Influenza A Virus Exacerbates *Staphylococcus aureus* Pneumonia in Mice by Attenuating Antimicrobial Peptide Production

Keven M. Robinson,^{1,2} Kevin J. McHugh,¹ Sivanarayana Mandalapu,¹ Michelle E. Clay,¹ Benjamin Lee,¹ Erich V. Scheller,¹ Richard I. Enelow,³ Yvonne R. Chan,² Jay K. Kolls,⁴ and John F. Alcorn¹

¹Department of Pediatrics, Children's Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania; ²Department of Medicine, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania; ³Department of Medicine, Dartmouth Medical School, Lebanon, New Hampshire and ⁴Richard K. Mellon Foundation Institute, Children's Hospital of Pittsburgh of UPMC, Pennsylvania

Influenza A represents a significant cause of morbidity and mortality worldwide. Bacterial complications of influenza A confer the greatest risk to patients. T_H17 pathway inhibition has been implicated as a mechanism by which influenza A alters bacterial host defense. Here we show that preceding influenza causes persistent *Staphylococcus aureus* infection and suppression of T_H17 pathway activation in mice. Influenza does not inhibit *S. aureus* binding and uptake by phagocytic cells but instead attenuates *S. aureus* induced T_H17 related antimicrobial peptides necessary for bacterial clearance in the lung. Importantly, exogenous lipocalin 2 rescued viral exacerbation of *S. aureus* infection and decreased free iron levels in the bronchoalveolar lavage from mice coinfecting with *S. aureus* and influenza. These findings indicate a novel mechanism by which influenza A inhibits T_H17 immunity and increases susceptibility to secondary bacterial pneumonia. Identification of new mechanisms in the pathogenesis of bacterial pneumonia could lead to future therapeutic targets.

Keywords. influenza A; *Staphylococcus aureus*; pneumonia; T_H17; host defense; coinfection.

Pneumonia is a leading cause of death worldwide, accounting for the highest mortality in children younger than 5 years of age, resulting in approximately 1.4 million deaths in 2010 [1]. In addition, pneumonia ranks 9th overall as a cause of death annually in the United States [2]. Numerous bacterial organisms cause pneumonia, including *Staphylococcus aureus*. The prevalence of *S. aureus* has increased in recent years with the emergence of methicillin-resistant *S. aureus*. MRSA currently accounts for 20%–40% of hospital-

acquired and ventilator-acquired pneumonias [3] and 9% of community-acquired pneumonias [4]. A primary cofactor involved in mortality due to community acquired MRSA infection is preceding influenza-like illness [5].

Influenza is a common respiratory illness that affects 5%–20% of the US population yearly and results in approximately 30 000 deaths annually. Although most cases of influenza are not fatal, complications such as bacterial pneumonia can have serious consequences. Increased intensive care admission, mechanical ventilation, and mortality have been described in children and young adults with influenza A and concomitant *S. aureus* infection compared to those with either influenza or *S. aureus* infection alone [6]. Tissue histologic specimens and bacteriologic samples recovered from autopsies performed during the 1918 pandemic reveal that most deaths were likely caused by secondary bacterial pneumonias [7]. Given the significant morbidity and mortality associated with influenza and secondary bacterial pneumonia, the identification of therapeutic immune targets to alleviate this copathogenesis will have substantial clinical benefits.

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Correspondence: John F. Alcorn, PhD, Division of Pulmonary Medicine, Allergy, and Immunology, Department of Pediatrics, Children's Hospital of Pittsburgh of UPMC, One Children's Hospital Dr 9127 Rangos, 4401 Penn Ave, Pittsburgh, PA 15224 (john.alcorn@chp.edu).

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The role of T cells in host defense against bacterial pneumonia is recently emerging. We have previously demonstrated that mice sequentially infected with influenza A and *S. aureus* resulted in influenza A-induced attenuation of *S. aureus* driven T_H17 pathway activation and increased susceptibility to bacterial pneumonia [8]. However, the breadth and kinetics of influenza A-induced inhibition of the T_H17 pathway and the explanation for the lung's increased susceptibility to bacterial infection remains unknown. In addition, the specific mechanism(s) by which the T_H17 pathway promotes *S. aureus* clearance has not been previously examined.

METHODS

Mice

Six to eight week-old male C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). Mice were maintained under pathogen-free conditions and studies performed on age- and sex-matched mice.

