Influenza A Inhibits Th17-Mediated Host Defense against Bacterial Pneumonia in Mice

Anupa Kudva[#*], **Erich V. Scheller**[#*], **Keven M. Robinson**[*], **Chris R. Crowe**[*], **Sun Mi Choi**[†], **Samantha R. Slight**[*], **Shabaana A. Khader**[*], **Patricia J. Dubin**[*], **Richard I. Enelow**[‡], **Jay K. Kolls**[*,†], and **John F. Alcorn**[*]

[*]Department of Pediatrics, Children's Hospital of Pittsburgh, University of Pittsburgh Medical Center, Pittsburgh, PA 15224

[†]Department of Genetics, Lousiana State University Health Sciences Center, New Orleans, LA 70112

[‡]Department of Medicine, Dartmouth Medical School, Lebanon, NH 03756

[#] These authors contributed equally to this work.

Abstract

Staphylococcus aureus is a significant cause of hospital and community acquired pneumonia and causes secondary infection after influenza A. Recently, patients with hyper-IgE syndrome, who often present with *S. aureus* infections of the lung and skin, were found to have mutations in STAT3, required for Th17 immunity, suggesting a potential critical role for Th17 cells in *S. aureus* pneumonia. Indeed, IL-17R$^{-/-}$ and IL-22$^{-/-}$ mice displayed impaired bacterial clearance of *S. aureus* compared with that of wild-type mice. Mice challenged with influenza A PR/8/34 H1N1 and subsequently with *S. aureus* had increased inflammation and decreased clearance of both virus and bacteria. Coinfection resulted in greater type I and II IFN production in the lung compared with that with virus infection alone. Importantly, influenza A coinfection resulted in substantially decreased IL-17, IL-22, and IL-23 production after *S. aureus* infection. The decrease in *S. aureus*-induced IL-17, IL-22, and IL-23 was independent of type II IFN but required type I IFN production in influenza A-infected mice. Furthermore, overexpression of IL-23 in influenza A, *S. aureus*-coinfected mice rescued the induction of IL-17 and IL-22 and markedly improved bacterial clearance. These data indicate a novel mechanism by which influenza A-induced type I IFNs inhibit Th17 immunity and increase susceptibility to secondary bacterial pneumonia.

Community-acquired and healthcare-associated pneumonia represent a significant cause of morbidity and mortality in the United States and the world. Pneumonia is the leading cause of death in children worldwide, resulting in nearly two million deaths per year (1). A

Address correspondence and reprint requests to Dr. Jay K. Kolls, Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA 70112. jkolls@lsuhsc.edu.

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number of causative organisms have been described in patients, including both Gram-positive and Gram-negative bacteria. Among these, *Staphylococcus aureus* infections have been found to be prevalent at rates as high as 45% (2). The recent increase in the occurrence of methicillin-resistant *S. aureus* (MRSA) has increased further the importance of understanding disease pathogenesis induced by this bacterium. The host response to bacteria is largely triggered by TLR ligands stimulating the production of inflammatory mediators and the recruitment of phagocytic cells to the lung. Several components of the innate immune system have been identified as key mediators of bacterial clearance (3–5). The role of T cells in bacterial pneumonia is more unclear. HIV patients with depleted CD4$^+$ T cells are more susceptible to bacterial infection in the lung (6, 7). In mice, CD4$^+$ and $\gamma\delta$ T cells have been shown to play a role in immunity versus *Klebsiella pneumoniae* (8, 9). In addition, vaccination strategies were effective in bacterial pneumonia (10), further suggesting a role for memory T cells. These data indicate that the host response in bacterial pneumonia requires multiple cell types and likely both arms of innate and adaptive immunity.

Recently, the Th17 subset of T cells has been described as producing high levels of the proinflammatory cytokines IL-17 and IL-22 (11-13). Th17 cells are characterized by high expression of the transcription factors retinoid orphan receptor (ROR)α and RORγT driven by IL-6 and TGF-β signaling through STAT3 and SMAD pathways, respectively (12). The cytokine IL-23 has been implicated in Th17 cell regulation, proliferation, and cytokine production. STAT3 activation, driven by IL-6 and IL-23, is required for terminal Th17 differentiation and IL-22 production (14, 15). A role for STAT3 in T cells in the context of bacterial infection has emerged recently. Patients with hyper-IgE syndrome (Job's syndrome) were shown to have STAT3 mutations (16). Consequently, these patients fail to develop Th17 cells or produce IL-17A, resulting in *S. aureus* infection of the skin and lung (17). These patients appear to have enhanced susceptibility to *S. aureus* due to a specific requirement for IL-17 and IL-22 signaling in the epithelium (18), suggesting a specific role for Th17 immunity in host defense against this pathogen.

A primary consequence of Th17 polarization and recruitment to the lung is production of IL-17 and IL-22. IL-17A, also termed IL-17, is known to signal through the heteromeric IL-17R complex to drive production of neutrophil growth factors and chemokines, including IL-6, G-CSF, keratinocyte chemoattractant, and MIP-2/IL-8. Thus, a primary consequence of IL-17A production in vivo is neutrophil accumulation, and IL-17R$^{-/-}$ mice are deficient in neutrophil recruitment to the airways in lung infection models (19). Overexpression of IL-17A by adenovirus augments the host response to *K. pneumoniae* (19). In addition to Th17 cells, lung-resident $\gamma\delta$T cells produce high levels of IL-17 after *Escherichia coli* challenge, and IL-17 is critical for neutrophil recruitment and bacterial clearance (20, 21). These data confirm a role for IL-17 in recruiting neutrophils to the airways and in the clearance of infection in the lung. Another Th17-derived cytokine, IL-22 signals though the activation of the STAT3 pathway by binding a heteromeric receptor consisting of IL-22R1 and IL-10R2 (22). IL-22 is known to induce the production of several classes of antimicrobial peptides, such as β-defensin 2 and 3, S100A7–9, RegIIIαβγ, and lipocalin 2 (13, 23-26). IL-22 also has been shown to be required for immunity against *K. pneumoniae*

(26). In that study, IL-22, along with IL-17, was shown to induce G-CSF, IL-6, and antimicrobial peptide production in the lung. These data suggest that IL-22 plays a critical role in regulating inflammation and promoting bacterial clearance in lung immunity. Although the role of IL-17A and IL-17F in promoting the clearance of *S. aureus* in mucocutaneous infection has recently been demonstrated recently (27), the impact of IL-17– and IL-22–mediated host defense against *S. aureus* pneumonia is unknown.

