by Tukey's multiple comparisons test.

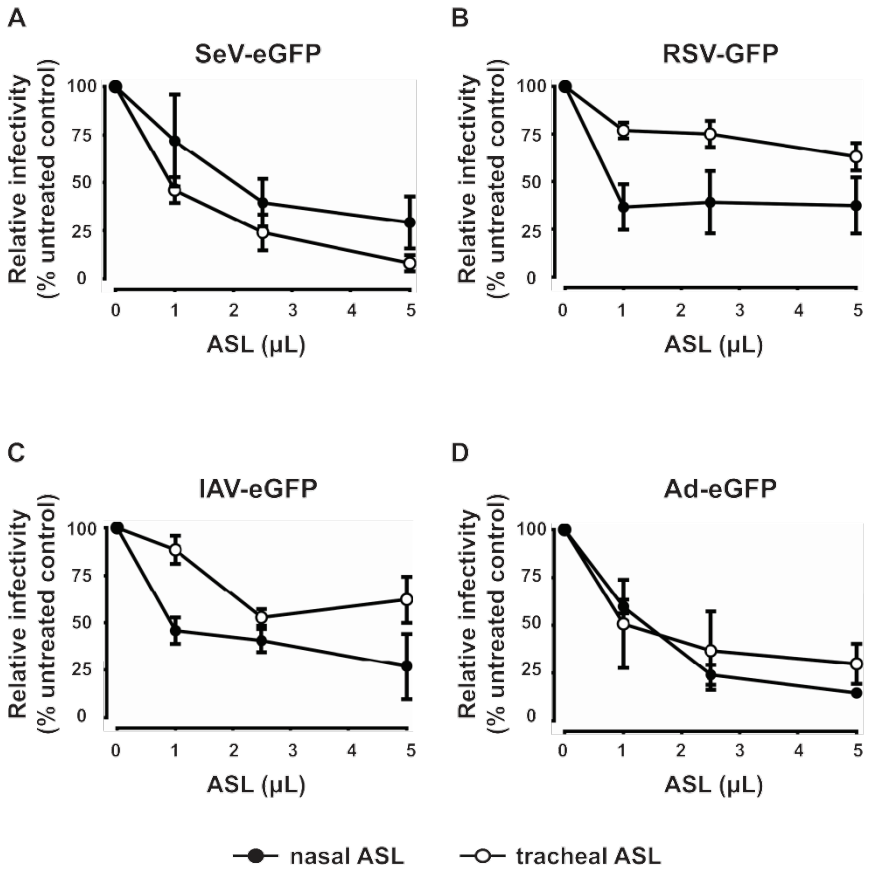
A
SeV-eGFP
B
RSV-GFP
C
IAV-eGFP
D
Ad-eGFP
Relative infectivity (% untreated control)
100
75
50
25
0
0
1
2
3
4
5
ASL (µL)
nasal ASL
tracheal ASL