S. aureus Infection

Methicillin-sensitive *S. aureus* (MSSA; ATCC 49775) and methicillin-resistant *S. aureus* (MRSA; USA 300) were used to inoculate mice with either 1×10^8 colony-forming units (CFU) of MSSA or 5×10^7 of MRSA (in 50 μ L sterile phosphate-buffered saline [PBS]) by oropharyngeal aspiration.

Influenza A Infection

Influenza A PR/8/34 H1N1 and Influenza A CA/07/2009 H1N1 [9, 10] were used to inoculate mice with 100 plaque-forming units (PFU) of influenza (in 40 μ L sterile PBS) by oropharyngeal aspiration. Viral burden was determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) on lung RNA for viral matrix protein as described [8].

Analysis of Lung Inflammation

Mouse lungs were lavaged with 1 mL sterile PBS for inflammatory cell counts. Lung homogenate was used for bacterial colony counting and cytokine analysis by Lincoplex (Millipore, Billerica, MA) or by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). RNA extraction was performed (Agilent Technologies, Santa Clara, CA), and RNA analysis was performed by RT-PCR using Assay on Demand Taqman probes and primers (Applied Biosystems, Foster City, CA).

Depletion Antibodies

BioXcell (West Lebanon, NH) antibodies clones RB6-8C5 (anti-Ly6G/C) and 1A8 (anti-Ly6G) and anti-IL-17 were used. Mice received 250 μ g (300 μ g anti-IL-17) of antibody 24 hours prior to *S. aureus* challenge.

Lipocalin 2 Protein

Lipocalin 2 (Lcn2) recombinant protein purification was performed as described elsewhere [11, 12] with modifications.

S. aureus Macrophage Binding and Uptake

S. aureus was labeled with fluorescein isothiocyanate (FITC; Life Technologies). FITC-labeled *S. aureus* were instilled into mice, and bronchoalveolar lavage (BAL) was performed 24 hours after challenge. BAL cells were stained with fluorescent conjugated antibodies against F4/80, Ly6G, Ly6G/C (GR-1), and Cd11c for flow cytometry. For in vitro studies, alveolar macrophages were isolated from the BAL of naive, PBS control, and influenza infected mice. Flow cytometry was performed to determine total number of FITC-positive cells and the total number of FITC-positive cells in the presence and absence of interferon γ and/or interferon β .

In Vitro Epithelial Cell Experiments

Mouse C10 cells were treated with interleukin 17A (IL-17A; 10 ng/mL), interleukin 22 (IL-22; 20 ng/mL), and/or tumor necrosis factor α (TNF- α ; 1 ng/mL) for 24 hours. Cell RNA was isolated by RNeasy kit (Qiagen, Valencia, CA) for RT-PCR analysis of gene expression.

Free Iron Quantification

At indicated time points, mouse lungs were lavaged with 1 mL sterile PBS and Ferrous (Fe²⁺) ions were measured using an iron assay kit (Abcam, Cambridge MA). Lavage samples were analyzed without assay buffer or iron reducing reagents in order to detect free iron.

Statistical Analysis

The data are presented as the mean $\pm$ standard error of the mean (SEM). Significance was tested by unpaired *t* test (for 2 means) or one-way analysis of variance (ANOVA; for multiple data groups) followed by Tukey post hoc test. Data were analyzed using GraphPad Prism and/or Microsoft Excel software.

RESULTS

Preceding Influenza A Infection Prolongs *S. aureus* Pneumonia and Attenuates Acute and Chronic T_H17 Pathway Activation by *S. aureus*

We have previously demonstrated that preceding influenza A infection results in increased susceptibility to bacterial pneumonia at 24 and 48 hours following bacterial challenge [8], but the persistence of bacterial colonization remains unknown. We challenged C57BL/6 mice with influenza A PR/8/34 H1N1 for 6 days followed by *S. aureus* (ATTC 49775) and after 6, 24, 48, 72, 96, and 120 hours, bacterial and viral clearance and lung inflammation were assessed. Preceding influenza infection resulted in decreased clearance of *S. aureus* in the lung up to 120 hours following bacterial challenge (Figure 1A). There was also significant exacerbation of lung inflammation for up to 120 hours (Supplementary Figure 1A). Histology of lungs coinfecting with influenza A and *S. aureus* at 120 hours postbacterial challenge revealed increased inflammation and lung damage