Influenza represents a highly contagious family of respiratory viruses that infect 5–20% of the U.S. population yearly and account for as many as 30,000 deaths annually. The majority of influenza A infection is not fatal; rather most patients appear to fully recover within 2 wk of infection. Due to this, there tends to be an assumption that the changes and damage that occur in response to infection are transient. However, it is well documented that influenza infection enhances susceptibility to secondary bacterial infections by altering bacterial adhesions (28), TLR expression (29, 30), and the pathogen-associated molecular pattern receptors (31) on epithelial cells. Recently, induction of type I or type II IFNs by influenza A has been shown to inhibit clearance of pneumococcal pneumonia in mice (32, 33). In addition, exacerbation of *S. aureus* pneumonia by preceding influenza A infection has been demonstrated (34, 35). These data indicate that influenza A infection enhances the lung's susceptibility to secondary bacterial pneumonia. Bacterial sepsis represents a significant cause of death in the United States, 200,000 deaths annually, and the primary cause and location of sepsis are Gram-positive bacteria and the lung (36, 37). Several studies have linked temporally the onset of influenza A infection and the incidence of *S. aureus* pneumonia (38). Furthermore, the presence of preceding influenza-like symptoms has been shown to correlate with increased mortality in MRSA-infected patients (39). These data suggest that a primary severe consequence of influenza A infection is secondary *S. aureus* pneumonia. However, the role of Th17 cells in promoting immunity against *S. aureus* in the context of influenza A infection has not been examined previously.

Materials and Methods

Mice

Six- to 8-wk-old wild-type (WT) C57BL/6 mice were purchased from Taconic. IFN-$\gamma^{-/-}$ and C57BL/6 control mice were purchased from The Jackson Laboratory. IL-17Ra$^{-/-}$ (40), IL-17A$^{-/-}$ (41), IL-17F$^{-/-}$ (Genentech), IL-22$^{-/-}$ (24), and IFN-αR$^{-/-}$ (42) have been reported previously. Mice were maintained under pathogen-free conditions. All of the studies were performed on age- and sex-matched mice. All of the animal studies were conducted with approval from the University of Pittsburgh Institutional Animal Care and Use Committee.

S. aureus infection

S. aureus (American Type Culture Collection 49775) producing γ-hemolysin and Panton-Valentine leukocidin was purchased from the American Type Culture Collection. *S. aureus* was cultured as detailed by American Type Culture Collection instructions in casein hydrolysate yeast extract containing-modified medium, overnight for 18 h to stationary

growth phase. Mice were inoculated with 10^8 CFU of *S. aureus* (in 50 μl sterile PBS) by oropharyngeal aspiration, and lungs were harvested 24 or 48 h later.

Influenza A PR/8/34 H1N1 infection

Influenza A PR/8/34 H1N1 was propagated in chicken eggs as described previously (43). Mice were infected with 100 PFU of 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, at which time mice received *S. aureus* inoculum or control PBS. After an additional 24 or 48 h, lungs were harvested. Viral burden was determined by quantitative real-time RT-PCR on lung RNA for viral matrix protein as described previously (44).

Adenoviral IL-23 infection

Generation of adenovirus expressing IL-23 has been described previously (45). Mice were infected with adenovirus (5×10^8 PFU in 50 μl sterile PBS) expressing IL-23 or enhanced GFP (EGFP) (control) by oropharyngeal aspiration 4 d after influenza A infection. After three additional days, lungs were harvested.

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-22 and IL-23 (R&D Systems). The middle and caudal lobes of the right lung were snap-frozen and homogenized under liquid nitrogen for RNA isolation by standard TRIzol extraction. RNA analysis was performed by standard RT-PCR using Assay on Demand Taqman probes and primers (Applied Biosystems, Foster City, CA). Mouse spleens were homogenized in sterile PBS and were used for bacterial colony counts.

IL-17 flow cytometry

For intracellular IL-17 staining, whole mouse lungs were digested with collagenase as described (44). Lung cells were stimulated with PMA (50 ng/ml) plus ionomycin (750 ng/ml) for 5 h at 37°C. Cells then were stained with fluorescent conjugated Abs for CD4, CD8, CD49b (DX5), and γδTCR (BD Biosciences). After surface marker staining, the cells were fixed and permeabilized (BD Biosciences), and intracellular staining was performed using anti–IL-17A Ab (BD Biosciences).

Bone marrow dendritic cell culture

Bone marrow dendritic cells were isolated from C57BL/6 mice as described previously (46). After 7 d in culture with MEM containing GM-CSF, nonadherent cells were used for assays. Dendritic cells were stimulated with peptidoglycan from *S. aureus* (Sigma-Aldrich).

CD11c⁺ dendritic cell isolation

Lungs from infected mice were cut into small pieces (~2 mm) and digested with collagenase as noted above. Digested lungs then were forced through a 70-μm filter, and RBCs were

lysed. CD11c$^+$ and CD11c$^-$ cells then were isolated using Miltenyi positive selection microbeads per the manufacturer's instructions (Mitlenyi Biotec, Auburn, CA). After isolation, RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA).

Statistical analysis

All of the data are presented as the mean ± SEM. Significance was tested by unpaired *t* test (for two means) or one-way ANOVA (for multiple data groups) followed by Tukey posthoc test. Data were analyzed using the Microsoft Excel software package. Mouse survival data were analyzed by log-rank test using the Graph Pad Prism software package.

