Influenza-induced Type I Interferon Enhances Susceptibility to Gram-negative and Gram-positive Bacterial Pneumonia in Mice

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Abstract

Suppression of Type 17 immunity by type I interferon (IFN) during influenza A infection has been shown to enhance susceptibility to secondary bacterial pneumonia. While this mechanism has been described in coinfection with Gram-positive bacteria, it is unclear if similar mechanisms may impair lung defense against Gram-negative infections. Furthermore, precise delineation of the duration of type I IFN-associated susceptibility to bacterial infection remains underexplored. Therefore, we investigated the effects of preceding influenza A virus infection on subsequent challenge with the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, and the temporal association between IFN expression with susceptibility to *Staphylococcus aureus* challenge in a mouse model of influenza and bacterial coinfection. Here, we demonstrate that preceding influenza A virus led to increased lung *E. coli* and *P. aeruginosa* bacterial burden, which was associated with suppression of Type 17 immunity and attenuation of antimicrobial peptide expression. Enhanced susceptibility to *S. aureus* coinfection ceased at day 14 of influenza infection, when influenza-associated type I IFN levels had returned to baseline levels, further suggesting a key role for type I IFN in coinfection pathogenesis. These findings further implicate type I IFN-associated suppression of Type 17 immunity and antimicrobial peptide production as a conserved mechanism for enhanced susceptibility to both Gram-positive and Gram-negative bacterial coinfection during influenza infection.

Keywords: Influenza A; *Escherichia coli*; *Staphylococcus aureus*; coinfection; type I interferon
Introduction

Influenza, a highly contagious group of RNA viruses of the family Orthomyxoviridae, is one of the most important causes of seasonal respiratory illness worldwide. The majority of infected patients resolve influenza infection without difficulty. However, a subset of patients suffers from severe disease or mortality. In the United States, influenza-related mortality can reach nearly 50,000 deaths in some seasons (1). In addition to the burden posed by seasonal influenza epidemics, the potential for catastrophic pandemic influenza A is a constant threat. Despite the availability of influenza vaccines, sub-optimal vaccine efficacy, uptake, and strain matching makes influenza infection an ongoing public health problem.

The association between influenza A virus (IAV) infection and secondary bacterial pneumonia, particularly during pandemic disease, is now well established. During the 1918 Spanish influenza pandemic, nearly all influenza-associated deaths were associated with evidence of bacterial super-infection, predominantly with upper respiratory tract bacterial pathogens (32). In recent years, an increasing role for bacterial super-infections as a cause of influenza-associated mortality during seasonal influenza epidemics, particularly due to community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), has been appreciated (14, 18, 36). Therefore, increasing focus has been paid towards understanding the immunologic mechanisms that enhance host susceptibility to secondary bacterial pneumonia during IAV infection, including the role of Type 17 immunity.

Activation of Type 17 immunity requires stimulation by IL-1β and IL-23, leading to IL-17 and IL-22 secretion by Type 17 T-cells following induction of the transcription factors ROR-α and ROR-γt (2). This signaling cascade results in two major effector functions: the proliferation and recruitment of inflammatory cells, and the elaboration of antimicrobial peptides (AMP) by the mucosal epithelium. We have previously demonstrated that attenuation of Type 17 immune responses by preceding IAV infection is an important component of increased susceptibility to
secondary Gram-positive bacterial pneumonia in mice, and that this mechanism was mediated at least in part by viral–induced type I interferon (IFN) via suppression of IL-23 (23). In humans, coinfection with Gram-negative bacterial species occurs less frequently than with Gram-positive organisms, but it is unclear if this is due to differences in host responses or bacteria-specific factors. While the importance of Type 17 immune responses to Gram-negative bacterial lung infections, such as *Klebsiella pneumoniae* and *E. coli*, has previously been demonstrated (3, 4, 19, 45, 46), it is unknown if similar mechanisms could suppress Type 17 immune responses to pulmonary challenge with Gram-negative bacteria during IAV infection. Furthermore, the kinetics of type I IFN-mediated suppression of Type 17 immunity remains under-explored. It would seem likely that a critical window period exists for susceptibility to enhanced secondary bacterial infection, a window that closes as type I IFN production during IAV infection resolves upon viral clearance.

