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Influenza Infection Leads to Increased Susceptibility to Subsequent Bacterial Superinfection by Impairing NK Cell Responses in the Lung

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Influenza viral infection is well-known to predispose to subsequent bacterial superinfection in the lung but the mechanisms have remained poorly defined. We have established a murine model of heterologous infections by an H1N1 influenza virus and *Staphylococcus aureus*. We found that indeed prior influenza infection markedly increased the susceptibility of mice to secondary *S. aureus* superinfection. Severe sickness and heightened bacterial infection in flu and *S. aureus* dual-infected animals were associated with severe immunopathology in the lung. We further found that flu-experienced lungs had an impaired NK cell response in the airway to subsequent *S. aureus* bacterial infection. Thus, adoptive transfer of naive NK cells to the airway of prior flu-infected mice restored flu-impaired antibacterial host defense. We identified that TNF- α production of NK cells played an important role in NK cell-mediated antibacterial host defense as NK cells in flu-experienced lungs had reduced TNF- α expression and adoptive transfer of TNF- α -deficient NK cells to the airway of flu-infected mice failed to restore flu-impaired antibacterial host defense. Defected NK cell function was found to be an upstream mechanism of depressed antibacterial activities by alveolar macrophages as contrast to naive wild-type NK cells, the NK cells from flu-infected or TNF- α -deficient mice failed to enhance *S. aureus* phagocytosis by alveolar macrophages. Together, our study identifies the weakened NK cell response in the lung to be a novel critical mechanism for flu-mediated susceptibility to bacterial superinfection. *The Journal of Immunology*, 2010, 184: 2048–2056.

Polyicrobial diseases also known as heterologous infections are acute or chronic infectious diseases that often occur as a result of prior viral infection predisposing to a secondary bacterial superinfection (1). Clinical and epidemiological evidence have indicated that, as far back as the Spanish influenza in 1918, more people could die of secondary bacterial pneumonia after influenza infection than from viral infection itself (2–4). Today, influenza and bacterial pneumonia combined are among the most deadly infectious diseases and are associated with significant morbidity and mortality worldwide. Furthermore, the incidence of multidrug resistance bacterial pathogens and the aging human populations have resulted in more individuals at risk (2, 5). Thus, as we constantly confront the threat of influenza pandemics and the ever-increasing rate of antibiotic-resistant bacteria, it is of importance to understand the mechanisms for increased susceptibility to bacterial superinfection after influenza infection in the lung.

In addition to *Streptococcus pneumoniae*, a significant portion of influenza-associated morbidity and mortality is attributed to bacterial pneumonia caused by *Staphylococcus aureus*, an extracellular Gram-positive bacterium (2, 4). *S. aureus* is normally carried by 10–35% of children and by ~35% of the general adult population. When there is a breach in host immune system, *S. aureus* may cause pneumonia that accounts for 20–30% of nosocomial infections and remains to be one of the leading causes of death during influenza epidemics (6, 7). Mounting evidence also indicates a high prevalence of community-acquired pneumonia during high influenza activity caused by multidrug resistance strains of *S. aureus* among otherwise healthy individuals (7, 8).

Innate cells play a major role in antiextracellular bacterial immune responses. Alveolar macrophages (AMs) have the ability to control bacterial infections by coordinating the innate immune response not only by producing proinflammatory cytokines, but also by recruiting and scavenging apoptotic polymorphonuclear cells (9, 10). In addition to AMs, NK cells also play a critical role in innate immunity (11, 12). Although NK cells are widely known to play an essential role in host defense at early stages of viral infection via killing of infected cells and production of cytokines, there is now mounting evidence to suggest a role for NK cells in host defense against extracellular bacterial pathogens (13). In vitro studies have demonstrated that NK cells may interact with macrophages to regulate macrophage-mediated bacterial clearance (14, 15). It was recently shown in vivo that NK cells are an important part of protective innate immunity in primary pulmonary staphylococcal infection (16).

However, the mechanisms of increased susceptibility by flu infection to subsequent bacterial superinfection still remain poorly understood. Earlier reports suggest that influenza virus can cause epithelial damage and/or surface receptor changes, which may increase bacterial colonization (17, 18). In contrast, altered

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Abbreviations used in this paper: AM, alveolar macrophage; BAL, bronchoalveolar lavage; ICS, intracellular cytokine staining; IP, inflammatory protein; i.t., intratracheal(ly); KO, knockout; PGN, peptidoglycan; TSA, tryptic soy agar; WT, wild type.

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ntrophil functions and excessive production of immunosuppressive IL-10 have been implicated in flu infection-increased susceptibility to secondary streptococcal infection in the lung (19–22). Recently, IFN- γ -mediated macrophage functional depression (23) or macrophage desensitization to bacterial ligand-triggered TLR signaling (20) was also found to play a role in this process. These findings suggest that the mechanisms underlying the flu-increased susceptibility to secondary bacterial superinfection are multifactorial and complex, and may differ greatly according to the disease settings and the nature of causative bacterial pathogen. In our current study, we have developed a murine model of heterologous infectious exposure to specifically address whether prior H1N1 influenza virus infection may predispose to secondary bacterial superinfection caused by *S. aureus* in the lung and if so, what are the underlying mechanisms. We have found that prior influenza infection predisposes to *S. aureus* superinfection in the lung via impairing NK cell responses. Thus, our study for the first time reveals that NK cell impairment is a critical mechanism for increased susceptibility to postflu bacterial pneumonia, upstream of functional depression of AMs. Our findings hold implications in designing effective preventive and therapeutic strategies combating influenza epidemics.

Materials and Methods

Mice

Eight- to ten-week-old female C57BL/6 mice were purchased Charles River Laboratories (Wilmington, MA). Age- and sex-matched TNF- α knockout (KO) mice raised on C57BL/6 background (24) were bred in the barrier facilities at McMaster University (Hamilton, Ontario, Canada). Animals were housed in specific pathogen-free level facilities. All experiments were conducted in accordance with the animal ethics research board of McMaster University.

Infectious agents

A mouse-adapted strain of influenza A/FM/1/47 (H1N1) virus was prepared and used as previously described (25). A clinical isolate of *S. aureus* B33349 strain was prepared as previously described (16). Briefly, it was inoculated in tryptic soy broth (Difco Laboratories, Frederick, MD) and incubated for 16 h at 37°C, 225 rpm. The bacteria were collected and resuspended in PBS. The bacterial concentration was determined as CFUs by plating 10-fold serial dilutions on tryptic soy agar (TSA), which was cultured for 24 h at 37°C. A virulent clinical isolate *S. aureus* strain Newman-expressing GFP (*S. aureus*-GFP kindly provided by Dr. David Heinrichs at the University of Western Ontario, Canada) was prepared as previously described (16) and used for macrophage phagocytosis assay. Chloramphenicol (10 $\mu\text{g ml}^{-1}$) was included in tryptic soy broth and TSA media for selection of *S. aureus*-GFP (16).