Figure 1

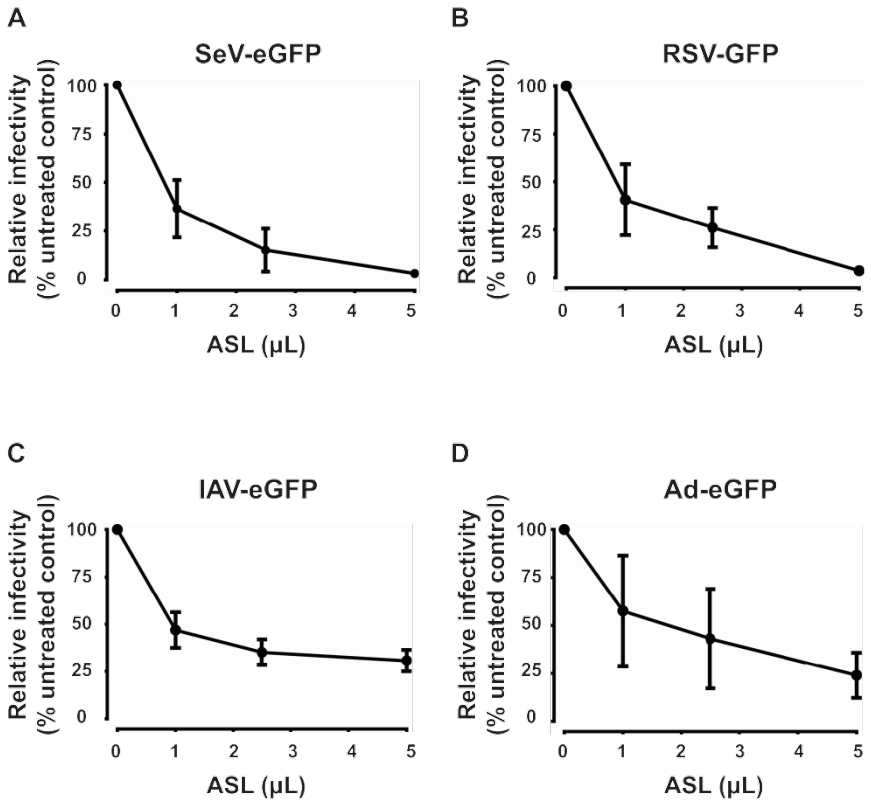
A
SeV-eGFP
Relative infectivity (% untreated control)
100
75
50
25
0
0 1 2 3 4 5
ASL (µL)
B
RSV-GFP
Relative infectivity (% untreated control)
100
75
50
25
0
0 1 2 3 4 5
ASL (µL)
C
IAV-eGFP
Relative infectivity (% untreated control)
100
75
50
25
0
0 1 2 3 4 5
ASL (µL)
D
Ad-eGFP
Relative infectivity (% untreated control)
100
75
50
25
0
0 1 2 3 4 5
ASL (µL)

Figure 2

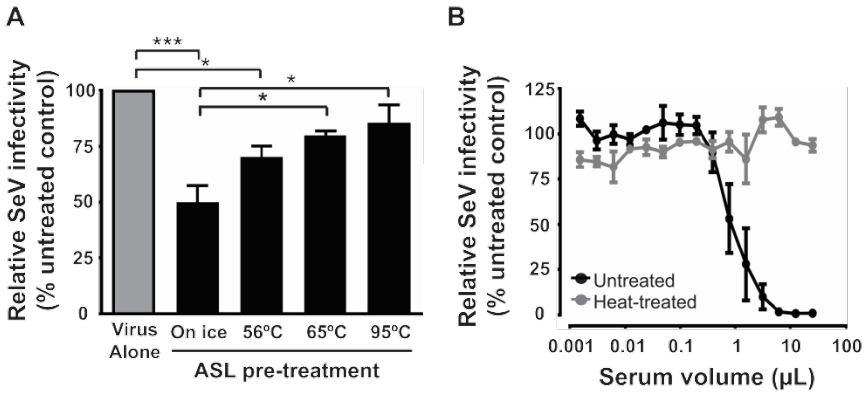
A
***
*
*
*
Relative SeV infectivity
(% untreated control)
100
75
50
25
0
Virus
Alone
On ice
56°C
65°C
95°C
ASL pre-treatment
B
Relative SeV infectivity
(% untreated control)
125
100
75
50
25
0
Untreated
Heat-treated
0.001
0.01
0.1
1
10
100
Serum volume (µL)

Figure 3

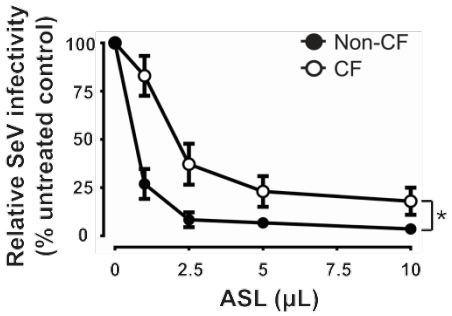
Relative SeV infectivity
(% untreated control)
100
75
50
25
0
Non-CF
CF
*
0
2.5
5
7.5
10
ASL (μL)