To examine Type 17 immune responses to Gram-negative bacteria during IAV coinfection and the kinetics of Type 17 immune suppression by IAV, we subjected both wild-type (WT) and IFN-α receptor (IFNAR) -/- mice to bacterial challenge with the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* following IAV infection. In addition, we assessed the kinetics of influenza-associated type I IFN induction in relation to Type 17 immune inhibition and susceptibility to pneumonia following challenge with *S. aureus*. 
Methods

Animals – WT six to eight week-old male C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). IFNAR-/- mice were generated as previously reported (16, 21). Mice were maintained under pathogen-free conditions by the University of Pittsburgh, Division of Laboratory Animal Resources. All studies were conducted on age and sex matched mice. All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Bacterial and Viral Infections – Panton-Valentine leukocidin and γ-hemolysin producing methicillin-susceptible S. aureus (American Type Culture Collection (ATCC) 49775) was grown overnight to stationary growth phase in CCY-modified medium according to ATCC instructions. Mice received 1x10^8 colony-forming units (CFU) of bacteria in 50 µl of sterile PBS. E. coli (DH5α genotype containing the pMAL-p2 plasmid, expressing ampicillin resistance) was obtained as a gift from Dr. Marc Jenkins (University of Minnesota) (10). This strain is a widely used non-enteropathogenic laboratory strain. E. coli was grown overnight to stationary growth phase in L-broth supplemented with 2 mg/ml glucose. Mice were infected with 1 x 10^7 CFU of bacteria in 50 µl of sterile PBS. P. aeruginosa strain PAO1 (ATCC BAA-47), a widely used laboratory strain descended from a pathogenic clinical isolate, was grown in 100 mL Luria Bertani (LB) broth for 12 hours. After 12 hours, 1 ml of culture was added to fresh LB broth and grown for an additional 12 hours. Bacteria were then pelleted, washed, and diluted to give a target inoculum in 50 µl PBS. Serial dilutions of bacteria were grown on LB plates and back calculated to confirm the inocula administered. In three separate experiments, mice received inocula of 4.5x10^5, 1.7x10^6, and 2.7x10^6 CFU. Influenza A/PR/8/34 was propagated in chicken eggs as described (8). Mice were infected with 100 plaque-forming units (PFU) of virus in 50 µl of sterile PBS. All
infections were delivered via oropharyngeal aspiration. Mice were sequentially challenged with influenza or vehicle for six or fourteen days followed by infection with bacteria or vehicle for one additional day. Resulting treatments groups consisted of influenza alone, bacteria alone, or coinfection.

Measurement of Lung Inflammation – One day following bacterial or vehicle challenge, mouse lungs were lavaged with 1 ml of sterile PBS. Cell differential counts were performed on bronchoalveolar lavage (BAL) fluid by cytospin and Protocol Hema 3 (Fisher Scientific, Kalamazoo, MI) staining. In BAL, LDH was measured by Cyto-Tox 96 Non-radioactive Cytotoxicity assay (Promega, Madison, WI) and total protein by Pierce BCA Protein Assay (Pierce Biotechnology, Rockford, IL), per manufacturer’s instructions. The cranial lobe of the right lung was homogenized in sterile PBS by mechanical grinding for quantification of bacterial burden by serial dilution plating and for cytokine production measurement by Lincoplex (Millipore, Billerica, MA) or Bio-plex (Bio-rad, Hercules, CA, for P. aeruginosa experiments). The middle and caudal lobes of the lung were snap-frozen in liquid nitrogen for later purification of RNA using the Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA). Gene expression was analyzed by RT-PCR utilizing Assay on Demand Taqman primer and probe sets (Applied Biosystems, Foster City, CA), using the \( \Delta \Delta CT \) method relative to the endogenous control gene hypoxanthine-guanine phosphoribosyltransferase.