Heterologous infection model

The influenza virus was delivered to the lung of anesthetized mice by intranasal instillation of 10^5 PFU dose/mouse in 25 μl PBS or PBS as control. Infected mice were left to recover for 7 d. Mice were then infected intratracheally (i.t.) with *S. aureus* using a previously described procedure (16). Briefly, mice were anesthetized by inhalation of isoflurane and a small incision was made at the midline of the neck to expose the trachea. A dose 5×10^7 CFU *S. aureus* dispersed in 40 μl PBS was instilled i.t. into the lungs of mice.

Survival and body weight monitoring

Mice were monitored daily after flu infection and were then monitored twice daily, after *S. aureus* infection for overt disease symptoms, such as ruffled fur, inactivity, and respiratory distress. Moreover, the extent of sickness was determined by comparing the body weight at various time points post-infection with the original body weight prior to infection. Moribund mice were terminated and considered dead when they lost $\geq 20\%$ of their initial weight, which was the endpoint set by the animal ethics board of McMaster University in Ontario, Canada. The mice that were dead in the cage at the time of monitoring were also recorded for survival rate determination.

Quantification of influenza virus or *S. aureus* bacterium in the tissue

Levels of virus in the lung were quantified 3, 7, and 10 d postflu infection by plaque forming assay as previously described (25). Briefly, Madin-Darby canine kidney cells were grown to 100% confluence in 6-well plates and

washed twice with PBS. Serial dilutions of lung homogenates were placed on Madin-Darby canine kidney cell monolayer for 30 min, followed by overlay with 1.3% agarose in $2 \times$ MEM F-11 with 1% penicillin-streptomycin, 1% L-glutamine. After 3 d, viral plaques were fixed in carnoys fixative (25% acetic acid, 75% methanol) and counted. Levels of staphylococcal burden were determined in the whole lung by plating 10-fold serial dilutions of tissue homogenates on TSA plates. After a 24-h incubation at 37°C, colonies were counted, calculated, and presented as CFU per organ (16).

Pulmonary histopathological evaluation

Mouse lungs were removed at various time points postinfection and fixed in 10% buffered formalin. The fixed lungs were then sectioned in 4-mm slices and stained with H&E. Multiple sections from the same lung of several animals/time points were assessed for designated pathological changes by using a semiquantitative scoring method ranging from 0 to 5, with 5 being the worst. Evaluation was carried out according to the severity of cellular infiltrates (mononuclear cells and polymorphonuclear neutrophils), neutrophil apoptosis, epithelium injury, and epithelial cell hyperplasia.

Cytokine quantification

Levels of cytokines in culture and bronchoalveolar lavaging (BAL) supernatants were determined using Quantikine murine ELISA from R&D Systems (Minneapolis, MN) or using multiplex Luminex kit (Luminex, Austin, TX) according to manufacturer's protocols.

Cell isolation

Spleens and lungs were removed aseptically. The airway luminal cells were removed by BAL five times with a total vol of 1.35 ml PBS. Subsequent to BAL, lungs were perfused through the left ventricle with HBSS (Life Technologies, Burlington, Ontario, Canada) to remove RBCs. To obtain mononuclear cells from lung tissue, the lungs were then cut into 1-mm pieces and treated with 150 U/ml collagenase type I (Sigma-Aldrich, St. Louis, MO) resuspended in HBSS for 1 h at 37°C, 200 rpm, and lung portions were then crushed through 40- μm basket filters and the remaining erythrocytes lysed with ACK lysis buffer (0.15 M NH_4Cl , 1.0 M KHCO_3 , 0.1 mM Na_2EDTA , pH 7.4) and washed with PBS. BAL cells were centrifuged and resuspended in cRPMI (10% FBS, 1% penicillin-streptomycin, 1% L-glutamine). Spleens were also processed for splenocyte isolation. All isolated cells were enumerated on a hemocytometer diluted in 0.5% trypan blue and resuspended to a given concentration in cRPMI.

Adoptive transfer of NK cells

NK cells were purified from the whole splenocytes and lung mononuclear cells of naive C57BL/6 or TNF- α KO mice and then 2×10^6 NK cells in 40 μl PBS or control PBS were adoptively transferred i.t. to the naive mice or mice infected with influenza virus 6 d prior. This i.t. procedure was carried out where the mice were anesthetized and hung from a high bar apparatus by their large front teeth. Tweezers were then used to pull the tongue of the mice aside and the NK cells were then instilled into the trachea. The next day, mice were infected with *S. aureus* by using a different i.t. procedure described previously.

Intracellular cytokine staining and flow cytometric analysis

Intracellular cytokine staining (ICS) and FACS were carried out as previously described (16). Briefly, for ICS, Golgi plugs (5 $\mu\text{g/ml}$ brefeldin A) (BD Biosciences, San Jose, CA) were added to all peptidoglycan (PGN)-stimulated cultures 6 h before the end of the 24-h incubation. Cells were subsequently washed and blocked for 15 min with CD16/CD32 in 0.5% BSA/PBS, then stained with the appropriate Abs against cell surface markers as stated previously. Cells were washed, permeabilized according to manufacturer's protocol (BD Biosciences), and subsequently ICS with FITC anti-TNF- α for 30 min. In other cases, cells were stained with biotin-conjugated anti-IL-15 mAb (PeproTech, Rocky Hill, NJ) at 4°C for 30 min, followed by the addition of streptavidin-PE cy7 (BD Pharmingen, San Diego, CA). Samples were washed and analyzed using LSRII. For FACS, single-cell suspensions were blocked with CD16/CD32 in 0.5% BSA/PBS for 15 min on ice and then stained for 30 min with the following specific Abs: PerCP-Cy5.5-anti-NK1.1 (clone PK136); FITC-anti-CD3 (clone SP34-3); PE-anti-CD49b/*Pan*-NK (DX5) or PE Cy7-anti-CD11b (clone M1/70); APC-anti-CD11c (clone HL3) purchased from BD Pharmingen and allophycocyanin-Alexa Fluor-750-anti-GR1 (clone RB6-8C5) purchased from eBioscience (San Diego, CA). Stained cells were analyzed using LSRII (BD Biosciences) where 250,000 events per sample were collected. The data of ICS/FACS was analyzed with FlowJo Software ver 6.3.4 TreeStar (Ashland, OR).