Results

Efficient clearance of S. aureus from the lung requires the Th17 pathway

Because hyper-IgE syndrome patients due to STAT3 mutations that impair Th17 immunity develop skin and lung *S. aureus* infections, we investigated the role of Th17 effector cytokines or their receptors in *S. aureus* pneumonia. To test this, we infected WT C57BL/6, IL-17A$^{-/-}$, IL-17F$^{-/-}$, IL-17R$^{-/-}$, and IL-22$^{-/-}$ mice with 10^8 CFU of *S. aureus* (Fig. 1, Supplemental Figs. 1, 2). *S. aureus* induced robust airway inflammation in WT mice characterized by neutrophil chemokine production and neutrophil recruitment to the airways (Fig. *1C*, 1*D*). Abrogation of IL-17R signaling resulted in significantly increased bacterial burden in the lung (48 h postinfection) and attenuated neutrophil chemokine production (at both 24 and 48 h postinfection) (Fig. 1*A*, 1*D*, Supplemental Fig. 1*C*). In addition, IL-17R deletion resulted in a trend toward increased bacterial dissemination to the spleen versus that in WT mice (Fig. 1*B*). A similar phenotype of impaired bacterial clearance was evident in IL-17A$^{-/-}$ and IL-17F$^{-/-}$ mice (Supplemental Fig. 2), suggesting that both of these IL-17 ligands contribute to this response. Next, we examined the role of IL-22 in immunity versus *S. aureus* pneumonia. IL-22$^{-/-}$ mice also had impaired clearance of *S. aureus* from the lung (48 h postinfection) and increased bacterial dissemination to the spleen (Fig. 1*E*, 1*F*). However, deletion of IL-22 did not impair neutrophil recruitment or cytokine production (Fig. 1*G*, 1*H*, Supplemental Fig. 1*E*, 1*F*). In addition, attenuation of IL-17 or IL-22 did not alter production of the proinflammatory cytokine TNF-α at the 24 h time point (Supplemental Fig. 3). These data suggest that the impaired clearance of *S. aureus* in Th17 pathway knockout mice is not solely due to a lack of neutrophil recruitment to the lung, because attenuation of IL-17 or IL-22 both result in decreased bacterial clearance despite differing effects on inflammation.

Preceding influenza A infection worsens S. aureus pneumonia

In a recent review, the presence of preceding influenza-like symptoms in patients with *S. aureus* pneumonia correlated with increased mortality (39). These data support the concept that superinfection with *S. aureus* may be a severe consequence of influenza. To test this hypothesis in an animal model, C57BL/6 mice were challenged with 100 PFU of influenza A PR/8/34 H1N1 for 6 d followed by 10^8 CFU of *S. aureus*; after 24 or 48 h, bacterial and viral clearance as well as lung inflammation were assessed. Preceding influenza A infection resulted in attenuated clearance of *S. aureus* in the lung (Fig. 2*A*). Interestingly, viral clearance also was inhibited in double-infected mice (Fig. 2*B*). Coinfection with influenza

and *S. aureus* increased both macrophage and neutrophil recruitment to the lung as well as IL-6 and MCP-1 production (Fig. 2*C*, 2*D*). These data suggest that coinfection suppresses both viral and bacterial immunity in the lung, despite increasing inflammation. Production of both type I and II IFNs were enhanced by coinfection (Fig. 2*D*, 2*E*). Previous data have shown an important role for IFNs in the suppression of *Streptococcus pneumoniae* immunity (32, 33); however, the impact of the IFN response in *S. aureus* coinfection is unknown. To further demonstrate the impact of preceding influenza A infection on *S. aureus* pneumonia, we challenged mice with influenza A or vehicle as described above followed by 5×10^8 to 2×10^9 CFU of *S. aureus* (Fig. 2*F*). The highest quantity of *S. aureus* induced mortality in both influenza-preinfected and control mice; however, influenza A increased mortality to *S. aureus* throughout the time course. These data indicate that Influenza A infection worsens the outcome of secondary *S. aureus* pneumonia.

Influenza A inhibits Th17 pathway activation by secondary bacterial challenge

Because the Th17 pathway was shown to be critical for host defense against *S. aureus* and influenza A worsened *S. aureus* pneumonia, we proposed that influenza A infection may inhibit Th17-mediated immunity in the lung. To test this, we challenged mice with influenza A followed by *S. aureus* as outlined in the above section. *S. aureus* infection alone induced a Th17 effector cytokine response (Fig. 3, Supplemental Fig. 4). *S. aureus* enhanced IL-17A and IL-22 protein and gene expression in the lung as well as production of Th17 cytokine-induced chemokines (Fig. 3). In addition, *S. aureus* increased the number of IL-17A$^+$ cells in the lung, and cell surface staining revealed that the majority of IL-17A–producing cells were CD4$^+$ or γδ T cells (Fig. 3*B*, 3*C*, Supplemental Fig. 4). *S. aureus* also induced expression of the Th17 transcription factor RORγT and the Th17-promoting cytokine IL-23 (Fig. 3*D*). IL-23 has been shown to be required for IL-17A production by both Th17 and γδ T cells (47, 48). These data confirm that *S. aureus* induces a Th17 pathway immune response in the lung, and data in Fig. 1 demonstrate that in the absence of the Th17 pathway bacterial clearance is delayed. Importantly, preceding influenza infection significantly inhibited the *S. aureus*-driven Th17 pathway activation. Influenza A infection resulted in substantially decreased IL-17A and IL-22 protein and gene expression, decreased IL-17A$^+$CD4$^+$ and γδ T cells, and decreased RORγT and IL-23 expression. These data illustrate a potential mechanism by which influenza A inhibits bacterial immunity by suppressing the Th17 response in the lung.