Measurement of Antimicrobial Peptide Production – Human bronchial epithelia (HBE) were cultured from excess pathological tissue after lung transplantation and organ donation under protocols approved by the Universities of Pittsburgh Investigational Review Boards (IRB970946). Primary cultures of HBE cells were prepared by enzymatic dispersion using
established methods as previously described (13, 26). Epithelial cells were dissociated and seeded onto collagen-coated, semipermeable membranes with a 0.4-µm pore size (Millicell-HA; surface area, 0.6 cm²; EMD Millipore, Billerica, MA). Cells were maintained in 2% Ultroser G medium at 37 °C with 5% CO₂. Twenty-four hours after seeding, the mucosal medium was removed and the cells were allowed to grow at the air-liquid interface. Only well-differentiated cultures (>4 weeks old) were used in these studies. The presence of tight junctions between differentiated epithelial cells was confirmed by trans-epithelial resistance using an electrical resistance system (ERS) meter (EMD Millipore; resistance >500 Ω·cm²). Cells were stimulated for 24 hours with IL-17A (10 ng/ml) and/or TNF-α (1 ng/ml) (R&D Systems, Minneapolis, MN). Gene expression was analyzed by TaqMan-based RT-PCR (ABI7700, Applied Biosystems, Foster City, CA) using the $\Delta\Delta$CT method relative to the endogenous control gene β–glucuronidase for the target BPIFA1, formerly known as PLUNC or SPLUNC1 (7). Human lipocalin 2 (LCN2) was assessed utilizing Assay on Demand Taqman primer and probe sets (Applied Biosystems, Foster City, CA), relative to the endogenous control gene β–actin. BPIFA1 concentration was measured in HBE cell culture supernatant following 24 hours of stimulation with cytokine by ELISA (Hycult Biotech, Plymouth Meeting, PA). In the mouse model, lung expression of mouse BPIFA1 and LCN2 was measured as described above.

Statistical analyses – All data are presented as mean ± standard error of the mean. Significance was determined by unpaired t-test or one-way ANOVA followed by post-hoc Tukey test for multiple comparisons, as appropriate. A $P$ value $\leq$ 0.05 was considered to be statistically significant. Data were processed using the Microsoft Excel and GraphPad Prism (GraphPad Software Inc., La Jolla, CA) software packages.
Results

To examine the effects of IAV on Type 17 immune responses during subsequent infection with Gram-negative bacteria, mice were challenged with influenza A/PR/8/34 or vehicle for six days prior to challenge with *E. coli* or vehicle. Twenty-four hours later, tissues were harvested and bacterial burden and inflammation were assessed. Preceding IAV significantly increased *E. coli* burden in the lung and resulted in increased BAL neutrophil counts compared to single pathogen-challenged mice (Figure 1A, B). Furthermore, coinfection resulted in significantly increased levels of the granulocyte promoting cytokines IL-6, G-CSF, KC, and MIP-1α compared to either challenge alone (Figure 1C). Similar to data found with *S. aureus* coinfection, IAV significantly attenuated subsequent IL-1β production in response to *E. coli* (Figure 1D). TNF-α was significantly increased by coinfection, while IFN-γ production was significantly increased during influenza and during coinfection compared to bacterial infection alone. Coinfected mice also had evidence of greater lung damage compared to mice infected with bacteria alone, with increased LDH (mean OD$_{490}$ 1.3 vs 0.28, p<0.0001, data not shown) and total protein as measured by BCA assay (mean, 3999 vs 592.2 μg/mL, p<0.0001, data not shown). These data show that *E. coli* burden is increased during IAV coinfection despite elevated innate immune cell recruitment to the lung and that coinfected mice have greater lung damage than mice with bacterial pneumonia alone.

The initial findings in the IAV, *E. coli* coinfection model were thus similar to those reported with *S. aureus* (23). We next examined if Type 17 immune activation by *E. coli* was suppressed by preceding IAV infection. IL-17 and IL-22 expression were markedly induced by *E. coli* challenge and this production was significantly attenuated by preceding IAV (Figure 2A). Similarly, IL-17 protein concentration was significantly increased in *E. coli* infection alone but not during coinfection (Figure 2B). Furthermore, *E. coli* significantly induced expression of the Type 17 immune cell transcription factors ROR-γt and ROR-α, as well as the promoting cytokine IL-
23. Preceding IAV infection inhibited expression of all three Type 17 markers, while type I IFN induction was significantly exacerbated by coinfection (Figure 2C).

