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Influenza A Exacerbates *Staphylococcus aureus* Pneumonia by Attenuating IL-1β Production in Mice

Keven M. Robinson,‡ Sun Mi Choi,‡ Kevin J. McHugh,*, Sivanarayana Mandalapu,*, Richard I. Enelow,§ Jay K. Kolls,‡ and John F. Alcorn*

Pneumonia is a leading cause of death worldwide. *Staphylococcal aureus* can be a cause of severe pneumonia alone or a common pathogen in secondary pneumonia following influenza. Recently, we reported that preceding influenza attenuated the Type 17 pathway, increasing the lung's susceptibility to secondary infection. IL-1β is known to regulate host defense, including playing a role in Th17 polarization. We examined whether IL-1β signaling is required for *S. aureus* host defense and whether influenza infection impacted *S. aureus*-induced IL-1β production and subsequent Type 17 pathway activation. Mice were challenged with *S. aureus* (USA 300), with or without preceding Influenza A/PR/8/34 H1N1 infection. IL-1R1<sup>-/-</sup> mice had significantly higher *S. aureus* burden, increased mortality, and decreased Type 17 pathway activation following *S. aureus* challenge. Coinfected mice had significantly decreased IL-1β production versus *S. aureus* infection alone at early time points following bacterial challenge. Preceding influenza did not attenuate *S. aureus*-induced inflammasome activation, but there was early suppression of NF-κB activation, suggesting an inhibition of NF-κB-dependent transcription of pro–IL-1β. Furthermore, overexpression of IL-1β in influenza and *S. aureus*-coinfected mice rescued the induction of IL-17 and IL-22 by *S. aureus* and improved bacterial clearance.

Finally, exogenous IL-1β did not significantly rescue *S. aureus* host defense during coinfection in IL-17RA<sup>-/-</sup> mice or in mice in which IL-17 and IL-22 activity were blocked. These data reveal a novel mechanism by which Influenza A inhibits *S. aureus*-induced IL-1β production, resulting in attenuation of Type 17 immunity and increased susceptibility to bacterial infection. The Journal of Immunology, 2013, 191: 5153–5159.

Influenza is a significant cause of morbidity and mortality worldwide. In the United States, influenza affects 5–20% of the population yearly and results in 30,000 deaths. Despite improvements in medical care, influenza pandemics continue to occur. Although most cases of influenza do not result in death, secondary bacterial infections can lead to increased mortality, particularly in previously healthy individuals. Increased intensive care admission, cost, and mortality were described in children and young adults with influenza and *Staphylococcus aureus* coinfection compared with those with either influenza or *S. aureus* infection alone (1). In addition, a primary cofactor associated with mortality in community-acquired methicillin-resistant *S. aureus* (MRSA) is preceding influenza-like illness (2).

An important role for T cells in host defense against bacterial infections has recently emerged with the discovery that patients with hyper-IgE syndrome have mutations in STAT3, a key transcription factor in the development of Th17 cells (3). Clinically, these patients have increased susceptibility to *S. aureus* infections of the lungs and skin. They are unable to produce Th17 cells and IL-17A, suggesting that the Type 17 pathway plays a critical role in the immune response against *S. aureus* (4). Th17 cells are a subset of CD4<sup>+</sup> T cells that produce high levels of the cytokines IL-17 and IL-22 (5–7). They have high expression of the transcription factors ROR<sup>γ</sup>t and ROR<sup>α</sup> driven by IL-6, TGF-β, and IL-1β signaling through STAT3, SMAD, and NF-κB pathways, respectively (6, 8–10). IL-23, a cytokine produced by APCs, is also important in Th17 cell regulation, proliferation, and cytokine production (11, 12).