Type I IFN induced by influenza A inhibits Th17 activation

To determine the mechanism by which influenza A inhibits Th17 immunity, we focused on the elevated production of type I and II IFNs during coinfection. This mechanism has been implicated in increased susceptibility to *S. pneumoniae* infection and may be related to the observations in this study. We observed that preceding influenza infection suppressed the production of IL-23 induced by *S. aureus* infection, which is required for the production of Th17 cytokines and the maintenance of Th17 cells in the lung. A likely source of IL-23 in the lung is dendritic cells that drive Th17 polarization. To further demonstrate influenza-induced suppression of dendritic cell IL-23 production, we compared IL-23 expression in CD11c$^+$ lung dendritic cells from *S. aureus* or coinfected mice. *S. aureus* induced IL-23 expression in CD11c$^+$ dendritic cells, and this was inhibited by preceding influenza A

infection (Fig. 4*A*). Next, we proposed that *S. aureus* TLR2 ligands, such as peptidoglycan or lipoteichoic acid, may stimulate IL-23 production in dendritic cells. Furthermore, we proposed that type I or II IFNs may inhibit IL-23 production by dendritic cells, thus attenuating the Th17 pathway. To test this mechanism in vitro, we derived bone marrow dendritic cells and stimulated them with peptidoglycan in the presence or absence of type I or II IFN (Fig. 4*B*). Peptidoglycan from *S. aureus* increased IL-23 production by dendritic cells; this induction was inhibited by the type I IFN IFN-β but not by the type II IFN IFN-γ. These data suggest that influenza A-induced type I IFN may inhibit the Th17 pathway in the lung. To then determine whether type I or II IFN was essential for the exacerbation of *S. aureus* pneumonia in the lung, we challenged IFN-αR$^{-/-}$ or IFN-γ$^{-/-}$ mice with influenza A followed by *S. aureus*. Deletion of either type I or II IFN resulted in suppressed lung inflammation during coinfection compared with that in WT mice (Supplemental Fig. 5). Type II IFN knockout mice still displayed exacerbation of *S. aureus* pneumonia in double-challenged mice (Fig. 4*D*). These data are consistent with a lack of suppression of IL-23 production in dendritic cells treated with IFN-γ. However, knockout of type I IFN signaling resulted in a loss of effects on *S. aureus* pneumonia, suggesting that type I IFNs are required for influenza A-induced worsening of bacterial immunity in the lung (Fig. 4*C*). Furthermore, preceding influenza A infection failed to inhibit IL-23 or IL-22 production in IFN-αR$^{-/-}$ mice (Fig. 4*E*, 4*F*). These data support a mechanism by which influenza A induces type I IFN, which through inhibition of IL-23 production attenuates Th17 immunity in the lung. The results of this molecular cascade lead to impaired antibacterial host defense and enhanced susceptibility to secondary infection.

Exogenous IL-23 rescues Th17 activation and improves S. aureus *clearance*

As our mechanism proposes, decreased IL-23 production in influenza A, *S. aureus*-coinfected mice leads to exacerbated bacterial pneumonia by inhibiting Th17 immunity. To test this pathway, we overexpressed IL-23 in influenza A, *S. aureus*-coinfected mice, which we would predict would rescue Th17 immunity and improve bacterial clearance. IL-23 overexpression resulted in decreased lung inflammation, increased production of IL-17A and IL-22 protein and mRNA, and increased Th17 cytokine-induced chemokines compared with those in control adenovirus-infected mice (Fig. 5). This elevation of Th17 pathway immunity increased the clearance of *S. aureus* in mice infected with both influenza A and bacteria. In addition, exogenous IL-23 did not significantly alter type I IFN production induced by influenza A (Fig. 5*D*). These data further provide evidence for the mechanism outlined in this study. Influenza A inhibition of the Th17 pathway was rescued by overexpression of IL-23, which restored bacterial immunity in the lung and prevented increased susceptibility to secondary infection. These data suggest a potential therapeutic benefit of boosting Th17 immunity in patients with primary influenza A infection to reduce the risk of complicating secondary infections.

Discussion

The findings of this study demonstrate a molecular and cellular mechanism by which influenza A infection impairs host defense against secondary *S. aureus* challenge. In addition, the data confirm a critical role for the Th17 pathway in promoting immunity versus

S. aureus pneumonia. The vast majority of severe and fatal influenza infections are related to secondary bacterial pneumonia (49), indicating the importance of understanding compromised immune defense in this context. Furthermore, recent findings during the current H1N1 pandemic have shown the presence of secondary *S. aureus* infections in fatal influenza A cases in young adults. In our study, type I IFN induced by influenza A inhibits IL-23 production, which is required for Th17 pathway activation. Exogenous IL-23 during influenza A infection increased Th17 pathway activation and improved the immune response to secondary *S. aureus* challenge. These observations may have implications for the attenuation of host defense against a number of pathogens, because the role of the Th17 pathway has been demonstrated in numerous bacterial challenge models and in fungal immunity.

The observation that influenza A worsens susceptibility to *S. aureus* was reported many years ago (50); however, the molecular mechanisms for this phenotype have remained unclear. Further, pathologic synergism between influenza A and *S. aureus* products has been identified (51-53). Recently, influenza A was shown to exacerbate secondary *S. aureus* infection in mice as measured by decreased bacterial clearance and increased mortality (34). In that study, influenza was shown to inhibit NK cell production of TNF-α, which resulted in impaired antimicrobial function of macrophages. In our *S. aureus* model, IL-17R$^{-/-}$ or IL-22$^{-/-}$ mice had no change in TNF-α production in the lung induced by *S. aureus* at 24 h postchallenge compared with that in control mice despite worsened clearance of *S. aureus*. It is possible that TNF-α levels differed at earlier time points after *S. aureus* challenge. Further investigation is required to determine the role of TNF-α in our model. In addition, we observed a greater neutrophil response to *S. aureus* compared with macrophage recruitment, suggesting a potential role for neutrophils in the host response. In the context of secondary *S. pneumoniae* infection, preceding influenza A infection inhibited neutrophil recruitment to the lung, potentially explaining the increased susceptibility to bacterial infection (32). In addition, it has been reported that influenza A results in lasting desensitization to TLR ligands, resulting in decreased proinflammatory cytokine production (30). However, in our study, we observed increased neutrophilia and macrophage recruitment in coinfected mice, indicating that inflammatory cell recruitment alone does not explain the differences in *S. aureus* clearance. These data suggest that alternate mechanisms for *S. aureus* clearance are being suppressed by influenza A infection, which will require further investigation.