To confirm that host responses were similar in the context of other Gram-negative bacterial infections, we challenged mice with influenza A/PR/8/34 or vehicle for six days prior to challenge with *P. aeruginosa*, an important, clinically relevant opportunistic human respiratory pathogen. We again observed significant exacerbation of lung bacterial burden in coinfected mice (Figure 3A), which was associated with increased BAL neutrophil counts (Figure 3B). The inflammatory cytokines IL-6, MCP-1, MIP-1α, and MIP-1β were significantly elevated in the lungs of coinfected mice, although no differences were observed for KC, G-CSF, or IL-1β (Figures 3C, D), and TNF-α was not detected (data not shown). IFN-γ was again significantly elevated in coinfected mice (Figure 3D). Similar to the *E. coli* model, coinfected mice had increased lung damage compared to mice infected with *P. aeruginosa* alone, with increased LDH (mean OD₄₉₀ 0.69 vs 0.26, p=0.013, data not shown) and total protein as measured by BCA assay (mean, 3525 vs 1767 μg/mL, p<0.0001, data not shown). Protein concentration of IL-23 (Figure 3E) and gene expression of ROR-α and ROR-γt (Figure 3F) were all decreased in the lungs of coinfected mice, although no differences were observed at this time point in IL-17, IL-22, or IFN-β (data not shown). Overall, consistent results were seen with either *P. aeruginosa* and with *E. coli*, with coinfection leading to increased bacterial lung burden, inflammation, lung damage, and attenuated Type 17 responses as measured by IL-23, ROR-α, and ROR-γt.

We previously demonstrated that AMP expression was attenuated in IAV, *S. aureus* coinfection compared to *S. aureus* infection alone (35). To see if similar effects could be observed with Gram-negative infection, we examined expression of AMPs with known activity against Gram-negative bacteria. First, we confirmed *in vitro* that IL-17 acts synergistically with
TNF-α to augment expression of both BPIFA1 (Figure 4A) and LCN2 (Figure 4B) as well as to stimulate the secretion of BPIF1A (Figure 4C) in HBE cells. In vivo, E. coli significantly induced BPIFA1 expression, which was inhibited by preceding IAV infection (Figure 4D), while LCN2 expression was unchanged (data not shown). P. aeruginosa significantly induced both BPIFA1 and LCN2 expression, which were both inhibited by preceding IAV infection (Figure 4E). These findings demonstrate that IL-17 is required for optimal induction of BPIFA1 and LCN2, but that preceding IAV attenuates production of these AMPs by Gram-negative bacteria.

We have previously implicated type I IFN signaling in the exacerbation of secondary S. aureus infection following IAV (23). Since we observed increased type I IFN production in IAV, E. coli co-infected mice, we investigated the role of type I IFN in susceptibility to bacterial infection. WT or IFNAR/- mice were challenged with influenza A/PR/8/34 or vehicle for six days prior to challenge with E. coli or vehicle for one additional day. IAV failed to significantly exacerbate E. coli bacterial burden in the lung in IFNAR/- mice (Figure 5A). Neutrophils in the BAL were significantly increased in co-infected WT mice compared to single-pathogen infection and were similarly increased in IFNAR/- mice (Figure 5B). IFN-γ and TNF-α production did not differ between WT and IFNAR/- mice (Figure 5C). IL-1β production was significantly suppressed during coinfection in WT mice compared to E. coli alone, but was similar in co-infected and E. coli infected IFNAR/- mice. Finally, IL-17 gene expression was clearly elevated in IFNAR/- mice compared to WT during influenza and coinfection, although these results did not reach statistical significance, while WT mice showed significant attenuation of IL-17 expression during coinfection compared to E. coli alone (Figure 5D). These data suggest that type I IFN is important in the exacerbation of E. coli infection by IAV, and the absence of interferon signaling may potentially rescue Type 17 immunity similar to what has been observed with Gram-positive bacteria.
These data suggest that during IAV infection, impaired host defense against both Gram-negative and Gram-positive bacteria are due to similar mechanisms, of which type I IFN signaling plays an important role. We thus sought to more precisely characterize the contribution of type I IFN during the course of IAV infection. We hypothesized that the window period of susceptibility to bacterial coinfection following IAV infection was transient and linked to the interferon-related anti-viral response. Since the majority of coinfection studies in the mouse model have focused on Gram-positive bacterial infection three to seven days following IAV infection (22-25, 30, 31, 34, 35, 38, 39), we again returned to the IAV, *S. aureus* coinfection model to test this hypothesis. Mice were challenged with influenza A/PR/8/34 or vehicle for fourteen days prior to challenge with *S. aureus* or vehicle. Bacterial burden and inflammation were then assessed one day after bacterial challenge. Preceding IAV failed to exacerbate *S. aureus* bacterial burden in the lung (Figure 6A). Neutrophil recruitment to the lung was still significantly elevated in coinfected animals versus either infection alone, with higher neutrophil counts observed during bacterial versus influenza infection (Figure 6B). Interestingly, co-infection no longer exacerbated production of cytokines (Figure 6C, D). In fact, production of IL-1α, IL-1β, IL-6, KC, IFN-γ, TNF-α, IP-10, MIP-1α, MIP-2, and G-CSF were decreased in co-infection compared to *S. aureus* infection alone. These data indicate that IAV no longer attenuated *S. aureus* clearance when challenged fourteen days after infection and that co-infection resulted in decreased lung cytokine production at this time point compared to bacterial infection alone.

