Type I Interferon Induction during Influenza Virus Infection Increases Susceptibility to Secondary Streptococcus pneumoniae Infection by Negative Regulation of γδ T Cells

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The majority of deaths following influenza virus infection result from secondary bacterial superinfection, most commonly caused by Streptococcus pneumoniae. Several models have been proposed to explain how primary respiratory viral infections exacerbate secondary bacterial disease, but the mechanistic explanations have been contradictory. In this study, mice were infected with S. pneumoniae at different days after primary influenza A (X31) virus infection. Our findings show that the induction of type I interferons (IFNs) during a primary nonlethal influenza virus infection is sufficient to promote a deadly S. pneumoniae secondary infection. Moreover, mice deficient in type I interferon receptor (IFNAR knockout [KO] mice) effectively cleared the secondary bacterial infection from their lungs, increased the recruitment of neutrophils, and demonstrated an enhanced innate expression of interleukin-17 (IL-17) relative to wild-type (WT) mice. Lung γδ T cells were responsible for almost all IL-17 production, and their function is compromised during secondary S. pneumoniae infection of WT but not IFNAR KO mice. Adoptive transfer of γδ T cells from IFNAR KO mice reduced the susceptibility to secondary S. pneumoniae infection in the lung of WT mice. Altogether, our study highlights the importance of type I interferon as a key master regulator that is exploited by opportunistic pathogens such as S. pneumoniae. Our findings may be utilized to design effective preventive and therapeutic strategies that may be beneficial for coinfect patients during influenza epidemics.

Clinical data have shown that the majority of deaths following influenza virus infection are due to secondary bacterial pneumonia superinfection during influenza pandemics (21). More than 90% of lung culture samples from autopsy records during 1918 pandemics were positive for bacteria, including Streptococcus pneumoniae, Staphylococcus aureus, and Haemophilus influenzae. Streptococcus was the most common pathogen and was responsible for over 50% of the deaths (27). Recently, similar findings were reported during the H1N1 influenza virus outbreak of 2009, with 30% of fatal cases attributed to combined influenza virus and bacterial infection (9). It is of urgent clinical importance to understand the mechanism for the increased susceptibility to secondary bacterial infection in light of the risk of influenza pandemics and the increasing prevalence of bacterial antibiotic resistance.

There have been several models proposed to explain the associations between primary viral and secondary bacterial infection, including an increased bacterial adherence to the respiratory tract, a dysregulated cytokine response, and impaired leukocyte migration after viral infection (3, 20, 22, 39, 41, 42, 44, 49, 50). Two innate cytokines have been shown to play crucial roles in exacerbating secondary bacterial infection following influenza virus infection: early type I interferon (IFN) and late gamma interferon (IFN-γ) produced by effector T cells. IFN-γ was shown to block the phagocytosis of S. pneumoniae by alveolar macrophages (44). Since intranasal inoculation of bacteria 3 days after virus infection is sufficient to cause severe superinfection (23) and T cell-produced IFN-γ is not produced until at least day 5, an alternative mechanism operating independently of IFN-γ must exist. Type I IFN was reported to account for defective neutrophil recruitment and bacterial killing during superinfection at day 5 after influenza virus infection; however, the detailed pathway underlying these defects was not ascertained (39).

Type I IFNs are indispensable mediators of the antiviral defense that induce the expression of proinflammatory cytokines and chemokines that both inhibit virus replication and boost T cell and B cell immunity (14, 24, 33). However, type I IFN complicates defense against a broad list of bacterial pathogens. Indeed, wild-type (WT) mice are more susceptible to Francisella tularensis and Listeria monocytogenes infection than IFN-α receptor knockout (IFNAR KO) mice (1, 8, 16, 32). The enhanced pathogenicity has been ascribed to a unique ability of certain bacterial strains such as Listeria and Mycobacteria to induce type I IFN mimicking a virus infection (37, 46, 48). Important targets of IFNAR signaling are innate cells producing the cytokine interleukin 17 (IL-17) that is known to participate in bacterial clearance. For instance, IFNAR KO mice are more resistant to F. tularensis infection, with a significant increase in IL-17 production by proliferating γδ T cells (16).

γδ T cells are specialized innate T cells that signal through a T cell receptor (TCR) formed by TCR-γ and TCR-δ chains (31, 45). In secondary and tertiary lymphoid organs, γδ T cells are able to respond directly to pathogen-associated molecular patterns (PAMPs) and cytokines in the absence of cognate TCR ligands. They kill the infected cells through pathways such as Fas or TRAIL and contribute to pathogen clearance by the release of cytotoxic and/or antimicrobial molecules (12, 13, 35). γδ T cells are rapidly activated, producing IFN-γ, tumor necrosis factor alpha (TNF-α), and IL-17 in several mouse models of bacterial infection with...
Mycobacterium bovis, Listeria monocytogenes, and Salmonella enterica (15, 38, 47).