Purification of NK cells and AMs

Single cell suspensions from lungs and spleens of naive or infected wild-type (WT) and TNF- α KO mice were isolated as stated above. NK cells were purified (~90% purity) from whole splenocytes using PAN-NK (CD49b⁺) positive selection kit from StemCell Technologies (Vancouver, BC, Canada) according to manufacturer's protocol and as previously described (16). To purify AMs, lungs from naive or infected mice were lavaged as stated previously and resuspended in cRPMI. BAL fluids were then plated and AMs allowed to adhere for 3 h at 37°C, 5% CO₂. More than 97% of adhered these cells were AMs as determined by differential cell counting and flow cytometric analysis.

In vitro coculture of AMs and NK cells

This was carried out as previously described (16). Briefly, AMs were isolated from naive or flu-infected mice and purified as stated previously. NK cells were purified from the spleen and lungs of naive or infected mice. Purified NK cells (1.5×10^5) were cultured in the presence or absence of AMs (4.5×10^5) (1NK/3AM ratio) with or without 4 μ g/ml staphylococcal PGN (Sigma-Aldrich) and 200 U/ml rIL-2 for 24 h at 37°C, 5% CO₂ as previously described (26). In some cases, prior to coculturing with AMs, purified NK cells were treated with 3 μ g/ml recombinant murine TNF- α and then incubated overnight at 37°C, 5% CO₂. TNF- α was removed by repeated washes before AMs were added. In separate experiments, purified NK cells were cultured in the presence or absence of AMs in 24-well plates or in transwells (BD Falcon; 0.4- μ m pore size) for 24 h. Transwells were used to address whether the effect of NK cells on AMs was cell-to-cell contact dependent.

Phagocytosis of *S. aureus* by AMs

Lungs from noninfected WT, flu-infected, and TNF- α KO mice were lavaged as described previously to obtain AMs. AMs (4.5×10^5 cells/well) were allowed to adhere in 24-well plates for 3 h at 37°C, 5% CO₂. The cells were then washed twice with PBS to remove nonadherent cells and resuspended to 500 μ l RPMI 1640 medium supplemented with 10% FBS, 1% glutamine, and 1% chloramphenicol. NK cells (1.5×10^5 cells/well) were purified from whole splenocytes and lung mononuclear cells of naive C57BL/6, flu-infected, and naive TNF- α KO mice and added to AMs (1NK:3AM ratio) for overnight incubation in 37°C, 5% CO₂. Recombinant *S. aureus*-GFP or *S. aureus* without expressing GFP was prepared as described previously and resuspended to 1×10^7 cells/ml. Opsonization was performed by adding 10% naive C57BL/6 serum to bacteria for 30 min at 37°C under slow rotation. Bacteria were then washed twice with PBS to remove excess serum and added to macrophage culture (macrophages to bacteria ratio = 1:20) for 3 h at 37°C. After incubation, internalization was stopped by placing cells on ice and treating with penicillin-streptomycin-containing media for 30 min at room temperature to remove any membrane-bound bacteria. Cells were then centrifuged and 1 ml distilled autoclaved H₂O was added for 30 min at room temperature to disrupt cell membrane of cells. After incubation, cells were centrifuged, resuspended in PBS, and subsequently plated in 10-fold serial dilutions on TSA plates containing chloramphenicol. Twenty-four hours postincubation, colonies were counted and determined as intracellular CFU of *S. aureus*. In separate experiments, AMs were treated with *S. aureus*-GFP and intracellular cell fluorescence (100,000 events) was measured using flow cytometry. The cells treated with the conventional, non-GFP-expressing *S. aureus* were also set up to control for the potential autofluorescence by macrophages. Naive AMs were identified as CD11c^{high} cells. The percentage of FITC-positive macrophages was used as a measure for phagocytic activity of these cells.

Statistical analysis

Statistics analyses were performed using either unpaired Student *t* test or one-way ANOVA for Tukey to determine the significant differences among infection groups. Significant differences in percent body weight losses between singly and heterologously infected mice were determined by repeated measures of one-way ANOVA for Tukey using Analyze-it, Excel (Analyze-it Software, Leeds, U.K.). In addition, Mantel-Cox survival curves were generated and differences in survival were analyzed with log-rank test. Any *p* values <0.05 were considered statistically significant.

Results

Prior flu infection causes increased susceptibility to bacterial superinfection in the lung

To determine the impact of prior influenza infection on host defense against a subsequent staphylococcal infection in the lung, C57BL/6 mice were infected intranasally with an H1N1 influenza A virus and

allowed to recover for 7 d at which time, the virus was almost entirely cleared from the lung (Supplemental Fig. 1). The 7 d time point postflu infection for the onset of bacterial superinfection was chosen also based on the clinical evidence that most bacterial superinfections in humans occur within the first 2 wk of influenza infection (19, 27). The mice were then infected i.t. with *S. aureus* bacteria at day 7 postflu infection (flu/staph) and as a control, naive mice were infected only with *S. aureus* (PBS/staph) or flu-infected mice were left uninfected with *S. aureus* (flu/PBS) (Fig. 1A). On monitoring body weight changes that reflected the severity of overall sickness, flu/PBS mice were found to have slightly gained body weight from day 7 and onward (Fig. 1B), whereas PBS/staph mice lost on average ~5% of their body weight over the course of 4 d after staphylococcal infection (Fig. 1B). In contrast, flu/staph mice lost significantly more body weight (15–20%) (Fig. 1B). Thus, many of these mice were moribund and dying or found dead in the cage and by 4 d after *S. aureus* inoculation, only ~40% of mice survived (Fig. 1C). Consistent with the severity of sickness, the lungs of flu/staph mice had markedly higher levels of bacterial burden, in particular at 6 and 24 h after *S. aureus* infection, than the lungs of PBS/staph mice (Fig. 1D). The latter had cleared much of the bacterial infection from their lungs by day 1 (Fig. 1D), which was associated with the regained body weight observed in these mice (Fig. 1B). Overall, these results

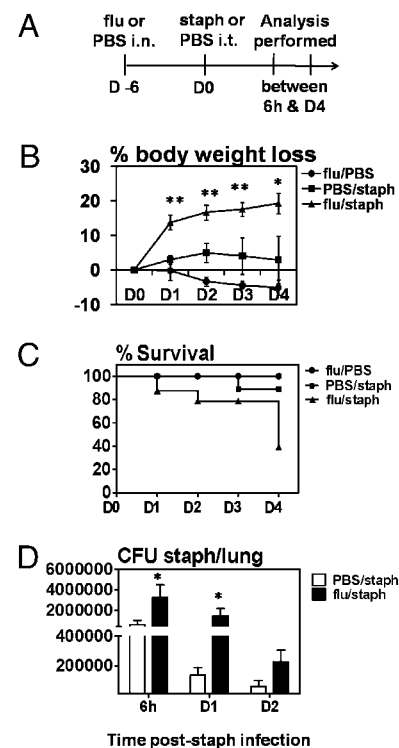


FIGURE 1. Influenza infection causes increased susceptibility to *S. aureus* superinfection. **A**, Experimental schema. **B**, Body weight was monitored from the point of *S. aureus* infection (day 7 postflu infection). Results were expressed as the mean percentage of body weight loss/gain \pm SEM of 5–10 mice representative of four independent experiments. **p* < 0.05, ***p* < 0.01 as compared with PBS/staph mice at the same time point postinfection. **C**, Percent survival of mice. Mice were deemed dead when reaching the experimental endpoint ($\geq 20\%$ body weight loss). Five to 10 mice per group were used and the results were representative of two independent experiments. **D**, Six hours, 1 and 2 d *S. aureus* postinfection, lungs were harvested from mice and levels of bacterial burden were determined by CFU assay. Data are expressed as the mean value \pm SEM of five mice/group, representative of two independent experiments. **p* < 0.05 compared with PBS/staph mice at the same time point.

indicate that prior influenza infection causes more severe sickness and increased susceptibility to secondary staphylococcal infection in the lung.