Since bacterial clearance was not affected by IAV fourteen days after challenge, we examined activation of Type 17 immunity at this time. *S. aureus* induced similar expression of IL-17 and ROR-γt in *S. aureus* and coinfected animals (Figure 7A). IL-17 protein production was not different between *S. aureus* and coinfected mice (Figure 7B). However, production of IL-22 and IL-23 were statistically lower in coinfected mice compared to *S. aureus* alone (Figure 7C). These data indicate that Type 17 immunity may only be partially functional during co-infection at
this later time point. Finally, we examined IFN-β and IFN-γ production in the lung during the IAV infection time course. IFN-β levels peaked at day four post infection and remained elevated through day ten (Figure 7D). By day fourteen after IAV infection, IFN-β expression was similar to sham treated mice. IFN-γ protein levels were highest on day six post-infection and were not significantly elevated at any other time point (Figure 7E). These data suggest that the lack of type I IFN at fourteen days post-influenza may explain the lack of exacerbation of S. aureus clearance.
These data demonstrate that preceding IAV infection impairs the host response to bacterial challenge with Gram-negative bacteria, similar to the attenuation previously described during IAV and Gram-positive bacterial coinfection (23, 25). Importantly, bacterial challenge in the setting of elevated type I IFN expression (i.e. during a specific window period during influenza infection) was associated with attenuation of Type 17 immunity in WT, but not IFNAR-/- mice. To our knowledge these are the first data that demonstrate attenuation of Type 17 immunity against Gram-negative bacteria in the setting of IAV infection. These findings support previously reported data suggesting the importance of type I IFN in the subversion of Type 17 immunity, increasing host susceptibility to infection from a broad range of extracellular bacteria, including bacterial coinfections complicating influenza.

In this study, the major effect of preceding influenza infection on bacterial infection was delayed clearance from the lung, as lethality was not assessed and neither *E. coli* nor *P. aeruginosa* exhibited increases in bacterial burden beyond the infecting inoculum that would suggest uncontrolled or overwhelming infection. A previous study showed similar delays in bacterial clearance in the context of influenza and *K. pneumoniae* coinfection, which was also associated with increased subsequent mortality (20). We previously demonstrated that bacterial clearance from the lung during influenza, *S. aureus* coinfection was significantly delayed compared to bacterial infection alone (35). In our current study, coinfection was associated with significantly increased inflammation and evidence of increased lung injury compared to bacterial infection alone, suggesting that delayed clearance might expose the host to a prolonged duration of inflammation and associated risk of greater lung injury. Similar to our previous findings, increased neutrophils and inflammatory cytokines and chemokines were observed in coinfected mice, suggesting that impaired bacterial clearance was not due to a defect in inflammatory cell recruitment to the lung (23, 34). Furthermore, neutrophil depletion has not
been shown to affect S. aureus lung bacterial burden, suggesting an alternate mechanism for impaired bacterial clearance (22, 35). This is in contrast to reports of neutrophil-dependent mechanisms during IAV, Streptococcus pneumoniae coinfection (31, 38). In one study, decreased levels of KC and MIP-2 were observed in coinfection and exogenous KC and MIP-2 rescued bacterial clearance, but restoration of neutrophil recruitment was not explicitly demonstrated (38). Furthermore, no attenuation of KC or MIP-2 was observed in our model. It is possible that species-specific bacterial effects may play some role, with differences in the murine response to S. pneumoniae and S. aureus.