Figure 1. Influenza A increases the susceptibility and prolongs *Staphylococcus aureus* pneumonia and inhibits T_H17 pathway induction by *S. aureus* following bacterial challenge. C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 days, mice were then challenged with 10^8 CFU of *S. aureus* for 6–120 hours. **A**, Bacterial colony counts in the lung ($n=6-7$). **B**, IL-17 protein production in lung homogenate as measured by ELISA ($n=6-7$). **C**, Representative histological sections of lung at 120 hours postinfection. **D**, IL-17 protein production in lung homogenate as measure by Linco-plex ($n=3-4$). C57BL/6 mice were infected with 100 PFU of influenza A CA/07/2009 or vehicle for 6 days, mice were then challenged with 10^8 CFU of *S. aureus* for 6–120 hours. **E**, Bacterial colony counts in the lung ($n=8$). * $P<.05$ vs *S. aureus* alone. Significance was tested by unpaired t test. Abbreviations: CFU, colony-forming units; ELISA, enzyme-linked immunosorbent assay; PFU, plaque-forming units.

compared to *S. aureus* alone (Figure 1C). These data show that preceding influenza A infection causes increased inflammation and *S. aureus* burden, as well as bacterial persistence in the lung. We proposed that there would be rapid and persistent attenuation of the T_H17 pathway in coinfecting mice following bacterial challenge. *S. aureus* induced a more robust T_H17 effector cytokine response up to 120 hours following bacterial challenge in mice infected with *S. aureus* alone compared to coinfecting mice (Figure 1B, Supplementary Figure 1B and C). Importantly, we examined early T_H17 cytokine responses and found that *S. aureus* failed to induce IL-17 (Figure 1D) or

interleukin 23 (IL-23; Supplementary Figure 1D) at early time points following challenge in mice previously infected with influenza. Finally, we examined whether 2009 pandemic influenza followed by *S. aureus* challenge would also result in decreased bacterial clearance compared to mice infected with *S. aureus* alone. We challenged C57BL/6 mice with influenza CA/07/2009/A H1N1 for 6 days followed by *S. aureus* (ATTC 49775) and after 24 hours, assessed bacterial clearance. Preceding 2009 H1N1 influenza infection resulted in decreased clearance of *S. aureus* in the lung following bacterial challenge (Figure 1E).

S. aureus induced production of the T_H17 cytokine-associated chemokines G-CSF, and KC was inhibited by influenza infection (Supplementary Figure 2). There was no change in IFN- γ production between the groups (Figure 2A), whereas interleukin 10 (IL-10) was increased coinfecting mice compared to those that received *S. aureus* alone (Figure 2B). Acute TNF- α production was also reduced in co-infected mice (Figure 2C). Interleukin 6 (IL-6) production was significantly elevated at 6 hours in the mice that received *S. aureus* only compared to the coinfecting mice but remained similar in the coinfecting mice at all time points, suggesting that preceding influenza infection does not result in suppression of all cytokines below *S. aureus* induced levels (Figure 2D). These data confirm that *S. aureus* induces a T_H17 immune response in the lung during bacterial challenge. Further, preceding influenza A infection significantly attenuates the *S. aureus*-driven T_H17 pathway as evidenced by decreased levels of T_H17 effector cytokines, T_H17 promoting cytokine, and T_H17 cytokine-induced chemokines as early as 30 minutes and up to 120 hours following bacterial challenge.

Neutrophils Are Not the Primary Mechanism by Which the T_H17 Pathway Promotes Bacterial Host Defense

A known function of IL-17A is to enhance inflammation through induction of neutrophil and macrophage chemokines

and growth factors [13]. We have published data that suggested the decreased clearance of *S. aureus* in T_H17 pathway knockout mice was not due to a lack of neutrophil recruitment [8]. In order to further investigate the contribution of neutrophils to *S. aureus* clearance in the lungs, C57BL/6 mice received neutrophil depletion antibodies anti-Ly6G (1A8) or anti-Ly6G/C (RB6-8C5 (RB6)) followed by administration of *S. aureus* (ATTC 49775) 24 hours later. Bacterial clearance and lung inflammation were assessed 24 hours following bacterial challenge. Mice that received RB6 antibody had severely attenuated clearance of *S. aureus*, whereas mice that received 1A8 antibody cleared *S. aureus* similar to control (Figure 3A). Both RB6 antibody and 1A8 antibody resulted in neutrophil depletion in the BAL, whereas only RB6 antibody resulted in macrophage depletion (Figure 3B). Mice that received RB6 or 1A8 antibody prior to bacterial challenge had increased proinflammatory and neutrophil recruitment cytokine production (Figure 3C–F). Histological sections of lungs confirmed suppression of inflammation in the mice that received 1A8 or RB6 (Figure 3G). Interestingly, although the RB6 exacerbated *S. aureus* infection, there was typical bacterial clearance in the mice that received the 1A8 antibody (neutrophil specific), suggesting that neutrophils are not the mechanism by which the T_H17 pathway promotes bacterial host defense.