Figure 4

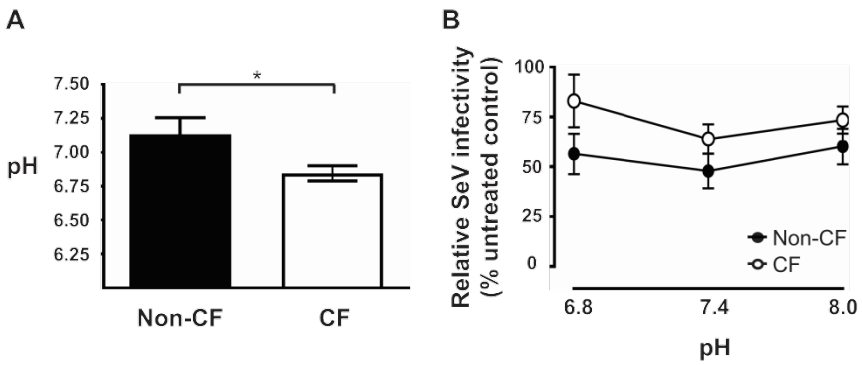
A
B
7.50
7.25
7.00
6.75
6.50
6.25
pH
*
Non-CF
CF
Relative SeV infectivity
(% untreated control)
100
75
50
25
0
Non-CF
CF
6.8
7.4
8.0
pH

Figure 5

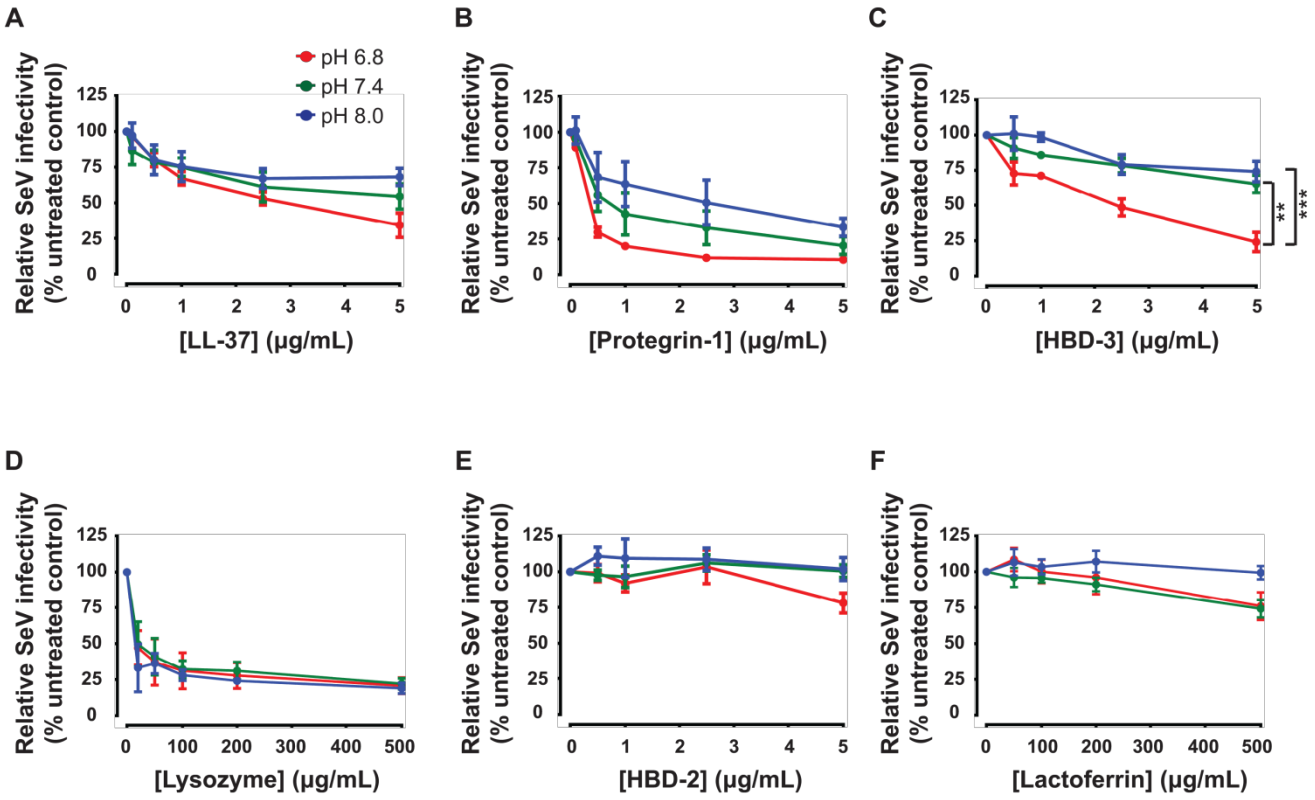
A
B
C
D
E
F
pH 6.8
pH 7.4
pH 8.0
Relative SeV infectivity (% untreated control)
[LL-37] (μg/mL)
[Protegrin-1] (μg/mL)
[HBD-3] (μg/mL)
[Lysozyme] (μg/mL)
[HBD-2] (μg/mL)
[Lactoferrin] (μg/mL)
**
***

Figure 6

Online Data Supplement

Airway surface liquid has innate antiviral activity that is reduced in cystic fibrosis

Abigail R. Berkebile, Jennifer A. Bartlett, Mahmoud Abou Alaiwa, Steven M. Varga, Ultan F. Power, Paul B. McCray, Jr.