As expected, infection with E. coli or P. aeruginosa alone was associated with increased IL-23, ROR-α, and ROR-γt, consistent with Type 17 immune activation. In E. coli infection alone, increased protein levels of IL-1β and IL-17A and increased expression of IL-17A and IL-22 were also detected. These responses were attenuated in mice infected with IAV alone and in coininfected mice, consistent with IAV-associated inhibition of Type 17 activity. As no defects in inflammatory cell recruitment were observed despite suppressed Type 17 activity, we sought to determine if AMP production was attenuated (35). In vitro, IL-17 alone was not sufficient for AMP expression, but IL-17 in the context of low levels of TNF-α clearly augmented AMP expression by HBE. In vivo, expression of BPIFA1 and LCN2 were suppressed during IAV, P. aeruginosa coinfection, with similar results seen for BPIFA1 during IAV, E. coli coinfection. Importantly, AMP expression in vivo did not appear to be modulated solely by TNF-α, which was significantly elevated in IAV, E. coli coinfection but was not detected at this time point in IAV, P. aeruginosa coinfection. BPIFA1 is secreted by secretory cells of the airway epithelium, and in mice has been shown to contribute to lung defense against Gram-negative bacteria, mainly due to inhibition of biofilm formation (6, 12, 26-28, 37). To our knowledge, this is the first assessment of the role of BPIFA1 in response to E. coli pneumonia in vivo. The role of LCN2 in mucosal defense against both Gram-positive and Gram-negative bacteria is well established (3, 9, 15,
where LCN2 exerts bacteriostatic activity via interference of siderophore-mediated bacterial iron acquisition (17). As neither BPIFA1 nor LCN2 are bactericidal, they alone are unlikely to be sufficient effectors of antimicrobial killing. Rather, they likely reflect activation of host responses that synergize to promote bacterial eradication. We have previously shown that exogenous LCN2 rescued the bacterial clearance defect in IAV, *S. aureus* coinfection, demonstrating the importance of AMP production in bacterial clearance, despite lack of direct bactericidal activity (35). These results further implicate inhibition of AMP as an important mechanism for IAV-mediated attenuation of bacterial lung defense, with Type 17 suppression a likely contributor to this effect.

Type I IFN signaling is an important mediator in suppression of Type 17 immunity following IAV infection (23, 25, 41). Here, IFNAR-/- mice were rescued from impaired lung clearance of *E. coli* during coinfection, which was associated with increased IL-1β and IL-17A. Although not tested directly (e.g. using IFNAR/IL-17 double knockout mice or using IL-17 neutralization), these results suggest rescue of Type 17 immunity and further support a role for type I IFN signaling in inhibition of Type 17 immunity. Previous studies in the *S. pneumoniae* model identified type II IFN (IFN-γ) as the main mediator of susceptibility to secondary bacterial infection (39). In our model, IFN-γ was elevated at 6 days post-IAV infection, suggesting a potential role in susceptibility. However, IFNAR-/- mice also had increased levels of IFN-γ during coinfection but efficiently cleared *E. coli*, suggesting that type I IFN signaling played a vital role.

The epidemiology of human influenza and bacterial coinfection demonstrates an overwhelming preponderance of infections with Gram-positive bacteria. During the 1918 influenza pandemic, up to three quarters of influenza-associated deaths involved coinfection with *Streptococcus* or *Staphylococcus* species (32). In contrast, Gram-negative bacteria are very infrequently encountered as community-acquired pathogens during influenza coinfection (33). While our results suggest that increased susceptibility to Gram-negative bacterial lung
infections proceeds through similar mechanisms, this may not translate to an increased risk of human disease, with infrequent exceptions. This most likely reflects the presumed pathogenesis of bacterial super-infection during IAV infection: contiguous spread or aspiration of bacteria from the upper to lower respiratory tract in the setting of a compromised respiratory epithelium (5, 29, 42). Nasopharyngeal acquisition and carriage of both *Streptococci* and *Staphylococci* is well established in humans and likely play a critical role in coinfection. Nasal carriage of *S. aureus* has previously been shown to be associated with pneumonia in critically ill patients (11). In contrast, *E. coli* is an enteric organism, while *P. aeruginosa* is an environmental organism that is an opportunistic, nosocomial human pathogen. Enteric or nosocomial Gram-negative bacteria infrequently colonize the upper respiratory tract of otherwise healthy hosts, the population disproportionately affected by IAV and Gram-positive bacterial coinfections, particularly those secondary to CA-MRSA (36). This makes them an unlikely source of natural lung infections in this population and likely explains the reduced frequency of community-acquired IAV, Gram-negative bacterial coinfections. However, our findings suggest the potential for pathological synergy between influenza and a broad range of extracellular bacteria, including typically non-pathogenic strains. This may have particularly important implications regarding susceptibility to hospital-acquired coinfections, where medical interventions remove typical barriers for Gram-negative pathogens to gain access to the respiratory tract. Recent data suggest that in patients admitted to the intensive care unit for influenza, recovery of Gram-negative bacteria rises substantially following admission (33). While the iatrogenic risks of ICU admission alone may contribute to this effect, preceding influenza likely modulates this risk.