While a role for γδ T cells in the response to primary bacterial infection has been documented, their contribution to recovery from superinfection following influenza virus infection has not been studied. Previous studies have shown that influenza virus inhibits type I IFN production in vivo and delays the initiation of lung inflammation until almost 2 days postinfection (dpi) in mice (25, 46). In the current study, we utilized a mouse model of superinfection and show that the increased susceptibility to S. pneumoniae infection is observed only after the delayed onset of the inflammatory burst and the release of type I IFN. In the absence of IFN signaling, mice showed resistance to superinfection. γδ T cells were pivotal in the observed resistance, and their IL-17 production was approximately 200-fold increased relative to wild-type (WT) mice. Altogether, these findings suggest novel directions for the design of preventative and therapeutic strategies to ameliorate secondary bacterial pneumonia associated with influenza virus infection.

MATERIALS AND METHODS

Mice. Eight-week-old female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Age- and sex-matched type I IFN-α receptor knockout (IFNAR KO) mice raised on a C57BL/6 background (a kind of gift from C. B. Wilson, Department of Immunology, Washington University) and type I and type III IFN receptor knockout (IFNOR KO) mice (Zymogenetics, Seattle, WA) were bred in the barrier facilities at Mount Sinai School of Medicine. Animals were housed in specific-pathogen-free facilities. All animal work was conducted in agreement with approved protocols by the Institutional Animal Care and Use Committee (IACUC) at the Mount Sinai School of Medicine (protocol numbers 96-301, 08-0951) and in accordance with guidelines in the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health (29). The program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

Infectious reagents and mice infection. Influenza virus A/H3N2 was grown in 10-day embryonated eggs (Spafas; Charles Rivers). Type 3 Streptococcus pneumoniae (ATCC 6303 clinical isolate with capsular serotype 3) was cultured in soy agar plate with 5% sheep blood (BD, Franklin Lakes, NJ). The colonies were picked and grown in Todd-Hewitt broth in 0.1% polysorbate 80 (Remel, Lenexa, KS) at 37°C in 5% CO₂ and protected mice from superinfection. Altogether, γδ T cells were pivotal in the observed resistance, and their IL-17 production was approximately 200-fold increased relative to wild-type (WT) mice. Altogether, these findings suggest novel directions for the design of preventative and therapeutic strategies to ameliorate secondary bacterial pneumonia associated with influenza virus infection.

Quantification of influenza virus or S. pneumoniae bacteria in the lung. Plaque assays for viral titration were performed as follows. Twenty-four-well tissue culture plates were seeded with a confluent monolayer of MDCK cells. The plates were incubated for 1 h with 200 μl of infected- lung homogenates (10-fold serial dilutions). After infection medium was removed, 500 μl of Dulbecco’s modified Eagle’s medium (DMEM)-F12 overlay containing 0.6% agar (Oxoid) and 1 μg/ml of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington) was added to the wells. Infected monolayers were incubated for 40 h at 37°C in 5% CO₂ and then fixed with 4% paraformaldehyde for 1 h. Agar overlays were then removed gently, and the fixed monolayers were stained with 0.1% crystal violet for 10 min. The numbers of influenza virus plaques were counted. Streptococcus was determined in the whole lung by plat- ing 10-fold serial dilutions of tissue homogenates on sheep blood-agar plates. After a 14- to 16-h incubation at 37°C in 5% CO₂, colonies were counted, calculated, and presented as the number of CFU per lung.

Pulmonary histopathology and cell isolation from whole lung. Mouse lungs were carefully removed at various time points postinfection (p.i.) and fixed in 10% buffered formalin. The fixed lungs were then sectioned in 6-μm slices and stained with hematoxylin and eosin (H&E). Whole lung from infected mice was perfused with PBS–2 mM EDTA to eliminate excess blood and dissociated in a C tube (Miltenyi Biotech, CA) in DMEM–1% fetal bovine serum (FBS) supplemented with 0.2 mg/ml Liberase (Roche, Almere, Netherlands) for 30 min at 37°C; the dissociated cell suspension was then passed through a 70-μm pore-size strainer (BD Biosciences, NJ). Single-cell suspensions were treated with red blood cell lysis buffer (BD Biosciences, NJ). γδ T cells were purified by using a mouse TCR γδ T Cell Isolation Kit (Miltenyi Biotech, CA). The antibodies anti-mouse CD49b, CD11b, and CD4 conjugated to biotin (Biolegend, CA) were used to bind NK cells, CD11b⁺ cells, and CD4⁺ T cells, and then cell populations were purified following the manufacturer’s instructions for antibiotin magnetic microbeads (Miltenyi Biotech, CA).