Materials and Methods

Viruses and tissue culture

Recombinant Sendai virus encoding the *eGFP* reporter gene (SeV-eGFP) (1, 2) was propagated by injecting the virus into the allantoic fluid of 10-day-old embryonated eggs. For infectivity assays with SeV-eGFP, LLC-MK2 cells were plated in 12-well plates at a density of 2×10^5 cells/well and maintained at 37°C, 5% CO_2, in Opti-MEM medium (Life Technologies, Madison, WI, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Influenza A virus (A/Puerto Rico/8/1934) encoding the *eGFP* gene in the NS segment (IAV-eGFP) (3) was kindly provided by Kevin Legge (University of Iowa, Iowa City, IA, USA). IAV-eGFP infection was assessed on A549 cells that had been seeded onto 48-well plates at a cell density of 4×10^4 cells/well. A549 cells were maintained in DMEM (Life Technologies) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C, 5% CO_2.

Recombinant respiratory syncytial virus encoding green fluorescent protein (RSV-GFP) upstream of the NS1 open reading frame (4) was propagated in HEp-2 cells. Recombinant, replication incompetent adenovirus expressing eGFP (Ad-eGFP) was prepared by the University of Iowa Viral Vector Core. RSV-GFP and Ad-eGFP infectivity were assessed by infecting HEp-2 cells seeded onto 48-well plates at 4×10^4 cells/well. HEp-2 cells were maintained in MEM medium (Life Technologies) containing 10% fetal bovine serum and 1% penicillin-streptomycin, at 37°C and 5% CO_2.

Animals

All animal protocols were approved by the University of Iowa Animal Care and Use Committee. For collection of nasal ASL and bronchoalveolar lavage, CF and non-CF piglets were obtained from Exemplar Genetics (Sioux Center, IA, USA) and samples were collected within 12 hours of birth.

Viral inactivation assays with heat-treated ASL and porcine serum

To test for heat lability of the antiviral activity present in airway secretions, tracheal ASL was heated to 56˚C, 65˚C, or 95˚C, or left on ice, for 30 minutes. For each of these conditions, 2.5 µL of treated ASL was then incubated with 10^6 ffu SeV-eGFP for 2 hours, and our standard viral inactivation assay was carried out. Briefly, virus:ASL mixtures were brought up to a 250 µL volume in 100 mM HEPES buffer (pH 7.4) and applied to LLC-MK2 cells. Cells were incubated with the virus:ASL mixtures for 1 hour at 37˚C, then mixtures were removed and replaced with cell culture medium. After overnight incubation at 37˚C and 5% CO_2, GFP-positive cells were quantified by flow cytometry.

To measure antiviral activity in porcine serum, serum from newborn pigs was serially diluted and incubated with 10^6 ffu SeV-eGFP at 37˚C for 1 hour. Samples were then brought up to 250 µL final volume in Opti-MEM (ThermoFisher Scientific,Waltham, MA, USA) and the viral inactivation assay was performed as described above. Where indicated, serum was heat-inactivated by incubating at 56˚C for 30 minutes prior to dilution in Opti-MEM.

Measurement of nasal secretion properties

Protein concentrations of newborn CF and non-CF porcine nasal ASL samples were measured immediately after collection, using the Pierce Coomassie protein assay kit (Life Technologies). The pH of CF and non-CF nasal ASL was measured at 37˚C and 5% CO_2, using a Dx-pH probe (Restech, San Diego, CA, USA) (5). pH measurements were made immediately prior to performing antiviral activity assays.