We next examined the histopathologic basis of severe illness observed in flu/staph mice and compared it with histopathologic findings in the lung of flu/PBS and PBS/staph mice. We found that by 8 or 9 d postflu infection (flu/PBS group) when the virus was cleared from the lung (Supplemental Fig. 1), the mice had resolved most of inflammatory responses with only some residual mononuclear cell infiltration seen in the perivascular and peribronchial areas (Fig. 2A, 2D, 2G, Table I). In comparison, *S. aureus* infection alone (PBS/staph group) caused primarily a neutrophilic response in the lung between 0.5 and 1 d postinfection, which switched over to a predominately mononuclear infiltration onward (Fig. 2B, 2E, 2H, Table I). There was no or only a minimum epithelium injury and hyperplasia seen. In contrast, *S. aureus* superinfection in prior influenza-infected animals (flu/staph group) caused much more severe inflammation and tissue injury in the lung than in flu/PBS and PBS/staph mice. We observed persisting tissue neutrophilia and mononuclear cell infiltration, and neutrophil apoptosis was also evident in the lung of flu/staph mice (Fig. 2C, 2F, 2I, Table I). Furthermore, there was inflammatory infiltration within the bronchial epithelium accompanied by epithelial sloughing and hyperplasia (Fig. 2C, 2F, 2I, Table I). Some airways were filled up with inflammatory “plugs” made up of apoptotic neutrophils, mucus, and cell debris (Fig. 2C, 2I). These findings suggest that prior influenza infection predisposes to developing severe bacterial pneumonia, tissue pathology, and illness.

Prior influenza infection decreases NK cell recruitment and activation on bacterial superinfection in the lung

Recently, airway luminal NK cells were found involved in host defense against primary acute *S. aureus* infection in the lung (16). To begin investigating whether prior influenza infection diminished antibacterial host defense via affecting NK cell responses in the lung, we examined and compared NK cell responses to staphylococcal infection in the lung with and without prior flu infection. Although we observed a marked influx of NK cells into the airway lumen of PBS/staph mice, flu-infected mice had a remarkably decreased NK cell response to *S. aureus* infection in the airway lumen when the NK cells were identified either as DX5⁺CD3⁻ or NK1.1⁺CD3⁺ cells (Fig. 3A, 3B). We next

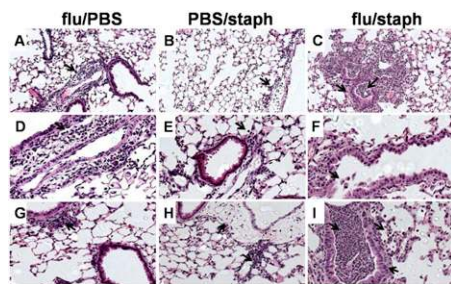


FIGURE 2. Influenza infection increases histopathologic responses to *S. aureus* superinfection. Mice were infected with influenza for 7 d and then superinfected with *S. aureus* (flu/staph; C, F, I) or treated with PBS (flu/PBS; A–C). The control mice were infected only with *S. aureus* (PBS/staph; B, E, H). At 24 h poststaphylococcal infection, the lungs (2–4 mice/group) were processed and assessed. Representative pictures are shown. Arrows indicate mononuclear infiltrates in A, D, and G, predominantly neutrophilic infiltration in B, E, and H, or mixed neutrophilic and mononuclear infiltration and intrabronchial inflammatory plug in C, epithelial sloughing in F or a large intrabronchial inflammatory plug in I. Original magnifications $\times 10$ (A–C) and $\times 20$ (D–I).

further assessed the activation status of recruited NK cells in the airway lumen by examining TNF- α production in NK cells. Indeed, we observed markedly reduced numbers and frequencies of TNF- α -producing NK cells in the airway lumen of flu/staph mice compared with those in the airway of PBS/staph mice (Fig. 3C).

As AM-derived IL-15 was shown to be a critical cytokine for airway luminal NK cell recruitment and activation on primary staphylococcal infection (16), we investigated whether there was a reduced IL-15 response in AMs in the lung of flu/staph mice. Indeed, by ICS analysis we found significantly reduced numbers and frequencies of IL-15-producing CD11b^{high}CD11c^{low} AM in the airway lumen of flu/staph mice, compared with those of PBS/staph mice (Fig. 3D). Furthermore, the reduced number of AMs producing IL-15 correlated well with reduced NK cells and their activation in flu/staph mice (Fig. 3A–C).

As we observed reduced NK cells and NK cell TNF- α production, we further examined the overall level of TNF- α protein in the BAL fluids. Indeed, there was a markedly reduced level of TNF- α in BAL (Fig. 4A) in flu/staph mice compared with PBS/staph mice. Because TNF- α is an alarm cytokine involved in the secondary induction of chemokines, we also measured the level of chemokines in BAL and found that there were also significant reduced levels of IP-10 and MIP-1 α in the airway lumen of flu/staph mice (Fig. 4B). These results together suggest that prior influenza viral infection has a profound negative effect on NK cell responses and on the signals involved in NK cell recruitment and activation on secondary bacterial infection in the lung.

Adoptive transfer of NK cells restores host defense against bacterial superinfection in the lung of prior influenza-infected mice

Having established an association of increased susceptibility to secondary staphylococcal infection with impaired NK cell responses within the airway lumen of flu/staph mice, we set out to determine the causal relationship between the two by addressing the question whether such prior flu infection-impaired host defense against *S. aureus* could be corrected by adoptive NK cell transfer to the airway lumen. To this end, we adoptively transferred NK cells isolated from naive C57BL/6 mice i.t. into the lung of prior flu-infected mice and subsequently challenged these mice with *S. aureus* (flu/NK/staph) (Fig. 5A). The control groups included the mice that were infected only with *S. aureus* (PBS/PBS/staph) and those that were prior flu-infected and subsequently infected with *S. aureus* without receiving NK cell transfer (flu/PBS/staph). The level of bacterial infection in the lung was assessed and compared. Consistent with the data shown earlier (Fig. 1D), prior flu infection caused a significant increase in bacterial burden in the lung of flu/PBS/staph mice (Fig. 5B). In contrast, adoptive NK cell transfer to the lung of previously flu-infected mice (flu/NK/staph) markedly reduced the bacterial burden to a level comparable to that in the mice infected only with *S. aureus* (Fig. 5B). These findings suggest that impaired NK cell responses caused by prior flu infection are causally linked to increased susceptibility to secondary staphylococcal infection in the lung.