Quantitative real-time RT-PCR. Lungs from infected mice were homogenized in 3 ml/sample of TRIzol reagent (Invitrogen, CA). RNA was extracted as indicated by the manufacturer. Total mRNA was converted to cDNA by reverse transcription-PCR (RT-PCR) using an oligo(dT) reaction (Roche). Quantitative PCRs (qPCRs) based on SYBR green detection were performed using LightCycler equipment (Roche). All reactions were normalized to those of Rsp11 and α-tubulin.

The following primers were used: Il-1β forward, 5'-AGGAAGACCT CAGCCATG-3' ; Il-1β reverse, 5'-CGTGGTTGTGAATGACTAC-3' ; Il-6 forward, 5'-ACAGAAGGATGGTCAAGGA-3' ; Il-6 reverse, 5'-CG CAGTACGTGTGCGGAGTA-3' ; Tnf-a forward, 5'-TCAAGGAGCC TCAAGGCTTCATGATG-3' ; Tnf-a reverse, 5'-GTGAGGAAGGGTGCTTCAGCTTGG-3' ; Kc forward, 5'-GCAACCAAACCGAAGTCA-3' ; Kc reverse, 5'-CTTGG GGGAGCCTTTCATTTA-3' ; Mip2 forward, 5'-CAGCCCACTCCACGCTG TAG-3' ; Mip2 reverse, 5'-AGGGTCTCTCGAGGATGAC-3' ; Gsf forward, 5'-CCCCGAAGTCTTCTCCATTA-3' ; Gsf reverse, 5'-CGAGTCTTG AGTGGGCACACA-3' ; Mx1 forward, 5'-CAACGTGAATCTCCTCGGA A-3' ; Mx1 reverse, 5'-GGCTGCTCTCCACGAAGTCA-3' ; Isg15 forward, 5'-CGGAGTACGCTCAGCAAC-3' ; Isg15 reverse, 5'-CTTCTGGGC AACTCTGCTTCT-3' ; Ifna1 forward, 5'-AGCCTTGGACATCCTGGTAC-3' ; Ifna1 reverse, 5'-IFNATA-3' ; Mx1 forward, 5'-TCAGTCTTGCGGCTGCAAT-3' ; Mx1 reverse, 5'-IFNATA-3' ; Tnf-a forward, 5'-CCGGCAGGCTTCATATAGT-3' ; Tnf-a reverse, 5'-CTCGCTGGGTTTGTAAGTTCACAC-3' ; A-tubulin forward, 5'-CTGAGTGTCTGCA-3' ; A-tubulin reverse, 5'-CCAGTCCCAG-3' -TCAACCGTTCCACGTC-3' ; Rsp11 forward, 5'-CTGGGTTTGTAAGTTCACAC-3' ; Rsp11 reverse, 5'-AGCCTTGGACATCCTGGTAC-3'.

Flow cytometry. For intracellular staining, single-cell suspensions were incubated for 6 h at 37°C in 5% CO₂ in RPMI medium–10% FBS supplemented with brefeldin A (Sigma, St. Louis, MO) at 5 μg/ml. Cells were harvested and incubated for 30 min on ice with anti-mouse TCR-γδ.
RESULTS

Influenza virus infection increased the susceptibility to the *S. pneumoniae* superinfection. To determine the effects of prior influenza virus infection on host defense against a subsequent *S. pneumoniae* infection in the lung, C57BL/6 mice were infected by aerosol with influenza A/X31 virus (H3N2) at a sublethal dose.

Influenza A/X31 virus is cleared by mice by day 8 after infection (4). Clinically, most secondary bacterial infections in humans occur within the first 2 weeks after primary influenza virus infection (5, 6, 11). The earliest time point reported for increased susceptibility to secondary bacterial challenge after primary influenza virus infection was day 3 (23). Therefore, in our primary experiments mice were challenged intranasally with *Streptococcus pneumoniae* at the dose of $4 \times 10^5$ to $2 \times 10^6$ CFU/mouse at day 4 after influenza virus infection. Mice infected with combined influenza virus and bacteria showed higher mortality and increased *S. pneumoniae* replication in their lungs than mice infected with bacteria alone (Fig. 1A and B). At 84 h after *S. pneumoniae* infection, 100% of mice in the influenza virus plus *S. pneumoniae* group died, whereas 75% of mice receiving *S. pneumoniae* alone survived. Bacteria in the lungs of mice infected with influenza virus followed by *S. pneumoniae* infection continued to replicate until mouse death/euthanasia. In contrast, peak titers were reached at 36 h in animals receiving bacteria alone, after which the bacteria were rapidly cleared. Strikingly, secondary bacterial infection influenced X31 virus clearance at both a high ($2 \times 10^5$ CFU/mouse) and a low ($4 \times 10^3$ CFU/mouse) bacterial inoculum (Fig. 1C). Indeed, 60 h after *S. pneumoniae* administration, mice had higher viral lung titers while titers for mice infected with X31 alone were declining rapidly, and by 84 h no virus could be detected.