We showed previously that mice coinfected with Influenza A and *S. aureus* have worsened bacterial burden and mortality compared with mice infected with *S. aureus* alone (13). Coinfected mice exhibit Influenza A–induced attenuation of *S. aureus*–driven Type 17 immunity and increased susceptibility to bacterial pneumonia. We also demonstrated that influenza suppressed *S. aureus*–induced IL-23 production by CD11c<sup>+</sup> cells; however, exogenous IL-23 partially rescued *S. aureus* host defense. The specific additional mechanisms by which influenza increases the lung’s susceptibility to *S. aureus* infection remains unknown. Prior studies showed that IL-1β plays a role in host defense against Influenza A and *S. aureus* through activation of the inflammasome (14–17). Because IL-1β is known to influence polarization of Th17 cells, we hypothesized that inhibition of *S. aureus*–induced IL-1β activation by preceding influenza infection may play a critical role in attenuation of Type 17 immunity and host defense against *S. aureus*. Materials and Methods

*Mice*

Six- to eight-week-old male wild-type (WT) C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). IL-1R<sup>1-/-</sup> and WT control mice
were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-1β was generated as previously described (18). Mice were maintained under pathogen-free conditions at the Children’s Hospital of Pittsburgh of University of Pittsburgh Medical Center, and experiments were conducted with approval from the University of Pittsburgh Institutional Animal Care and Use Committee. All of the studies used age- and sex-matched mice.

**S. aureus infection**

Methicillin-sensitive *S. aureus* (ATCC 49775) producing γ-hemolysin and Pantone-Valentine leukocidin was purchased from the American Type Culture Collection. MRSA (USA 300) was provided as a gift by Dr. Alice Prince (Columbia University, New York, NY). *S. aureus* was cultured as detailed by American Type Culture Collection instructions in casein hydrolysate yeast extract-containing modified medium for 18 h to stationary growth phase. Mice were inoculated with either methicillin-sensitive *S. aureus* (1 × 10^6 or 4 × 10^6 CFU) or MRSA (5 × 10^8) in 50 μl sterile PBS by oropharyngeal aspiration, and lungs were harvested 30 min to 120 h later. Mice in all studies received *S. aureus* 6 d following influenza infection, as described below.

**Influenza A/PR/8/34 H1N1 infection**

Influenza A/PR/8/34 H1N1 was propagated in chicken eggs, as previously described (19). Mice were infected with 100 PFU Influenza A/PR/8/34 H1N1 (in 40 μl sterile PBS) from a frozen stock or control PBS by oropharyngeal aspiration. Infected mice were incubated for 6 d and then received *S. aureus* inoculum or control PBS. After an additional 30 min to 120 h, lungs were harvested.

**Adenoviral IL-1β infection**

E1- and E3-deleted adenoviral vector encoding EGFβ was constructed as described (20, 21) by Cre-lox recombination with reagents generously provided by S. Hardy (Somatix, Alameda, CA). Briefly, a SnaBI-HpaI fragment containing part of the CMV promoter, the EGFβ CDNA, and part of the SV40 poly(A) sequence was inserted in the pAdlox shuttle plasmid. E1-substituted recombinant adenovirus was generated by cotransfection of SfiI-digested pAdlox-EGFβ and φ5 helper virus DNA into the adenoviral packaging cell line CRE8, propagated, and purified as described (21). The E1- and E3-deleted adenovirus encoding mouse IL-1β was constructed by cloning the mIL1β cDNA as a Sall-NotI fragment in the pAdlox shuttle plasmid. E1-substituted recombinant adenovirus was generated by cotransfection, as described above. Mice were infected with adenovirus expressing IL-1β (2.5 × 10^5 PFU in 50 μl sterile PBS) or EGFβ (1 × 10^3 PFU in 50 μl sterile PBS) (control) by oropharyngeal aspiration 3 d after Influenza A infection.

**Analysis of lung inflammation**

At the indicated time points, mouse lungs were lavaged with 1 ml sterile PBS for inflammatory cell differential counts. The cranial lobe of the right lung was homogenized in sterile PBS by mechanical grinding. The resulting lung homogenate was used for bacterial colony counting and cytokine analysis by Lincoplex (Millipore) or by ELISA for IL-18 (R&D Systems) (BioLegend). The middle and caudal lobes of the right lung were snap-frozen and homogenized under liquid nitrogen for RNA extraction by either standard TRIzol extraction or using an RNA isolation kit (Agilent Technologies). RNA analysis was performed by standard RT-PCR using Assay on Demand TaqMan probes and primers (Applied Biosystems).