Infection with *S. aureus* on day 14 of IAV infection showed no differences in bacterial burden one day following challenge. Surprisingly, while total inflammatory cells were elevated in the BAL of mice co-infected with *S. aureus* at this time point, levels of pro-inflammatory cytokines were paradoxically lower than in mice infected with *S. aureus* alone. The reasons for
this finding remain unclear. It is conceivable that at this point during the influenza recovery period, the balance of pro- versus anti-inflammatory signals had shifted towards suppression of inflammation while the cellular infiltrate had not yet resolved. Interestingly, IL-22 and IL-23 expression remained attenuated at this time point in coinfection, but expression of ROR-γt and IL-17 levels did not differ, suggesting only a partial defect in Type 17 immunity. In contrast to earlier time points, IL-17 was preserved when type I IFN expression had returned to baseline, suggesting that the effect of type I IFN may be more specific to IL-17 than IL-22 and that restoration of IL-17 alone may be sufficient to rescue bacterial clearance.

Numerous studies have demonstrated that increased susceptibility to secondary bacterial pneumonia due to *S. aureus* or *S. pneumoniae* began following elevations in type I IFN production, typically three to seven days following IAV infection, while IFNAR-/- were rescued from impaired bacterial clearance or mortality (23-25, 30, 31, 34, 35, 38-40). Few studies however have demonstrated when restoration of normal bacterial clearance resumes following IAV infection. Our findings support a window period of three to 14 days of enhanced susceptibility to secondary *S. aureus* challenge following IAV infection (22), which correlates precisely with the presence of increased type I IFN expression. Others have reported enhanced susceptibility to *S. pneumoniae* still occurred 14 days following IAV infection (20, 30, 43). No type I IFN data was presented in these studies, so it is difficult to draw comparative conclusions. While definitive data regarding type I IFN levels and risk of coinfection in humans are lacking, the striking clinical and epidemiological observations of the association between IAV and secondary bacterial pneumonia, particularly in cases of influenza-associated death, suggest that a similar, critical risk window exists in human populations.

In conclusion, these results provide evidence that support a common mechanistic pathway for attenuation of host lung defense and Type 17 immunity against both Gram-positive and Gram-negative bacteria during IAV coinfection. We show that enhanced susceptibility
coincides with the period of peak type I IFN production, supporting a role for IAV-induced type I IFN as a key mediator in this attenuation. Further insights into the mechanisms underlying the ability of type I IFN to inhibit Type 17 immunity are needed to further elucidate the pathogenesis of IAV and bacterial coinfection. These may yield novel strategies for the management and prevention of secondary bacterial pneumonia complicating IAV infection, reducing morbidity and mortality.
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References


Figure Legends

Figure 1 – Preceding influenza infection exacerbates secondary Gram-negative pneumonia with *Escherichia coli*. Mice were challenged with influenza A/PR/8/34 (100 pfu) or vehicle for 6 days followed by infection with *E. coli* (1x10^7 cfu) for 24 hours. EC, *E. coli* only; FLU/EC, influenza and *E. coli*; FLU, influenza only. A) bacterial burden in the lung (n=7,8). B) bronchoalveolar lavage inflammatory cells (n=8). C,D) inflammatory cytokines in lung homogenate (n=8). *p<0.05 versus FLU and EC; †p<0.05 versus FLU and FLU/EC; ‡p<0.05 versus FLU/EC and EC

Figure 2 – Preceding influenza infection attenuates type 17 immunity during secondary Gram-negative pneumonia with *Escherichia coli*. Mice were challenged with influenza A/PR/8/34 (100 pfu) or vehicle for 6 days followed by infection with *E. coli* (1x10^7 cfu) for 24 hours. EC, *E. coli* only; FLU/EC, influenza and *E. coli*; FLU, influenza only. A,C) cytokine gene expression in lung (n=4). B) inflammatory cytokine in lung homogenate (n=8). *p<0.05 versus FLU and EC; †p<0.05 versus FLU and FLU/EC; ‡p<0.05 versus FLU