Impaired NK cell responses in prior influenza-infected mice lead to altered antibacterial activities of AMs

AMs are considered to be one of the main effector cells in host defense responsible for controlling extracellular bacterial infections and previous studies have suggested a critical role of NK cells in the regulation of macrophage functions (15, 16, 28, 29). Thus, we examined whether impaired NK cell responses in influenza-infected animals led to altered AM responses to secondary bacterial infection. We found that there were by and large comparable

Table I. Assessment of histopathologic changes in the lung

Histopathological Parameters	Flu/PBS				PBS/ <i>S. aureus</i>				Flu/ <i>S. aureus</i>			
	12 h	D 1	D 2	D 4	12 h	D 1	D 2	D 4	12 h	D 1	D 2	D 4
Neutrophilia	ND	-	-	ND	++	+++	+	-	++	+++	++	+
Mononuclear infiltration	ND	++++	+++	ND	-	-	+++	+++	++	+++	++++	++++
Apoptotic neutrophils	ND	-	-	ND	-	-	+	-	+++	++++	++	++
Epithelial injury	ND	-	-	ND	+	+	-	-	+++	++++	+++	++++
Epithelial hyperplasia	ND	++	+	ND	-	+	+	-	+	+++	++	+++

The histopathological parameters including tissue neutrophilia, mononuclear infiltration, apoptosis of neutrophils, epithelial injury (epithelial sloughing, epithelial infiltration, and intrabronchial inflammatory plug formation) and epithelial hyperplasia were each semiquantitatively scored.

For each time point, the lungs of multiple animals were examined and the mean score of each of the five histological parameters calculated where possible. Data were representative of two independent experiments.

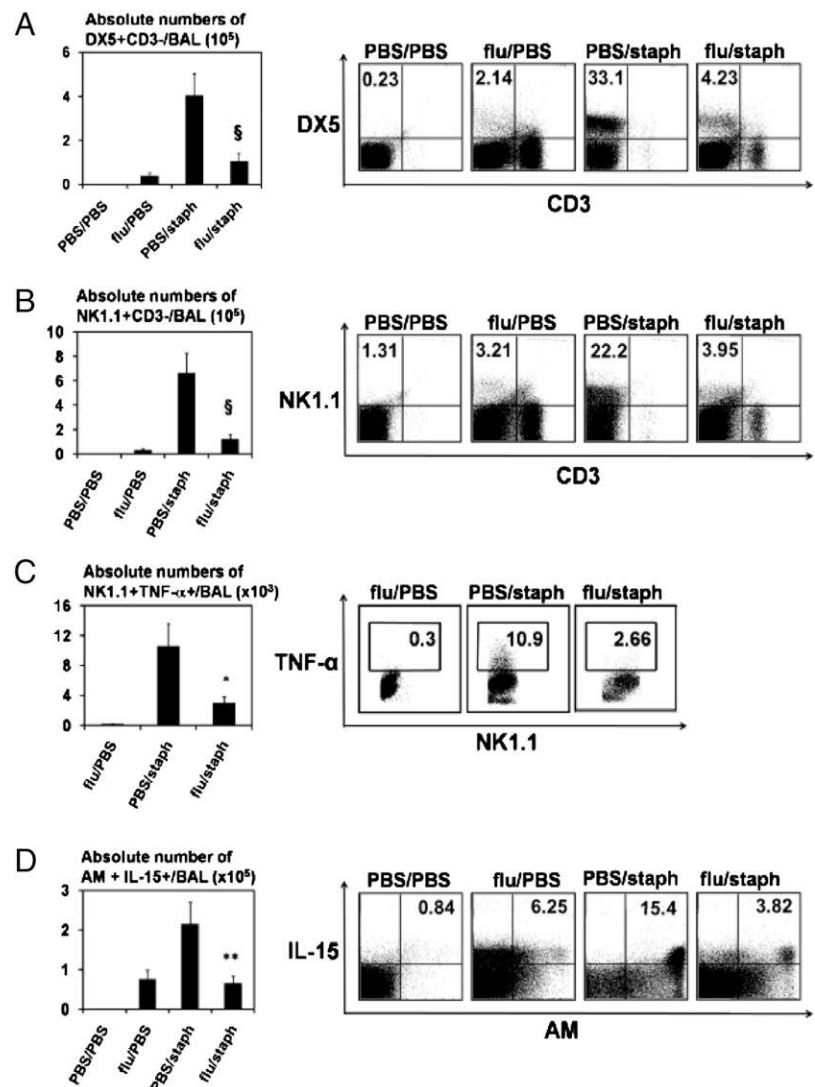
-, absent; +, minimal; ++, slight; +++, moderate; +++++, marked; and +++++, severe.

numbers of AMs in the airway of both PBS/staph and flu/staph groups (Fig. 6A). However, there was a significant reduction in the frequency of activated AMs capable of TNF- α production in the lung of flu/staph animals (Fig. 6B), which coincided with diminished levels of IL-15-producing AMs (Fig. 3D). Based on these observations, we also examined the phagocytosis rate of staphylococci by AMs by incubating AMs with a strain of *S. aureus* expressing GFP (*S. aureus*-GFP) or non-GFP-expressing *S. aureus* (*S. aureus* without GFP). Indeed, we found that the AMs

isolated from flu-infected lungs were less capable of phagocytosing *S. aureus*-GFP than their naive counterparts (Fig. 6C).

To investigate whether decreased AMs antibacterial activities observed in flu/staph infected mice resulted directly from impaired NK cell responses, we isolated AMs from the lung of naive and flu-infected mice and assessed their respective bacterial phagocytic capabilities in the presence of NK cells isolated from naive (naive NK) or flu-infected (flu NK) mice. We found that phagocytosis of *S. aureus* by AMs from naive mouse lungs were enhanced by the

FIGURE 3. Influenza infection impairs NK cell responses to *S. aureus* superinfection. Mice were infected with influenza for 7 d and then superinfected with *S. aureus* (flu/staph) or treated with PBS (flu/PBS). The control mice were infected only with *S. aureus* (PBS/staph). Noninfected mice (PBS/PBS) were used as negative controls. At 24 h poststaphylococcal infection, mice were sacrificed and the lungs were lavaged. The cells in BAL fluids were collected, immunostained, and analyzed by FACS. **A** and **B**, Representative dotplots and absolute numbers of NK cells that were either NK1.1⁺CD3⁻ or DX5⁺CD3⁻ in BAL. **C**, Representative dotplots and absolute numbers of NK cells producing TNF- α (NK1.1⁺CD3⁻TNF- α ⁺) in BAL. **D**, Representative histogram and absolute numbers of AMs (CD11b^{high}CD11c^{low}Gr1^{low}) positive for IL-15. The results in all bar graphs are expressed as the mean value \pm SEM of 3–5 mice/group, representative of three independent experiments. [§]*p* < 0.005; **p* < 0.05; ***p* < 0.01 compared with PBS/staph mice.