**Protein quantification**

Whole-cell lysates were extracted from homogenized liquid N2 snap-frozen middle and caudal lobes of the right lung. Equal amounts of protein (20 μg) were separated on NuPAGE 4–12% BisTris gels (Novex) and transferred to a nitrocellulose membrane. Target proteins were detected by Western blot and immunostaining with specific primary Ab, followed by HRP-labeled secondary Ab. The specific immunoreactive bands were detected by chemiluminescence. The caspase-1 p20 and NF-κB–p65 Abs were purchased from Santa Cruz Biotechnology, and the Phospho–NF-κB p65 Ab was from Cell Signaling. Quantification of band intensity (pixel density) was performed using National Institutes of Health ImageJ software.

**Caspase-1 activity**

Caspase-1 activity was measured using Abcam’s (Cambridge, MA) Caspase-1 Colorimetric assay kit. The assay measures spectrophotometric detection (at 405 nm) of chromophore p-nitroanilide after cleavage from labeled substrate UVAD-p-NA.
preceding influenza has similar inhibitory effects on IL-18 levels in our model. C57BL/6 mice were challenged with 100 PFU of Influenza A/PR/8/34 H1N1 for 6 d, followed by 5 × 10⁷ CFU of *S. aureus* (USA 300). At 6 and 24 h following bacterial challenge, there was no decrease in IL-18 protein levels or gene expression (Fig. 2A, 2B) in coinfected mice compared with mice that received *S. aureus* alone. In contrast, there was increased gene expression of IL-18 in coinfected mice at 6 h following bacterial challenge and increased protein levels in coinfected mice at 24 h following bacterial challenge. These data suggest that influenza does not cause overall inflammasome suppression. To examine this further, caspase-1 expression, cleavage, and activity were assessed. C57BL/6 mice were challenged with 100 PFU of Influenza A/PR/8/34 H1N1 for 6 d, followed by 5 × 10⁷ CFU of *S. aureus* (USA 300). Caspase-1 expression in the lung was not reduced in coinfected mice compared with *S. aureus* challenge alone; in fact it was significantly increased (Fig. 2C). Next, we examined caspase-1 cleavage (an activation marker) and activity in lung homogenate. There were no differences in caspase-1 p20 protein levels or enzymatic activity (Fig. 2D, 2E) between *S. aureus*–infected mice and coinfected mice. These data suggest that caspase-1 suppression does not play a role in Influenza A inhibition of IL-1β.

**Preceding Influenza A infection suppresses *S. aureus*–induced NF-κB activation, leading to attenuation of IL-1β production in the lung**

Because inflammasome activation did not appear to be impaired during coinfection, we investigated whether influenza was inhibiting transcriptional activation of IL-1β. We measured IL-1β mRNA in the lung and found there was significant inhibition of expression at 6 h following bacterial challenge in coinfected mice (Fig. 3A). To determine whether NF-κB–dependent transcription of pro–IL-1β is the mechanism by which Influenza A inhibits IL-1β production, C57BL/6 mice were challenged with influenza and *S. aureus*, as described in the previous section, and protein levels were assessed after 1 and 2 h. There was decreased phosphorylated NF-κB RelA (p65) in the coinfected mice versus mice infected with *S. aureus* alone (Fig. 3B, 3C). Of note, naive mice (labeled as control) had decreased phosphorylated NF-κB compared with mice receiving either influenza and *S. aureus* or *S. aureus* alone. There was no difference in nonphosphorylated NF-κB protein levels between naive and infected mice (Fig. 3B, 3C). These data support a mechanism by which ongoing Influenza A infection suppresses subsequent NF-κB activation triggered by *S. aureus*, leading to inhibition of IL-1β transcription and subsequent attenuation of Type 17 immunity in the lung.