Effects of pH on the antiviral properties of ASL and antimicrobial proteins/peptides

To assess the effect of pH on airway secretions, we pre-incubated 1 μL of CF or non-CF nasal ASL with 25 μL 100 mM HEPES buffer at pH 6.8, 7.4, or 8.0 for 5 minutes. SeV-eGFP (10^6 ffu) was then added to the buffered ASL and incubated for 2 hours at 37°C, 5% CO_2. After this 2 hour incubation, the volume of each mixture was brought up to 250 μL with 100 mM HEPES buffer at the appropriate pH (6.8, 7.4, or 8.0), and the ASL:virus mixtures were used to infect LLC-MK2 cells as described previously.

To investigate the antiviral activity of specific antimicrobials, the SeV inactivation assay was performed as described above, using increasing amounts of human LL-37 (Anaspec, Fremont, CA, USA), porcine protegrin-1 (Anaspec), recombinant human beta-defensin 2 (HBD2) (PeproTech, Rocky Hill, NJ, USA), HBD3 (PeproTech), recombinant human lysozyme (Sigma-Aldrich, St. Louis, MO, USA), and human lactoferrin (Sigma-Aldrich) in 100 mM HEPES buffer at pH 6.8, 7.4, or 8.0.

References

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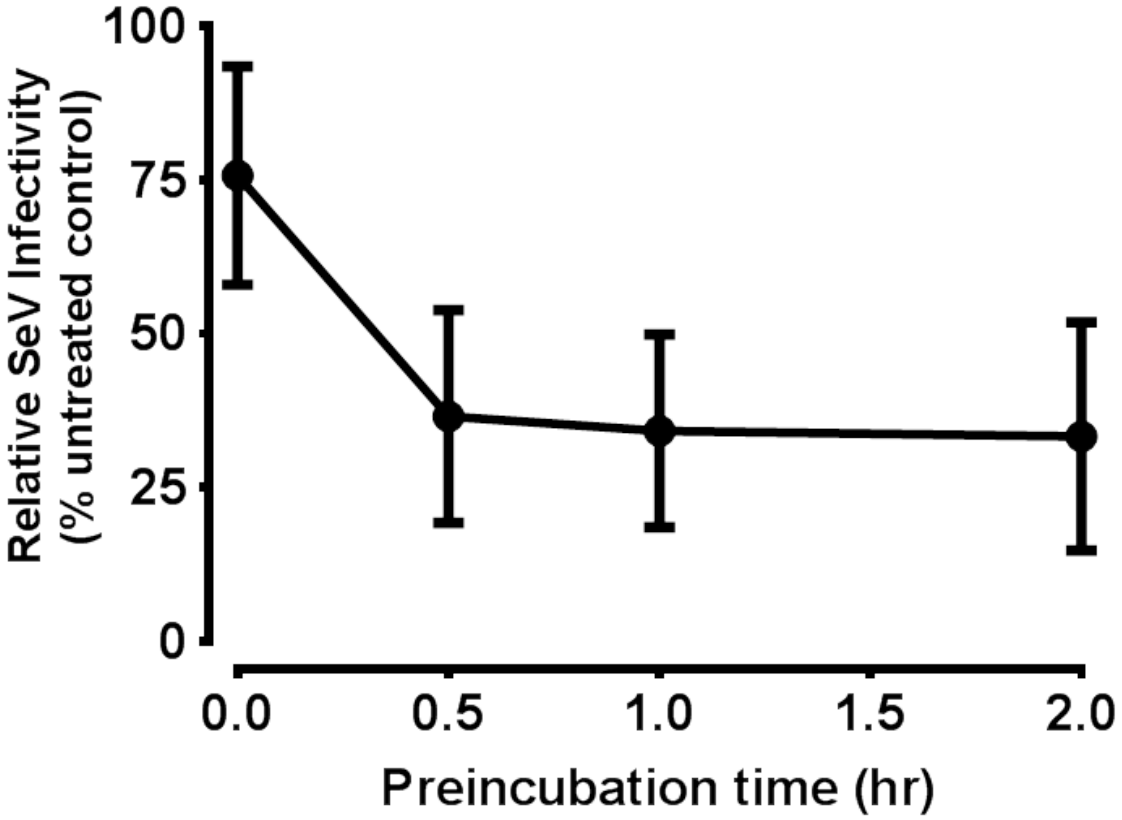
E2. Villenave R, Touzelet O, Thavagnanam S, Sarlang S, Parker J, Skibinski G, Heaney LG, McKaigue JP, Coyle PV, Shields MD, et al. Cytopathogenesis of sendai virus in well-differentiated primary pediatric bronchial epithelial cells. *J Virol* 2010;84(22):11718-11728.