Previous work in our laboratory confirmed that IL-17 is required for acute lung injury during influenza A infection (44). IL-17R$^{-/-}$ mice displayed decreased lung inflammation, decreased weight loss, and decreased mortality after a lethal influenza A challenge. Despite the suppression of lung injury, IL-17R$^{-/-}$ mice did not fail to clear the virus from the lung, suggesting a therapeutic benefit of IL-17 neutralization in viral pneumonia. However, in light of our current data, inhibition of IL-17 during viral infection may lead to enhanced susceptibility to secondary bacterial challenge. These data indicate that modulation of the IL-17 pathway in the context of influenza A infection may have opposing effects on lung injury and susceptibility to further infection. In our study, exogenous IL-23 given 4–6 d after influenza A resulted in enhanced Th17 activation and suppressed airway neutrophilia. Increased Th17 activation may have been expected to increase inflammation, and the

mechanism for the observed inhibition is unclear. These data suggest that the timing of Th17 pathway modulation may be critical to achieving the desired effect of reducing the risk of infection and minimizing lung injury.

Two recent studies have shown a role for type I or II IFNs in inhibiting the clearance of secondary *S. pneumoniae* infection after influenza, suggesting a critical mechanism for the repression of bacterial immunity (32, 33). In our study, the type II IFN pathway had no effect on IL-23 production or bacterial clearance. However, type I IFN robustly inhibited IL-23 levels produced by dendritic cells in response to *S. aureus* ligands. In addition, attenuation of type I IFN signaling resulted in a loss of influenza A exacerbation of *S. aureus* pneumonia and increased Th17 pathway activation. In support of this finding, the inhibition of Th17 polarization by type I IFN has been reported in human T cells (54). These data also suggest a broader mechanism by which other mucosal pathogens that induce type I IFN responses may inhibit Th17 immunity worsening secondary infections due to a variety of bacterial and fungal pathogens. Furthermore, a recent study has shown that highly virulent MRSA strains directly induce type I IFN production (55). This may represent a novel pathway by which *S. aureus* can directly inhibit host defense via Th17 pathway inhibition.

Due to the often severe consequences of secondary bacterial infections after influenza A on both lung function and the development of sepsis, a better understanding of the molecular pathways involved in host defense is critical. The impact of the Th17 pathway in promoting immunity against *S. aureus* appears clear; however, the molecular mechanisms involved remain elusive. Future studies will define the specific pathways and cell types critical for bacterial clearance. Inhibition of Th17 immunity in the lung by influenza A could have an impact on susceptibility to a variety of pathogens, expanding the impact of these studies presented in this work. In addition, the role of Th17 immunity in additional mucosal tissues is well described, suggesting a broader application of these data beyond the lung. The therapeutic potential of manipulating Th17 immunity in this context is yet to be determined and will require future investigation. This approach may be exploited to improve patient outcomes in severe cases of viral and bacterial coinfection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used in this article

EGFP   enhanced GFP

| MRSA | methicillin-resistant Staphylococcus aureus |
| ROR | retinoid orphan receptor |
| WT | wild-type |