Figure 2. Influenza A alters inflammatory cytokine production following bacterial challenge with *Staphylococcus aureus*. C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 days, mice were then challenged with 10^8 CFU of *S. aureus* for 6–120 hours. A–D, cytokine concentrations in lung homogenate measured by Lincplex (n = 6–7). * $P < .05$ vs *S. aureus* alone. Significance was tested by unpaired t test. Abbreviations: CFU, colony-forming units; PFU, plaque-forming units.

Figure 3. Specific neutrophil depletion does not significantly attenuate *Staphylococcus aureus* clearance. C57BL/6 mice received 250 µg of RB6-8C5 (anti-Ly6G/C) or 1A8 (anti-Ly6G) antibody 24 hours prior to challenge with 10⁸ CFU of *S. aureus* for 24 hours. **A**, Bacterial colony counts in the lung (n = 10). **B**, Bronchoalveolar lavage cell differential counts (n = 10). **C–F**, T_H17 pathway cytokine concentrations in lung homogenate measured by Lincoplex (n = 10). **G**, Representative histological sections of lung. *P < .05 vs *S. aureus* alone. Significance was tested by unpaired *t* test. Abbreviation: CFU, colony-forming units.

Alveolar Macrophage and Neutrophil Binding and Uptake of *S. aureus* Are Not Impaired by Preceding Influenza Infection

It is possible that preceding influenza infection directly attenuates innate immune cell killing of *S. aureus* in the lung (despite increased cell numbers), increasing susceptibility to secondary

bacterial pneumonia. To test this, we infected mice with FITC-labeled *S. aureus* for 24 hours and performed flow cytometry. Examination of the *S. aureus* positive macrophages revealed no difference in the total number of FITC-positive cells during co-infection (Figure 4A and 4B). There was a decrease in the

Figure 4. Influenza A infection does not impair alveolar macrophage or neutrophil binding and uptake of *Staphylococcus aureus*. C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 days, mice were then challenged with 1×10^8 CFU FITC-labeled *S. aureus* or vehicle for 24 hours. **A** and **B**, Total FITC-positive macrophages and neutrophils from *S. aureus* and co-infected lungs by flow cytometry ($n = 6-8$). C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 days, macrophages were then collected and suspended with 1×10^7 CFU FITC-labeled *S. aureus* to allow for phagocytosis. **C**, Total FITC-positive macrophages from control and influenza-infected lungs as determined by percent positivity and mean fluorescence by flow cytometry ($n = 8$). Naive alveolar macrophages were stimulated with IFN- β (100 U/mL) and/or IFN- γ (5 ng/mL) prior to stimulation with FITC-labeled *S. aureus*. **D**, Total FITC-positive cells in lung by flow cytometry ($n = 8-17$). * $P < .05$ vs *S. aureus* alone, ** $P < .05$ vs control. Significance was tested by unpaired *t* test (for 2 means) or one-way ANOVA (for multiple data groups) followed by Tukey post hoc test. Abbreviations: ANOVA, analysis of variance; CFU, colony-forming units; FITC, fluorescein isothiocyanate; IFN, interferon; PFU, plaque-forming units.

percentage of *S. aureus* positive neutrophils in influenza coinfected lungs, however, coinfecting mice had elevated neutrophil numbers compared to *S. aureus* only infected animals. The total number of *S. aureus* positive neutrophils was not different between groups (data not shown). Further, we isolated alveolar macrophages from influenza infected or control lungs and performed flow cytometry. Macrophages from influenza-infected lungs were able to bind and take up significantly more *S. aureus* than naive macrophages as determined by percent positivity

and mean fluorescence (Figure 4C and Supplementary Figure 3A). Finally, interferons have been suggested to inhibit macrophage uptake of bacteria during influenza co-infection [14]. We treated naive alveolar macrophages with IFN- β and/or IFN- γ prior to in vitro flow cytometry. Interferon treatment did not alter macrophage binding and uptake of *S. aureus* (Figure 4E and Supplementary Figure 3B). These data suggest that there is no innate cellular defect in influenza-infected lungs that results in aberrant *S. aureus* clearance.