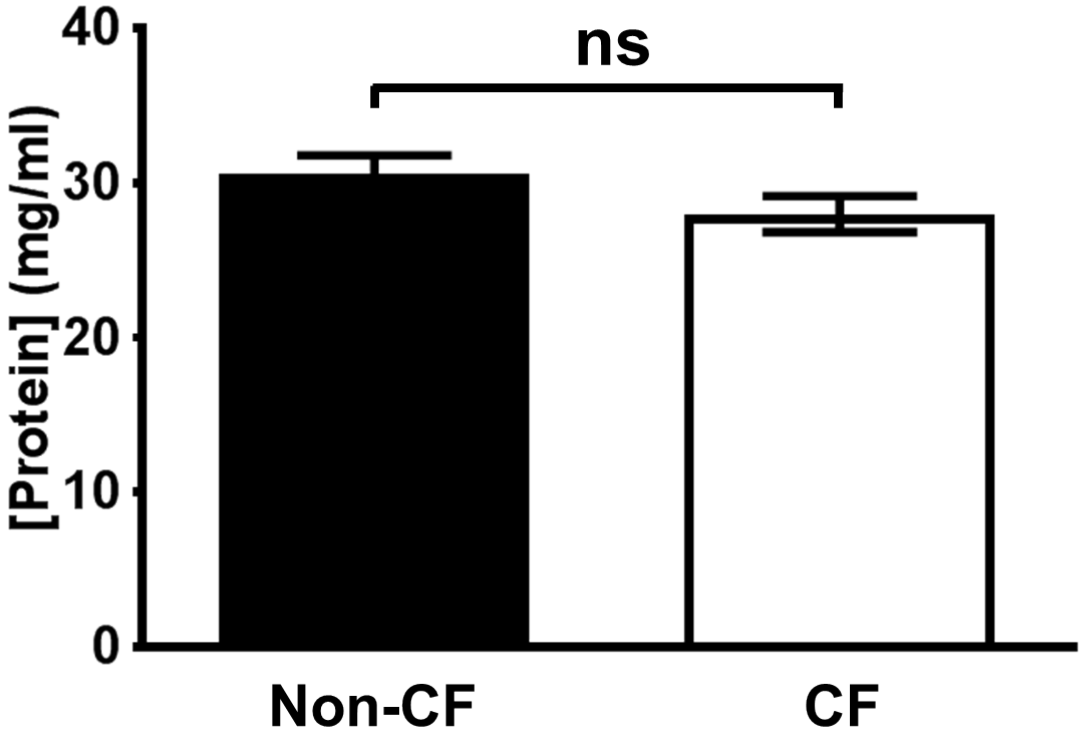
E3. Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, Garcia-Sastre A. Analysis of in vivo dynamics of influenza virus infection in mice using a gfp reporter virus. *Proc Natl Acad Sci U S A* 2010;107(25):11531-11536.

E4. Hallak LK, Spillmann D, Collins PL, Peeples ME. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. *J Virol* 2000;74(22):10508-10513.

E5. Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, Karp PH, Wohlford-Lenane CL, Haagsman HP, van Eijk M, et al. Reduced airway surface ph impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 2012;487(7405):109-113.



Supplemental Figure E1. Time dependence of anti-SeV activity in porcine nasal ASL. SeV-eGFP (10^6 ffu) was pre-incubated with 2.5 µL porcine nasal ASL for 0, 0.5, 1, or 2 hours at 37°C, 5% CO_2. Virus:ASL mixtures were then brought up to 250 µL volume in 100 mM HEPES buffer (pH 7.4) and used to infect LLC-MK2 cells. SeV-eGFP infectivity was quantified 24 hours post infection via flow cytometry analysis of eGFP expression. Infectivity is expressed as the eGFP fluorescence for a given condition as a percentage of the fluorescence seen for the untreated (virus alone) condition. Results are shown as mean ± SE for samples collected from 3 individual pigs.



Supplemental Figure E2. Total protein concentrations in newborn CF and non-CF pig nasal secretions. Protein concentrations were measured using the Pierce Coomassie protein assay kit. (n = 11 non-CF, 13 CF) Results shown as mean ± SE. ns = not statistically significant as determined by Student's *t*-test