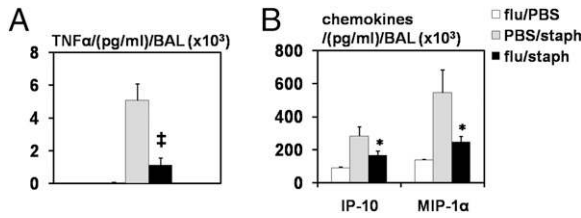


FIGURE 4. Modulation by influenza infection of proinflammatory cytokine responses to *S. aureus* superinfection in the lung. Mice were infected with influenza for 7 d and then superinfected with *S. aureus* (flu/staph) or treated with PBS (flu/PBS). The control mice were infected only with *S. aureus* (PBS/staph). At 24 h poststaphylococcal infection, mice were sacrificed and the lungs were lavaged. BAL fluids were then collected and measured for the content of TNF- α (A) and IP-10/MIP-1 α (B) protein by ELISA and Luminex assay, respectively. Data are expressed as the mean value \pm SEM of six mice/group from two independent experiments (A) or of 10–20 mice/group from three independent experiments (B). * p < 0.05; † p < 0.001 compared with PBS/staph group.

presence of naive NK cells, but was markedly inhibited by the presence of flu NK (Fig. 6D). In contrast, although the AMs from influenza-infected lungs were less capable of *S. aureus* phagocytosis than their naive counterparts, this diminished phagocytosis was restored by the presence of naive NK cells but not by flu NK cells (Fig. 6D, 6E). The previously described results suggest that impaired NK cell functionality by prior flu infection negatively influences the antistaphylococcal activities of AM.

TNF- α production by NK cells is critical to NK cell activation, host defense, and antibacterial activities of AMs

To investigate the molecular mechanism of reduced NK cell activation and its linkage to anti-*S. aureus* host defense and macrophage function, we examined the role of NK cell-derived TNF- α . Not only was NK cell-derived TNF- α shown to be important to NK cell functions (13), but as shown in Figs. 3 and 4, NK cells in the airway of flu/staph lung displayed impaired TNF- α responses that were associated with overall reduced TNF- α levels in the airway lumen of these mice. Thus, we first examined the role of NK cell-derived TNF- α in NK cell-mediated protection against *S. aureus* superinfection in influenza-infected lung. To

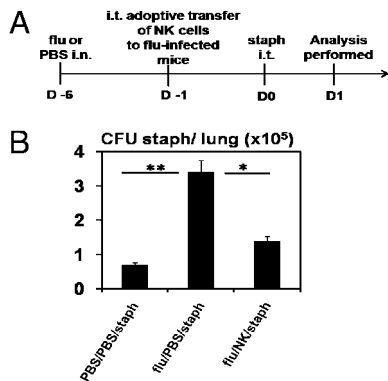


FIGURE 5. Adoptive transfer of NK cells restores host defense against *S. aureus* superinfection in the lung of influenza-infected mice. A, Experimental schema. Mice were infected with flu for 7 d and then superinfected with *S. aureus* (flu/PBS/staph) or *S. aureus* alone (PBS/PBS/staph). A separate group of flu/staph mice received the transferred NK cells 1 d before *S. aureus* infection (flu/NK/staph). B, Twenty-four hours after *S. aureus* superinfection, lungs were harvested and levels of bacterial burden were determined by CFU assay. Data are expressed as the mean value \pm SEM of 4–5 mice/group, representative of two independent experiments. * p < 0.05; ** p < 0.01.

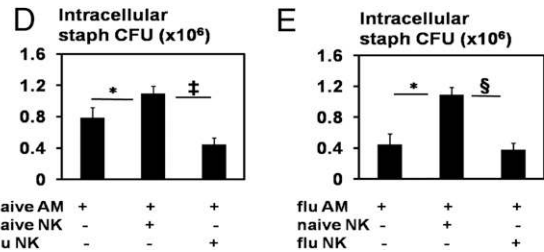
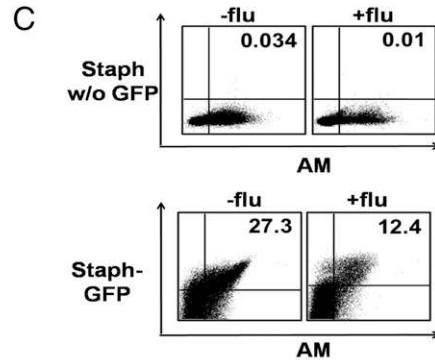
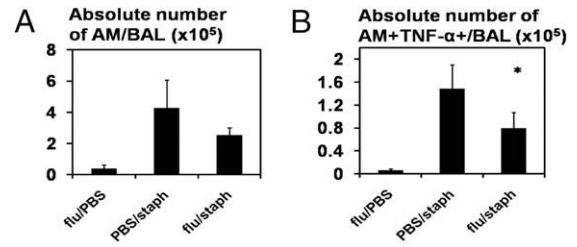


FIGURE 6. NK cells modulate antibacterial activities of AMs. A and B, Mice were infected with influenza for 7 d and then superinfected with *S. aureus*. Mice were sacrificed at 24 h *S. aureus* postinfection. A, Lungs were lavaged and BAL cells were collected, immunostained and analyzed by FACS to determine the number of AM (CD11b^{hi}Gr1^{low}CD11c^{low}). B, BAL cells were stimulated with *S. aureus* PGNs, immunostained and processed by ICS for TNF- α production. Data are expressed as the mean value \pm SEM of 3–10 mice/group from three independent experiments. * p < 0.05 compared with PBS/staph mice. C, Opsonized *S. aureus*-GFP or *S. aureus* without GFP expression was added to AMs isolated from naive or flu-infected mouse lung and phagocytosis was allowed to occur for 3 h at 37°C. Penicillin-streptomycin was added to samples for 30 min to remove the membrane-bound bacteria and the cells were then immunostained and analyzed by FACS. Data are from duplicate determinations, representative of three independent experiments. D and E, NK cells were purified either from naive or flu-infected mice and cultured with or without AMs isolated from naive or flu-infected mice for 24 h. Opsonized *S. aureus*-GFP was subsequently added to the coculture and phagocytosis was allowed to occur for 3 h at 37°C. Penicillin-streptomycin was added to samples for 30 min to remove the membrane-bound bacteria and then H₂O for 30 min at room temperature to disrupt the membrane of AMs. Cells were subsequently analyzed for quantification of phagocytosed bacteria. Data are expressed as the mean value \pm SEM of triplicate determinations, representative of two independent experiments. * p < 0.05; † p < 0.005; ‡ p < 0.001.