***S. aureus* Induces T_H17 Pathway Associated Antimicrobial Peptides**

Because innate immune cell uptake of *S. aureus* was similar in *S. aureus* and coinfecting mice, we examined alternative pathways by which T_H17 activation may promote bacterial clearance. To test if the T_H17 pathway stimulates antimicrobial peptides (AMPs), C10 airway epithelial cells were stimulated with combinations of IL-17A, IL-22, with or without TNF- α . It has been shown previously that T_H17 cytokines and TNF- α have synergistic effects on epithelial cells [15]. We found that TNF- α , IL-17A + TNF- α , and IL-17A + IL-22 + TNF- α treatment synergistically induced Lcn2 expression in C10 cells (Figure 5A). T_H17 cytokines induced expression of RegIII β (Figure 5B) and CAMP in all groups stimulated with TNF- α (Figure 5C). To determine whether *S. aureus* induces T_H17 pathway associated AMPs in vivo, C57BL/6 mice were challenged with influenza A followed by *S. aureus*. *S. aureus* enhanced production of IL-17 and IL-22 associated AMPs: Lcn2, RegIII $\alpha\beta\gamma$, CAMP, and S100A8-9 (Figure 5D–H), particularly at acute time points (6, 24 hours). Preceding influenza infection resulted in marked suppression of AMP production. To test whether IL-17 is required for *S. aureus* induction of AMPs in vivo, C57BL/6 mice were challenged with MRSA following the administration of anti-IL-17 antibody. At 24 hours following bacterial challenge, there was overall decreased expression of AMPs (Figure 5I). These data support that *S. aureus* induces T_H17 pathway associated AMPs and illustrate a potential mechanism by which the T_H17 pathway may promote bacterial immunity in the lung. Further, these findings demonstrate suppression of AMP production by influenza A, a novel mechanism for exacerbation of secondary bacterial pneumonia.

Exogenous Lcn2 Improves *S. aureus* Clearance in the Lung and Rescues Influenza Exacerbation During Co-infection

We proposed that Lcn2 might aid in lung clearance of *S. aureus*. To test this, C57BL/6 mice received Lcn2 or a control protein and 4 hours later were challenged with *S. aureus* (USA 300); 24 hours after bacterial challenge, bacterial burden and lung inflammation were assessed. *S. aureus* clearance was increased in mice that received exogenous Lcn2 compared to control (Figure 6A). There was also decreased lung inflammation (Figure 6B). Next, mice were coinfecting with influenza A and MRSA in the presence of Lcn2 or control protein. There was increased bacterial clearance in the mice that received exogenous Lcn2 compared to control (Figure 6C–D). Thus, exogenous Lcn2 rescued *S. aureus* clearance in both mice infected with *S. aureus* alone and coinfecting with influenza A. Finally, there were lower levels of ferrous (Fe²⁺) ions in the *S. aureus* challenged and coinfecting mice that received exogenous Lcn2 compared to control (Figure 6E). These data provide evidence that T_H17 pathway induction of AMPs is a likely mechanism used to aid bacterial killing. In addition, these data suggest that

Lcn2 depletes iron in the lung microenvironment, inhibiting the growth of *S. aureus*.

DISCUSSION

Our findings demonstrate a novel mechanism by which preceding influenza infection impairs immunity against bacterial pneumonia. The data substantiate a crucial role for the T_H17 pathway in host defense against *S. aureus* pneumonia. Here we show that preceding influenza in mice causes persistent *S. aureus* infection and suppression of the T_H17 pathway. Our study shows that influenza does not inhibit inflammation or *S. aureus* binding and uptake by phagocytic cells. Rather, influenza inhibits *S. aureus* induced T_H17 pathway associated AMPs and attenuates bacterial clearance in the lung. Importantly, exogenous Lcn2 improved *S. aureus* clearance and rescued viral exacerbation of infection. In addition, exogenous Lcn2 decreased levels of free iron in BAL fluid. These data indicate a key mechanism by which T_H17 pathway activation results in *S. aureus* clearance in the lung. Exogenous AMPs may be useful as a therapeutic target in co-infection.