**IL-1β overexpression rescues Type 17 activation and improves *S. aureus* clearance during coinfection**

If the defect in early IL-1β observed in coinfected mice is indeed critical to impaired *S. aureus* clearance, then restoration of IL-1β production should provide a host defense benefit. To test this, we overexpressed IL-1β in influenza and *S. aureus*–coinfected mice, proposing that it would rescue Type 17 immunity and aid bacterial clearance. Surprisingly, IL-1β overexpression resulted in decreased bronchoalveolar lavage (BAL) inflammation, specifically

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**FIGURE 1.** IL-1β signaling is critical to Type 17 pathway activation and host defense against *S. aureus*. C57BL/6 and IL-1R1−/− mice were infected with 4 × 10⁸ CFU of *S. aureus* for 24 h. (A) Bacterial colony counts in lung homogenate (*n* = 5). (B and C) Type 17 pathway gene expression in lung RNA (*n* = 5). (D) Mortality curve for C57BL/6 and IL-1R1−/− mice infected with 1 × 10⁸ CFU of *S. aureus* (*n* = 8). C57BL/6 mice were infected with 100 PFU of Influenza A/PR/8/34 or vehicle for 6 d and then challenged with 10⁸ CFU of *S. aureus* for 6–120 h. (E) IL-1β cytokine concentrations in lung homogenate (*n* = 6–7). *p* < 0.05 versus *S. aureus* alone, **p** < 0.05 versus WT, ***p*** < 0.10 versus WT.

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macrophages, neutrophils, and lymphocytes (Fig. 4A). However, IL-1β treatment significantly increased the levels of IL-17A and IL-22 mRNA (Fig. 4B) compared with those in mice infected with control adenovirus. IL-1β also significantly increased lipocalin 2 expression (Fig. 4C), an IL-17–associated antimicrobial peptide. In addition, overexpression of IL-1β also significantly increased production of the Type 17 cytokine-associated cytokines G-CSF and KC (Fig. 4D). There was no change in IL-6 levels between the two groups and decreased TNF-α levels in the mice that received exogenous IL-1β, suggesting that exogenous IL-1β does not have generalized effects on inflammation (Fig. 4E). As predicted by our hypothesis, IL-1β treatment significantly improved S. aureus clearance in coinfected mice (Fig. 4F). In these mice, bacterial titers were similar to those in mice that received S. aureus alone.

**FIGURE 2.** Influenza A does not impact S. aureus–induced inflammasome activation. C57BL/6 mice were infected with 100 PFU of Influenza A/PR/8/34 or vehicle for 6 d and then challenged with $5 \times 10^7$ CFU of S. aureus for 6–24 h. (A) IL-18 protein concentration in lung homogenate, as measured by ELISA ($n = 6$). (B) IL-18 gene expression in lung RNA ($n = 7-8$). (C) Caspase-1 gene expression in lung RNA ($n = 7-8$). (D) Western blot analysis for anti-caspase-1 p20 cleaved product. (E) Caspase-1 enzyme activity ($n = 6$). *$p < 0.05$ versus S. aureus alone.

**FIGURE 3.** Influenza A suppresses NF-κB activation and IL-1β expression in the lung. C57BL/6 mice were infected with 100 PFU of Influenza A/PR/8/34 or vehicle for 6 d, mice were then challenged with $5 \times 10^7$ CFU of S. aureus for 1-24 h. (A) IL-1β gene expression in lung RNA ($n = 7-8$). (B) Western blot analysis for anti-phosphorylated NF-κB and anti-nonphosphorylated NF-κB p50 and p65. (C) Densitometry for the Western blot bands. *$p < 0.05$ versus S. aureus alone.
In addition, we measured IL-1β levels at the time mice would have received S. aureus in our coinfection model. Mice that received IL-1β adenovirus had increased IL-1β levels (375.2 ± 3.24 pg/ml versus 270.9 ± 42.56 pg/ml) in lung homogenate compared with those that received control adenovirus. Influenza A attenuation of the Type 17 pathway was rescued by IL-1β, perhaps leading to improved bacterial immunity in influenza coinfection.