this end, either WT NK cells (WT NK) or TNF- α -deficient NK cells (TNF- α KO NK) were adoptively transferred to the lung of prior flu-infected mouse lung and these mice were then challenged with *S. aureus* (Fig. 7A). As a control, mice were infected with flu and *S. aureus* without receiving NK cells (flu/PBS/staph). We found that although adoptive transfer of WT NK cells enhanced antibacterial host defense in the lung of prior flu-infected mice, consistent with the data shown in Fig. 5, adoptive transfer of TNF- α -deficient NK cells was incapable of

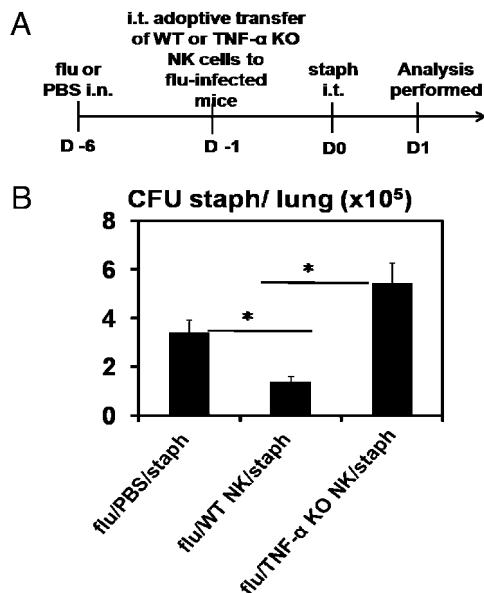


FIGURE 7. NK cells require TNF- α to mediate host defense against *S. aureus* superinfection in vivo. **A**, Experimental schema. Mice were infected with flu for 6 d and then received adoptive transfer of either WT NK cells (flu/WT NK/staph) or TNF- α -deficient NK cells (flu/TNF- α KO NK/staph) or treated only with PBS (flu/PBS/staph). On the following day, these mice were then superinfected with *S. aureus*. **B**, Twenty-four hours *S. aureus* postinfection, lungs were harvested and levels of bacterial burden were determined by CFU assay. Data are expressed as the mean value \pm SEM of 3–5 mice/group, representative of two independent experiments. * p < 0.05.

doing so (Fig. 7B). These findings indicate that autocrine TNF- α production of NK cells plays a critical effector role in host defense against staphylococcal superinfection.

To further understand the relationship between NK cell-derived TNF- α , NK cell activation, and macrophage antibacterial function, we cocultured naive AMs with naive NK, flu NK, or TNF- α -deficient NK cells and then assessed macrophage phagocytosis rate of live *S. aureus*. We found that consistent with the data in Figs. 5 and 6D, naive AMs cocultured with naive NK cells demonstrated a high rate of phagocytosis, whereas naive AMs cocultured with flu NK cells showed a markedly decreased rate (Fig. 8). In support of the in vivo findings (Fig. 7), naive AMs cocultured with naive NK cells from naive TNF- α KO mice (TNF- α KO naive NK) also demonstrated a markedly reduced level of AM phagocytic capacity (Fig. 8). Thus, in terms of AM-activating capability, the TNF- α -deficient NK cells behaved similarly as the NK cells from flu-infected mice, suggesting an important role for NK cell-derived TNF- α in rendering NK cells the ability to activate macrophage antistaphylococcal activities. To further support this conclusion, we pretreated flu NK cells with recombinant TNF- α protein and then cocultured these cells with AMs, and found that this could also restore the phagocytic capacity of AMs (Fig. 8). Furthermore, reactivation of flu NK cells by pretreatment with recombinant IL-15 resulted in a similar outcome (data not shown). Taken together, these results suggest that influenza infection reduces antistaphylococcal host defense by impairing NK cell TNF- α production and crippling their ability to activate antibacterial activities of AMs.

Previous reports suggest that the cross-talk between NK cells and dendritic cells as well as macrophages requires the physical contact (11, 14, 15, 28). We have identified the impaired NK cell function as the reason for reduced AM antistaphylococcal function. Very likely, the regulation of AM function by airway NK cells also

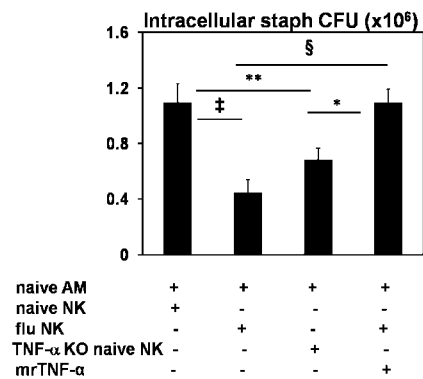


FIGURE 8. NK cells require TNF- α for their ability to modulate antibacterial activities of AMs. AMs were isolated from naive mice and incubated for 24 h in the presence of NK cells purified from flu-infected (flu NK), naive WT (naive NK) or naive TNF- α -deficient (TNF- α KO naive NK) mice. Under separate conditions, NK cells from flu-infected mice were treated first with murine recombinant TNF- α protein overnight before AMs were introduced. *S. aureus*-GFP was then added to AMs and NK cells and phagocytosis was allowed to occur for 3 h at 37°C. Cell cultures were then treated and processed for phagocytosis assay. Data are expressed as the mean value \pm SEM of triplicate determinations, representative of two independent experiments. * p < 0.05; ** p < 0.01; § p < 0.005; ‡ p < 0.001.

involves the physical communication given the both cell types coexisting within the airway luminal compartment in our model. Indeed, by using a transwell system, we found that flu NK cell-mediated inhibition of AMs activation required the cell-to-cell contact (data not shown).