Multiple mechanisms for increased susceptibility to bacterial infections following viral infection have been investigated [16]. Our current findings demonstrate that the impairment of *S. aureus* clearance and attenuation of the T_H17 pathway by preceding influenza A is both an acute and chronic response of the immune system. The early inhibition of IL-17 production likely reflects $\gamma\delta$ T cell suppression as well as T_H17. Mice challenged with *S. aureus* alone induced high levels of IL-17 in the lung that remained elevated following bacterial clearance. Influenza coinfection reduced this T_H17 pathway activation throughout the study. Numerous influenza A viruses cause disease in mice and humans. We observed impaired *S. aureus* clearance during coinfection with 2 different strains of murine H1N1 influenza A infections. Different types of influenza viruses may elicit differing responses of the innate immune response and future studies will have to be completed in order to address these differences. Although we observed influenza A attenuation of the *S. aureus*-driven T_H17 pathway, there was increased IL-10 in the coinfecting mice compared to those that received *S. aureus* alone. Previous studies have shown that influenza infection induces IL-10 [17] and that IL-10 expression suppresses the development of T_H17 cytokines during influenza infection [18]. Influenza-induced IL-10 has also been reported to enhance susceptibility to secondary pneumococcal infection [19], although a recent study showed that IFN- γ attenuation of *S. pneumoniae* clearance in mice was IL-10 independent [14]. In that study, IFN- γ levels were elevated in coinfecting mice resulting in suppression of macrophage uptake of bacteria. We found no difference in IFN- γ levels in coinfecting mice vs *S. aureus* alone, suggesting a minimal role for IFN- γ in the *S. aureus* model. In addition, we observed decreased TNF- α levels induced by

Figure 5. T_H17 cytokines induce antimicrobial peptides in vitro, Influenza A inhibits *Staphylococcus aureus* induced antimicrobial peptides in vivo. C10 airway epithelial cells were stimulated with combinations of IL-17A, IL-22, and TNF- α ($n=6$). A–C, T_H17 pathway associated antimicrobial peptide gene expression in cell RNA. * $P<.05$ vs control, *** $P<.05$ vs TNF- α . C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 days, mice were then challenged with 10^8 CFU of *S. aureus* for 6–120 hours. D–H, T_H17 pathway associated antimicrobial peptide gene expression in lung RNA ($n=6-7$). C57BL/6 mice received 300 μ g of anti-IL-17 antibody 24 hours prior to challenge with 5×10^7 CFU of *S. aureus* for 24 hours. I, T_H17 pathway associated antimicrobial peptide gene expression in lung RNA ($n=8$). * $P<.05$ vs control, ** $P<.05$ vs *S. aureus* alone. Significance was tested by unpaired t test (for 2 means) or 1-way ANOVA (for multiple data groups) followed by Tukey post hoc test. Abbreviations: ANOVA, analysis of variance; CFU, colony-forming units; IL-17A, interleukin 17A; IL-22, interleukin 22; PFU, plaque-forming units; TNF- α , tumor necrosis factor α .

Figure 6. Exogenous Lcn2 rescues *Staphylococcus aureus* clearance. C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 days, then received 100 μ g of lipocalin 2 or BSA control and after 6 hours, mice were then challenged with 5×10^7 CFU of *S. aureus* for 24 hours. **A**, Bacterial colony counts in the lung of mice infected with *S. aureus* alone ($n = 8$). **B**, Bronchoalveolar lavage cell total counts ($n = 8$). **C**, Bacterial colony counts in the lung of mice co-infected influenza A and *S. aureus* ($n = 8$). **D**, Bronchoalveolar lavage cell total counts ($n = 8$). **E**, Ferrous (Fe^{2+}) levels in the bronchoalveolar lavage ($n = 7-8$). * $P < .05$ vs control, ** $P < .10$ vs control. Significance was tested by unpaired t test. Abbreviation: CFU, colony-forming units.

S. aureus in the lungs of coinfecting mice. TNF- α production by natural killer (NK) cells has been suggested to be required for *S. aureus* killing in the influenza coinfection model [20]. It is possible that this suppression of TNF- α in coinfecting mice plays a role in impaired host defense independent of the T_H17 pathway, although TNF- α is known to synergize with IL-17 in promoting immunity.