**IL-17 and IL-22 play an important role in IL-1β’s rescue of S. aureus clearance**

To determine whether IL-1β’s restoration of bacterial immunity was IL-17 dependent, we overexpressed IL-1β or control EGFP in influenza and S. aureus–coinfected IL-17RA−/− mice. IL-1β failed to significantly improve bacterial clearance (Fig. 5A), although there was a trend toward improved bacterial clearance in the mice that received exogenous IL-1β (p = 0.38). In addition, there was no decrease in lung inflammation in IL-17RA−/− mice (Fig. 5B). Although there was no difference in the production of IL-17A and IL-23 mRNA, there was a significant increase in IL-22 mRNA in the mice that received exogenous IL-1β (Fig. 5C). As expected, the two groups had similar lipocalin 2 expression (Fig. 5D). Overexpression of IL-1β also significantly increased the production of the Type 17 cytokine-associated chemokines G-CSF and KC (Fig. 5E). Similar to WT mice, there was no change in IL-6 between the two groups and decreased TNF-α levels in the mice that received exogenous IL-1β (Fig. 5F). Because of the trend toward improved bacterial clearance in the IL-17RA−/− mice that received exogenous IL-1β and the significant increase in IL-22 mRNA in these mice, we proposed that IL-1β’s partial (nonsignificant) restoration of bacterial immunity may be dependent upon both IL-17 and IL-22. To test this, we overexpressed IL-1β or control EGFP in influenza and S. aureus–coinfected WT mice that had received anti–IL-17R and anti–IL-22 Abs prior to bacterial challenge. In these mice, IL-1β failed to significantly improve bacterial clearance (p = 0.27) (Fig. 5G), although we again observed a trend toward improved bacterial clearance in the mice that received exogenous IL-1β. In both IL-17RA−/− mice and WT mice in which IL-17R and IL-22 were blocked, exogenous IL-1β did not prevent secondary bacterial pneumonia to the same degree as its effect on WT mice, suggesting that the Th17 axis plays an important role in IL-1β’s effect on influenza and S. aureus coinfection.

**Discussion**

These findings demonstrate a mechanism by which preceding influenza infection impairs Type 17 immunity and allows for increased susceptibility to secondary bacterial infection in the lung. We demonstrate that IL-1β signaling is required for S. aureus host defense and, further, for activation of Type 17 cytokines and downstream target genes. Preceding influenza infection markedly attenuates acute IL-1β production induced by S. aureus. Our study shows that Influenza A infection suppresses S. aureus–induced NF-κB activation in mice, leading to inhibition of IL-1β production and subsequent attenuation of the Type 17 pathway. IL-1β overexpression rescued Type 17 pathway activation, enhancing the immune response to secondary bacterial challenge. In addition, the Type 17 axis was found to play an important role in IL-1β’s restoration
FIGURE 5. IL-17 and IL-22 play an important role in IL-1β’s rescue of influenza-induced *S. aureus* pneumonia. IL-17R/−/− mice were infected with 100 PFU of Influenza A/PR/8/34. On day 3, they received adenovirus (2.5 × 10^6 PFU in 50 μl sterile PBS) expressing IL-1β or EGFP (control) and were challenged on day 6 with 5 × 10^8 CFU of *S. aureus* for 24 h. (A) Bacterial colony counts in the lung (n = 6). (B) BAL fluid cell counts (n = 6). (C) Type 17 pathway expression in lung tissue measured by RT-PCR (n = 6). (D) Lipocalin 2 expression in lung tissue measured by RT-PCR (n = 6). (E and F) Type 17–associated cytokine production in lung homogenate (n = 6). C57BL/6 mice were infected with 100 PFU of Influenza A/PR/8/34. They received adenovirus expressing IL-1β (2.5 × 10^6 PFU in 50 μl sterile PBS) or EGFP (1 × 10^8 PFU in 50 μl sterile PBS) (control) on day 3 and 10 μg of IL-17R Fc chimera and 50 μg of anti-IL-22 (in 100 μl sterile PBS) on day 5. They were challenged on day 6 with 5 × 10^7 CFU of *S. aureus* for 24 h. (G) Bacterial colony counts in the lung (n = 7–8). *p < 0.05 versus control.

of bacterial immunity. These data indicate a key mechanism by which influenza infection attenuates host defense against secondary infection and could provide a potential therapeutic target in the future.