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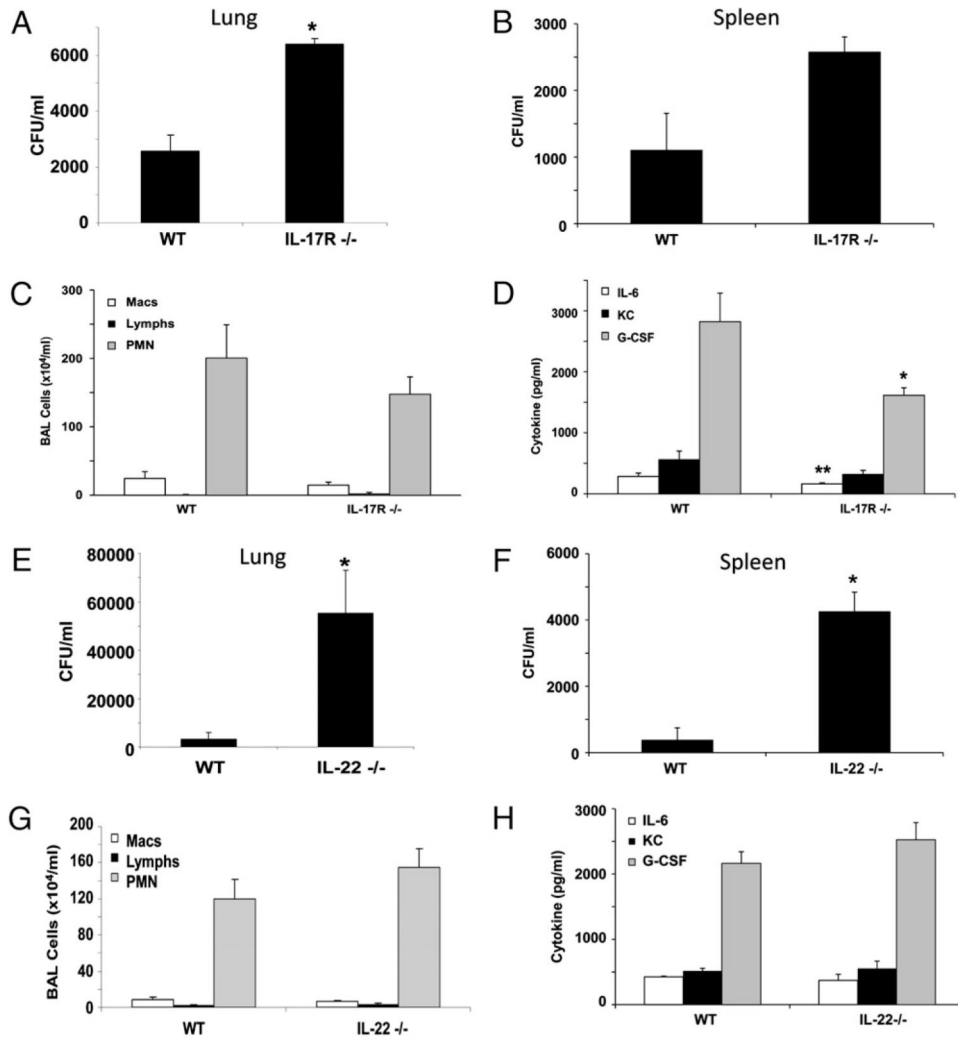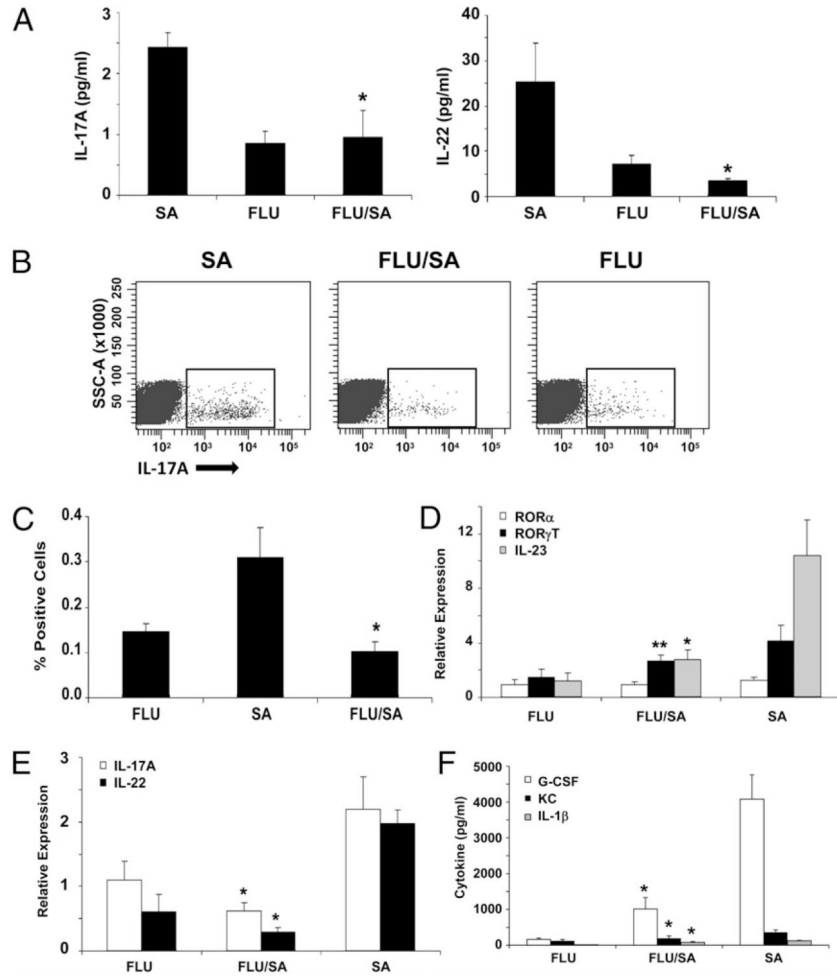
FIGURE 1.
IL-17 and IL-22 are required for efficient clearance of *S. aureus* from the lung. C57BL/6, IL-17R$^{-/-}$, or IL-22$^{-/-}$ mice were challenged with 10^8 CFU of *S. aureus* for 24 (*C*, *D*, *G*, *H*) or 48 h (*A*, *B*, *E*, *F*). All of the experiments were repeated once; representative data are presented. *A* and *E*, Bacterial colony counts in the cranial lobe of the right lung (*n* = 4). *B* and *F*, Bacterial colony counts in the spleen (*n* = 4). *C* and *G*, Lavage cell differential counts (*n* = 6 IL-17R$^{-/-}$, *n* = 4 IL-22$^{-/-}$). *D* and *H*, Th17-induced cytokines in lung homogenate (*n* = 6 IL-17R$^{-/-}$, *n* = 4 IL-22$^{-/-}$). *p< 0.05; **p< 0.10 versus WT.
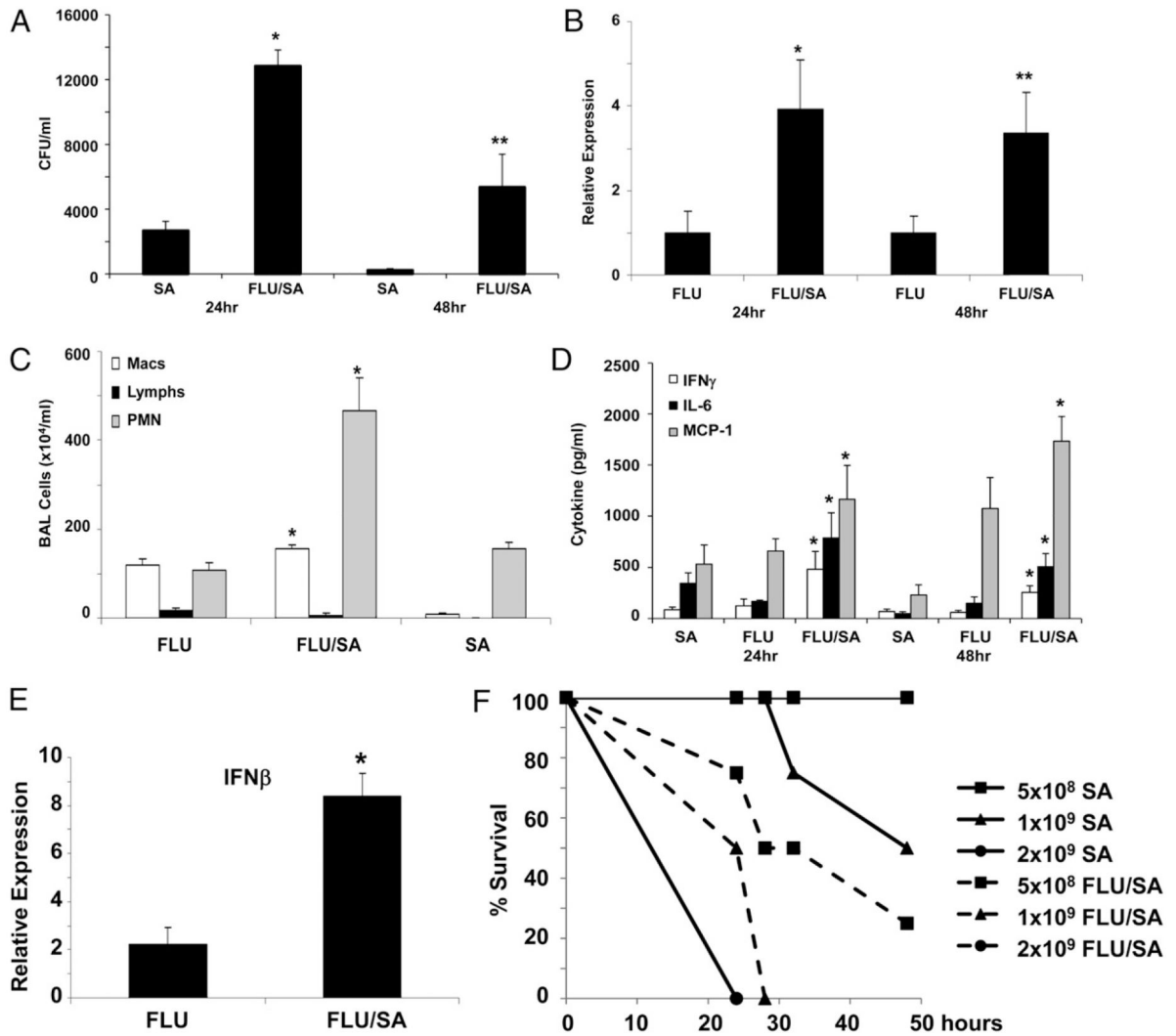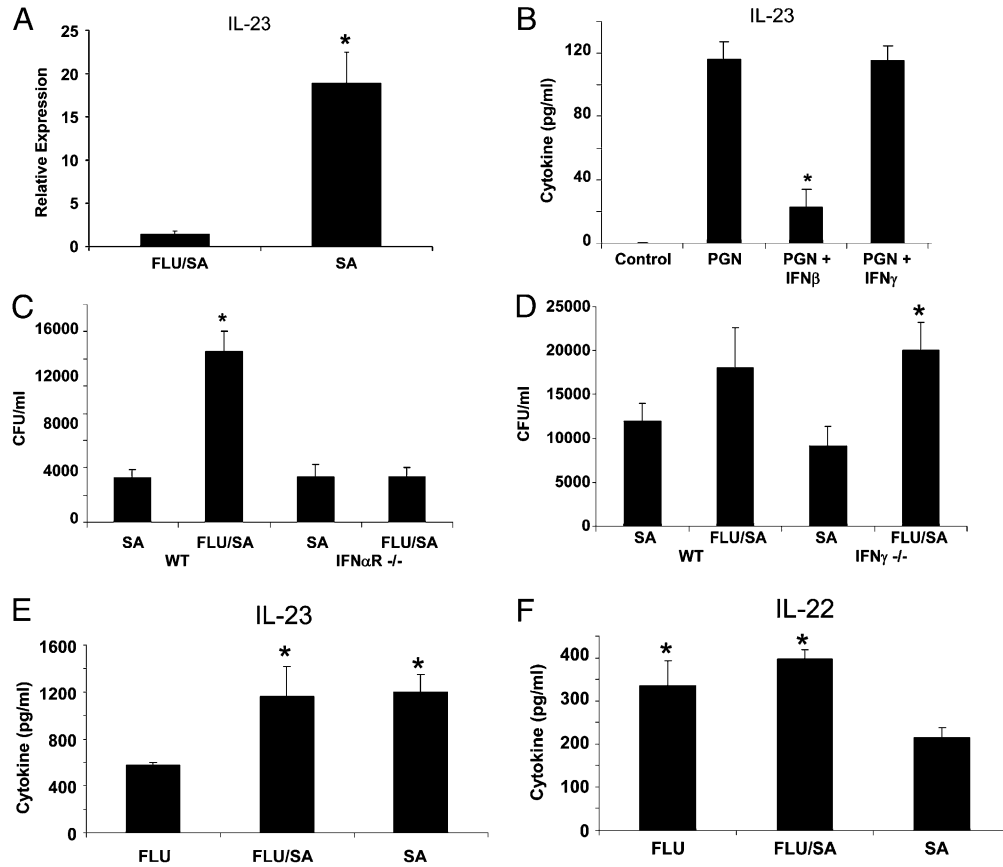
FIGURE 2.
Influenza A infection increases the susceptibility to *S. aureus* pneumonia. C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 d, mice then were challenged with 10^8 CFU of *S. aureus* for 24 or 48 h ($n = 4$), all of the experiments were repeated twice, and