Figure 3 – Preceding influenza infection exacerbates secondary Gram-negative pneumonia with *Pseudomonas aeruginosa*. Mice were challenged with influenza A/PR/8/34 (100 pfu) or vehicle for 6 days followed by infection with *P. aeruginosa* (range, 4.5x10^5 – 2.7x10^6 cfu) for 24 hours. PA, *P. aeruginosa* only; Flu/PA, influenza and *P. aeruginosa*. A) bacterial burden in the lung (n=12). B) bronchoalveolar lavage inflammatory cells (n=12, 11). C,D,E) inflammatory cytokines in lung homogenate (n=12). F) cytokine gene expression in lung (n=4, representative data from one experiment shown, repeated twice). *p<0.05 versus PA; †p<0.05 versus Flu/PA Mac and Flu/PA Lymph
Figure 4 – IL-17 augments antimicrobial peptide (AMP) expression, but preceding influenza attenuates AMP expression during Gram-negative bacterial challenge. EC, *E. coli* only; FLU/EC, influenza and *E. coli*; FLU, influenza only; PA, *P. aeruginosa* only; Flu/PA, influenza and *P. aeruginosa*, LCN, lipocalin-2. A,B) AMP gene expression in cultured human bronchial epithelial cells (n=2 donors, each condition performed in triplicate). C) AMP protein in cultured human bronchial epithelial cells (representative data from one human donor, each condition in triplicate, repeated once). D) AMP gene expression in lung (n=8). E) AMP gene expression in lung (n=4, representative data from one experiment shown, repeated twice). * p<0.05 versus Control; † p<0.05 versus TNF-α + IL-17A; ‡ p<0.05 versus FLU and FLU/EC; § p<0.05 vs PA

Figure 5 – Bacterial clearance is improved in IFNAR-/- mice during coinfection. WT or IFNAR-/- mice were challenged with influenza A/PR/8/34 (100 pfu) or vehicle for 6 days followed by infection with *E. coli* (1x10^7 cfu) for 24 hours. EC, *E. coli* only; FLU/EC, influenza and *E. coli*; FLU, influenza only. A) bacterial burden in the lung (n=6). B) bronchoalveolar lavage inflammatory cells (n=4-6). C) inflammatory cytokines in lung homogenate (n=4-6). D) cytokine gene expression in lung (n=4-6). * p<0.05 versus FLU and EC; † p<0.05 versus FLU and FLU/EC; ‡ p<0.05 versus FLU/EC and EC; ** p<0.05 versus EC

Figure 6 – Fourteen days post challenge, influenza fails to exacerbate S. aureus coinfection. Mice were challenged with influenza A/PR/8/34 (100 pfu) or vehicle for 14 days followed by infection with *S. aureus* (1x10^8 cfu) for 24 hours. SA, *S. aureus* only; FLU/SA, influenza and *S. aureus*; FLU, influenza only. A) bacterial burden in the lung (n=12). B) bronchoalveolar lavage inflammatory cells (n=12). C,D) inflammatory cytokines in lung homogenate (n=12). * p<0.05 versus FLU; † p<0.05 versus FLU/SA; ‡ p<0.05 versus FLU and SA
Figure 7 – In the absence of increased IFN-β induction, influenza infection only partially inhibits Type 17 immunity. Mice were challenged with influenza A/PR/8/34 (100 pfu) or vehicle for 14 days followed by infection with S. aureus (1x10^8 cfu) for 24 hours. SA, S. aureus only; FLU/SA, influenza and S. aureus; FLU, influenza only. A,C) cytokine gene expression in lung (n=11-12). B) inflammatory cytokine in lung homogenate (n=8). D) cytokine gene expression in lung (n=3-4). E) cytokine concentration in lung homogenate (n=4). * p<0.05 versus all other groups; † p<0.05 versus all groups except day 8.
Figure 1

A

CFU/ml (x10^2)

FLU/EC

EC

B

BAL Cells (x10^5/ml)

FLU

FLU/EC

EC

C

Cytokine (pg/ml)

FLU

FLU/EC

EC

D

Cytokine (pg/ml)

FLU

FLU/EC

EC
Figure 2

A

Relative Expression

IL-17A
IL-22

B

IL-17A (pg/ml)

FLU
FLU/EC
EC

C

Relative Expression

IFN-β
ROR-α
ROR-γt
IL-23

FLU
FLU/EC
EC

* † ‡
Figure 4

A. BPIFA1

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B. Lipocalin 2

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E. Relative Expression

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