IL-1β is a proinflammatory cytokine that is produced following activation of pattern recognition receptors by microbial products, which initiates IL-1β gene expression and synthesis of pro–IL-1β. IL-1β induces recruitment of neutrophils and macrophages, activates the release of other cytokines important to host defense (TNF-α and IL-6), and drives Type 17 pathway differentiation of naive and innate T cells (8, 9). Multiple studies showed that IL-1β plays a role in host defense against Influenza A through activation of the inflammasome and induction of IL-1β and IL-18 (14–16). Influenza was shown to activate the NLRP3 and ASC inflammasome, and IL-1R, caspase-1, and ASC are required for protection against influenza infection in mice. In addition, it was reported that inflammasome-mediated IL-1β production is important to immunity against cutaneous *S. aureus* infection (17). *S. aureus* pneumonia activates the NLRP3 inflammasome, leading to the release of IL-1β and IL-18, possibly contributing to pulmonary pathology. In our study, we observed that IL-1β is crucial to host defense against *S. aureus* in the lung through activation of the Type 17 pathway during the acute phase of *S. aureus* infection.

Although the inflammasome activation of caspase-1 converts pro–IL-1β to IL-1β, IL-1β production is also regulated by induction of NF-kB–dependent transcription of pro–IL-1β. Our coinfection model suggested that preceding influenza does not suppress *S. aureus*–induced activation of the inflammasome but, alternatively, suppresses NF-kB activation, leading to inhibition of IL-1β. In our current study, exogenous IL-1β given 72 h prior to *S. aureus* resulted in enhanced Type 17 pathway activation during influenza and *S. aureus* coinfection in WT mice. We observed an increase in expression of Type 17 effector cytokines and the Type 17–associated antimicrobial peptide lipocalin 2. IL-1β promoted the clearance of *S. aureus* and reduced BAL inflammation in WT mice. Inflammatory cells and epithelial cells produce IL-1β during infection (24, 25). IL-17 is secreted by both Th17 cells and γδT cells in response to IL-1β (26). In our experimental coinfection model, IL-1β acts quickly and within 24 h following bacterial challenge to restore Type 17 pathway activation and restore bacterial immunity. IL-17 is likely being secreted from Th17 cells and γδT cells, as well as other innate T cell subsets in response to IL-1β.

IL-1β’s impact on *S. aureus* host defense was found to be regulated, in part, by IL-17 and IL-22, as evidenced by the decrease in restoration of bacterial immunity by exogenous IL-1β in IL-17R/−/− mice and WT mice in which IL-17R and IL-22 were blocked. IL-1β overexpression resulted in increased IL-22 production in IL-17RA−/− mice but not IL-17 mRNA. IL-17 mRNA likely was not increased by IL-1β because its expression is already elevated during lung infection in IL-17RA−/− mice, as previously reported (18). Interestingly, there was increased production of the Type 17 cytokine–associated chemokines G-CSF and KC in IL-17RA−/− mice that received IL-1β adenovirus compared with control. Although G-CSF and KC are typically associated with...
IL-17 effector function, Numasaki et al. (27) showed that G-CSF can be induced by IL-1β in lung endothelial cells in the absence or presence of IL-17. In this study, the presence of IL-17 enhanced IL-1β’s induction of G-CSF. Unexpectedly, we also showed decreased production of TNF-α in both WT and IL-17RA−/− mice that received IL-1β adenovirus compared with control. This may be due to decreased bacterial burden or lessened BAL inflammation in WT mice. Regardless, it is interesting that decreased TNF-α was not associated with exacerbation of S. aureus.

Bacterial coinfection is a serious, often life-threatening consequence of influenza infection. Influenza pandemic strains have been shown to be associated with decreased bacterial burden or lessened BAL inflammation in WT mice. This finding underscores the potential importance of influenza infection in the development of bacterial coinfection.

References

The authors have no financial conflicts of interest.

Disclosures

First and last names of all authors are included in the reference list. No other disclosures were reported.