representative data are presented. *A*, Bacterial colony counts in the upper right lobe of the lung. *B*, Influenza matrix protein expression in lung RNA. *C*, Lavage cell differential counts (24 h). *D*, Cytokine concentrations in lung homogenate. *E*, IFN-β gene expression in lung RNA (24 h). *$p < 0.05$; **$p < 0.10$ versus influenza A or *S. aureus* alone. *F*, Mortality curve for *S. aureus* challenged (solid lines) versus influenza A, *S. aureus*-coinfected (dashed lines) mice ($n = 4$). $p < 0.05$ versus *S. aureus* alone.
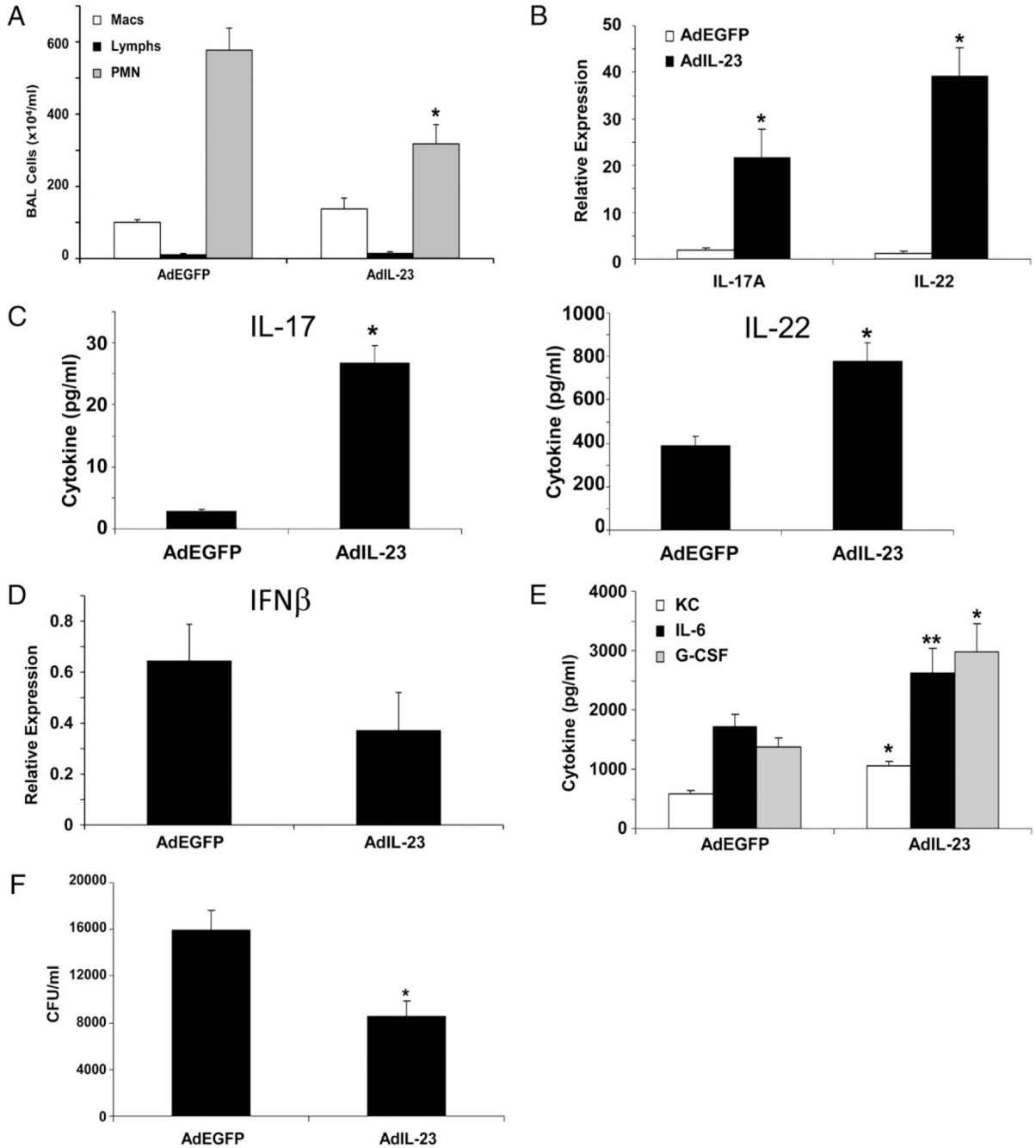
FIGURE 3.
Influenza A inhibits the Th17 pathway induction by *S. aureus*. C57BL/6 mice were infected
with 100 PFU of influenza A PR/8/34 or vehicle for 6 d, mice then were challenged with 10^8
CFU of *S. aureus* for 24 h ($n = 4$), all of the experiments were repeated twice, and
representative data are depicted. *A*, IL-17 and IL-22 protein concentrations in lung
homogenate. *B* and *C*, Total IL-17A–producing cells in lung by flow cytometry. *D* and *E*,
Th17 pathway gene expression in lung RNA. *F*, Th17 pathway cytokine concentrations in
lung homogenate. *$p< 0.05$; **$p< 0.10$ versus *S. aureus* alone.