Discussion

The innate immune system in the lung requires nonspecific, well coordinated cellular and cytokine responses to effectively control extracellular bacterial infection (30). Yet, previous exposure to a viral infection may imprint on the lung innate immune system and modulate its responses to subsequent bacterial superinfection. One of the likely outcomes of such modulation is increased susceptibility to subsequent bacterial superinfection, which accounts for an important portion of morbidity and mortality associated with influenza infection. Indeed, clinically, the majority of flu patients diagnosed with bacterial superinfection were found to have just recovered or were still recovering from the flu (19, 27). However, the underlying mechanisms have remained largely to be elucidated. In our current study, by using an experimental system, we found that prior influenza infection led to increased susceptibility to subsequent bacterial superinfection by *S. aureus*. Compared with naive mouse lungs, flu-experienced lungs had an impaired ability to mount a potent NK cell response in the airway to subsequent *S. aureus* challenge. We identified that TNF- α production of NK cells played a key role in NK cell-mediated antibacterial host defense as adoptive transfer to flu-infected lungs of WT NK cells, but not TNF- α -deficient NK cells, restored antibacterial host defense blunted by prior flu infection. In contrast to the WT naive NK cells but similar to the NK cells from flu-infected lung, TNF- α -deficient NK cells failed to enhance antibacterial activities of AMs. Our study for the first time has identified the weakening of NK cell function in the lung to be an important mechanism, upstream of depressed AM function, underlying increased susceptibility to bacterial superinfection after influenza virus infection.

We found that reduced NK cells and NK activation in the airway lumen of flu-infected mice on *S. aureus* infection was correlated with decreased IL-15 expression by AMs and an overall reduction

in MIP-1 α and IP-10 production in the airway. These findings thus support the current understanding that IL-15 is a key cytokine involved in the chemotaxis and activation of NK cells (31, 32). AMs are a main source of IL-15 during lung extracellular bacterial infection and IL-15 deficiency leads to severely impaired NK cell responses in the airway and weakened host defense (16). In addition to IL-15, other chemokines, such as MIP-1 α , were also found to participate in optimizing NK cell recruitment in the airway (33). The downstream event of reduced NK cell recruitment and activation within the airway lumen is the decreased NK functionality required for the subsequent activation of important innate phagocytes, such as AMs, in the course of bacterial infection. Our current study further identified TNF- α to be an effector molecule critically required for optimal NK cell activation and its function to activate AMs. Thus, our findings suggest that NK cells serve as a master switch in antistaphylococcal host defense in the lung and prior flu infection may switch NK cells off to impair their function to activate the ultimate antistaphylococcal phagocytes, AMs. Although neutrophils may also play a role in antistaphylococcal infection, we found little evidence to suggest a major defect in neutrophil responses. Rather, we observed an undiminished and somewhat prolonged neutrophilic response in the lung and airways of flu-*S. aureus*-infected animals.

Our observations are in line with a recent study by Sun and Metzger who also demonstrated an increased susceptibility to *S. pneumoniae* superinfection in the mice that were prior flu-infected for 7 d (23). Such impaired host defense against bacterial superinfection by prior flu infection appears persisting as Didierlaurent et al. found similarly increased susceptibility to *S. pneumoniae* superinfection in the mice that were prior flu-infected for 14 d (20). In this study, AMs were found to remain desensitized to bacterial TLR stimulation, thus contributing to increased susceptibility to streptococcal superinfection (20). The study by Sun and Metzger has also identified AMs to be an ultimate culprit in a murine model of flu-increased susceptibility to *S. pneumoniae* infection (23). Although the cellular and molecular mechanisms upstream of defective macrophage responses still remain largely unclear, increased IFN- γ production in prior flu-infected lung in response to bacterial challenge was shown accountable for decreased antibacterial activities of AMs in this study (23). In addition to the lack of information on the cellular sources of IFN- γ in the study, several major differences were noted between this and our current studies. The study by Sun and Metzger used a different strain of H1N1 influenza virus. Moreover, it used *S. pneumoniae* for bacterial superinfection. Thus, it is expected that the host responses in prior flu-infected lung to streptococci may be different from those to staphylococci and, of importance, the nature of protective immune mechanisms against primary streptococcal and staphylococcal infection in the lung may also differ. Indeed, different from the study by Sun and Metzger, we detected a minimum level of IFN- γ in flu-infected lung after *S. aureus* superinfection (data not shown). Furthermore, to date, NK cells have only been found to contribute to host defense against primary staphylococcal infection (16, 34). In contrast, although exaggerated IL-10 responses to streptococcal superinfection in the flu-infected lung was reportedly linked to increased susceptibility (22, 35), we did not detect any measurable levels of IL-10 responses to staphylococcal superinfection in the flu-infected lung (data not shown). These contrasting observations may be due to a number of differences between these and our studies including different strains of flu virus and extracellular bacterial species used and different time intervals between prior flu infection and bacterial superinfection. A couple of previous studies also demonstrated the exaggerated proinflammatory cytokine and chemokine responses

to streptococcal superinfection in the airway of the flu-infected lung (36–39). In contrast, we observed a depressed proinflammatory cytokine/chemokine response to staphylococcal superinfection in the airway of the flu-infected lung. Together, these findings suggest the complexity of the immune mechanisms underlying increased susceptibility to bacterial superinfection by flu infection and that different mechanisms may be involved, depending largely on the nature of both bacteria and protective immunity.

Our current study has firmly established a critical role of NK cells in host defense against staphylococcal superinfection and their vulnerability to the history of prior influenza infection in the lung. Our study implies that reviving NK cell function would be a potential therapeutic strategy to combat influenza epidemics preventing or treating antibiotic-resistant bacterial superinfection. For instance, as we have now found depressed TNF- α production by NK cells to be a key feature in this process, conceivably TNF- α - or IL-15-based therapy locally in the lung would be beneficial. Depressed TNF- α responses in flu-infected lung may reflect a way by which the virus evades host defense mechanisms as we have recently found TNF- α -deficient mice are more susceptible to flu-induced immunopathology (unpublished data). At the current time, the mechanisms by which flu infection alters NK cell responses to bacterial superinfection still remain incompletely understood. Although we have obtained the evidence in our current study that downregulation of IL-15 responses in the lung represents one mechanism, it remains to be elucidated how flu infection affects IL-15 expression in macrophages. The recent study by Didierlaurent et al. suggests that influenza infection history is able to imprint on innate cells, such as macrophages, in the lung rendering them insensitive to bacterial ligand stimulation perhaps in an attempt to limit the level of tissue immunopathology (20). Our results strongly suggest that flu infection may also desensitize NK cells and that the desensitized/reduced NK cells may be an important mechanism leading to subsequent macrophage desensitization. Indeed, during the resolution phase of an infection, such as flu infection, NK cells are believed to be suppressed or rendered capable of inhibitory activities to limit immunopathology in the lung (13, 33, 40). This belief may explain why as we observed, “flu-imprinted” NK cells inhibited the macrophage phagocytosis of *S. aureus* in a cell-to-cell contact-dependent manner. But apparently, what is initially a “good” intent weakens the preparedness of the flu virus-experienced lung to cope with a secondary bacterial hit. Our current study indicates that the outcome of the lack of such preparedness is increased susceptibility and lung immunopathology after bacterial superinfection.

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Disclosures

The authors have no financial conflicts of interest.

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