IL-17 is known to induce neutrophilia in response to infection. We have previously observed decreased *S. aureus* clearance in T_H17 pathway knockout mice was not solely due to a lack of neutrophil recruitment to the lung [8]. Other groups have suggested influenza-induced defects in innate immune cell uptake of bacteria as a mechanism for viral exacerbation of secondary infection [14]. We investigated the role of neutrophils

through antibody depletion. RB6 antibody depletes neutrophils, macrophages, and dendritic cells, whereas 1A8 antibody specifically depletes neutrophils. We observed standard clearance of *S. aureus* in mice that received the 1A8 antibody, confirming that there are alternate mechanisms by which influenza A inhibits *S. aureus* immunity. When monocytes were depleted with RB6 antibody, we observed a large increase in bacterial burden, suggesting a role for antigen presenting cells in *S. aureus* host defense. Decreased phagocytosis of bacteria by neutrophils in the context of influenza and *S. pneumoniae* infection has been reported [21]. We performed in vitro and in vivo studies to determine if inflammatory cells in the lung were capable of phagocytosis of *S. aureus* in the presence and absence of preceding influenza infection and found that there was no change in the binding and

uptake of *S. aureus* by macrophages or neutrophils. Further, interferon treatment did not alter macrophage binding and uptake of *S. aureus* in vitro. This mechanism has been suggested in the context of influenza, streptococcal infection [14]. Of note, the assay we used to determine phagocytosis measures both extracellular bound and ingested bacteria. Our data suggest that no innate cellular defect in influenza-infected lungs is present during *S. aureus* coinfection. Future studies will have to be performed in order to differentiate between extracellular bound and ingested *S. aureus* by macrophages.

Because influenza coinfecting mice have elevated innate immune cell recruitment compared to *S. aureus* alone and there was no evidence of a defect in bacterial binding and uptake by these cells, we examined the ability of *S. aureus* to induce T_H17 pathway associated AMPs. *S. aureus* drove the expression of several AMPs; however, this was attenuated in influenza coinfecting mice. IL-17 and IL-22 can induce production of several AMPs including serum amyloid A, Lcn2, β -defensins, S100 proteins, and RegIII $\alpha\beta\gamma$ [13, 22–26]. Specifically, Lcn2 can inhibit bacterial growth by sequestering enterobactin and depriving bacteria of iron essential for growth [27]. Although previous studies have shown that Lcn2 is important to host defense against gram-negative and gram-positive bacteria, no prior evidence has shown that Lcn2 aids in immunity against MRSA pneumonia [28–31]. Here we show that IL-17, IL-22, with or without TNF- α can induce epithelial production of AMPs. Further, *S. aureus* induction of these peptides requires IL-17. Our data suggest that preceding influenza A infection attenuates the T_H17 pathway and subsequent production of AMPs, allowing for a T_H17 dependent mechanism for *S. aureus* clearance. In support of this finding, exogenous Lcn2 rescued *S. aureus* clearance in mice infected with *S. aureus* alone and coinfected with influenza A, confirming the role of AMPs as a mechanism for bacterial killing. Although a prior investigation by Flo et al has shown that Lcn2 deficiency has no effect on bacterial killing of *S. aureus* [28], these experiments were performed in vitro rather than in vivo. They also found that Lcn2 deficiency had no effect on survival of mice infected with methicillin-sensitive *S. aureus* intraperitoneally. This conflicting evidence may be a result of different strains of *S. aureus* or different locations of infection. In our studies, Lcn2 rescued the bacterial clearance of *S. aureus* independent of rescuing the T_H17 pathway (data not shown), suggesting that AMP production occurs downstream of IL-17. Exogenous Lcn2 decreased levels of ferrous ions in the BAL fluid of mice coinfecting with *S. aureus* and influenza A, suggesting that Lcn2 treatment results in iron sequestration and inhibits bacterial growth of gram-positive bacteria. These findings are significant given the morbidity and mortality associated with influenza and secondary bacterial pneumonia. These data expand our current knowledge regarding host defense, and future studies may allow for the identification of exogenous AMPs as therapeutic targets. The role of

T_H17 immunity is well described in other sites of mucosal host defense, allowing for broader relevance beyond the lung and its epithelium. In addition, influenza A inhibition of T_H17 immunity may be important to a spectrum of pathogens in addition to *S. aureus*. The identification of T_H17 effector mechanisms involved in bacterial clearance provides a novel therapeutic target in viral, bacterial coinfection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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