FIGURE 4.
Inhibition of the Th17 response by influenza A requires type I IFN. Mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 d followed by 10^8 CFU of *S. aureus* for 24 h. CD11c$^+$ dendritic cells were isolated from the lung of *S. aureus* or coinfected mice, and IL-23 expression was determined by RT-PCR. *A*, CD11c$^+$ dendritic cell IL-23 expression (*n* = 7, 6, respectively). Bone marrow-derived dendritic cells were stimulated for 48 h with *Staphylococcus* peptidoglycan (20 μg/ml) in the presence or absence of IFN-β (10 U/ml) or IFN-γ (5 ng/ml) (*n* = 3), all of the experiments were repeated twice, and representative data are presented. *B*, IL-23 production in the media as measured by ELISA. C57BL/6, IFN-αR$^{-/-}$, or IFN-γ$^{-/-}$ mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 d, mice then were challenged with 10^8 CFU of *S. aureus* for 24 h (*n* = 4), all of the experiments were repeated once, and representative data are presented. *C* and *D*, Bacterial colony counts in the upper right lobe of the lung. *E* and *F*, IL-22 and IL-23 production in lung homogenate from IFN-αR$^{-/-}$ mice as measured by ELISA. *p< 0.05 versus *S. aureus* alone (A versus influenza A/*S. aureus*; B versus peptidoglycan; D versus influenza A).

FIGURE 5.
Overexpression of IL-23 rescues the influenza-induced defect in Th17 induction and improves clearance of *S. aureus*. C57BL/6 mice were infected with 100 PFU of influenza A PR/8/34 or vehicle for 6 d, on Day 4 mice were inoculated with 5×10^8 PFU of adenovirus expressing IL-23 or EGFP, mice then were challenged with 10^8 CFU of *S. aureus* for 24 h (*n* = 6), all of the experiments were repeated twice, and representative data are presented. *A*, Lavage cell differential counts. *B*, IL-17A and IL-22 gene expression in lung RNA. *C*, IL-17A and IL-22 protein production in lung homogenate. *D*, IFN-β expression in lung

tissue by RT-PCR. *E*, Th17 pathway cytokine levels in lung homogenate. *F*, Bacterial colony counts in the upper right lobe of the lung. *$p< 0.05$; **$p< 0.10$ versus adenoviral EGFP (AdEGFP).