Influenza virus infection increases the pathological effects of *S. pneumoniae* in the lungs. Consistent with the degree of illness and the increasing bacterial/virus titers, the mice superinfected with influenza virus plus *S. pneumoniae* displayed more severe...
pathology in their lungs than the mice infected with S. pneumoniae or influenza virus alone (Fig. 1D). In addition to the significant epithelium damage and hyperplasia, at 12 h following superinfection, a massive mononuclear cell infiltration was observed below the epithelium with cell sloughing and hyperplasia. Furthermore, these mice had airways heavily congested with apoptotic neutrophils, mucus, and cell debris at 72 h postsuperinfection. S. pneumoniae infection alone caused a neutrophilic response in the lung that could be observed as early as 12 h p.i. and consisted of predominantly mononuclear cells 72 h after S. pneumoniae inoculation. Only a minimal inflammatory response with some residual mononuclear cell infiltration was seen in the section from X31-infected mice. Altogether, these data show that prior influenza virus infection predisposes to bacterial infection and results in an exacerbated and lethal lung immunopathology.

IL-17 production triggered by bacterial infection is inhibited in superinfected mice. Diverse cytokines and chemokines are released in lungs during the response to microbial invasion (7), which contributed to initiation of the acute-phase response and the recruitment of effector cells with the concomitant clearance of the pathogen. In order to identify changes to the lung cytokine milieu that may account for the enhanced bacterial replication, cytokines/chemokines/growth factors were measured from lung homogenates of mice infected with virus alone, bacteria alone, or influenza virus plus bacteria. The results show that by 24 h after bacterial inoculation, there is a significant increase in inflammatory gene transcription of IL-1, IL-6, TNF-α, keratinocyte-derived chemokine (KC), macrophage inflammatory protein 2 (MIP-2), and granulocyte colony-stimulating factor (G-CSF) in superinfected mice relative to mice given virus or bacteria alone (Fig. 2B). During the same period, bacterial replication in the lungs was significantly higher in this group than in mice receiving S. pneumoniae alone, and this may account for the increased response (Fig. 2A). In contrast, type I IFN (IFN-α and IFN-β) levels are identical in coinfected and virus-infected mice, indicating that bacteria neither inhibits nor enhances the production of type I IFNs (Fig. 2C). Strikingly, even though bacterial replication is much higher in superinfected mice, secretion of IL-17 is significantly reduced in superinfected mice compared to levels in mice infected with S. pneumoniae alone (Fig. 2C), indicating that IL-17 is negatively regulated by prior influenza virus infection.

The initiation of influenza virus-mediated lung inflammation correlates with enhanced bacterial growth. In previous studies we observed that the initiation of inflammation after aerosol virus infection is delayed for 40 to 48 h (stealth phase), after which a rapid release of immunologically active proteins occurs (25, 46). We hypothesized that if the increased permissiveness for bacterial growth in superinfected mice was dependent upon the innate response to the virus, enhanced bacterial replication should be more pronounced when bacteria are inoculated at least 2 days after virus infection. Animals were inoculated with S. pneumoniae alone on the day of virus X31 infection (Fig. 3, Flu + S.p, 0d), 1 day after X31 infection (Fig. 3, Flu + S.p, 1d), or 2 days after X31 infection (Fig. 3, Flu + S.p, 2d). Lungs were collected at 4, 24, and 48 h after S. pneumoniae inoculations, and the induction of cytokines and replication of bacteria were measured at all time points. As
expected, IFN-β message was observed only in lungs collected at least 48 h after viral infection and was not present in animals given bacteria alone (Fig. 3A and B). Enhanced bacterial replication was observed only in animals infected with X31 at least 2 days earlier and correlated perfectly with the release of IFN-β (Fig. 3A, Flu + S. p. 2d). Twenty-four hours after S. pneumoniae inoculation, the production of IFN-β was detected in animals superinfected 1 or 2 days after virus infection (Fig. 3B, Flu + S. p. 1d and Flu + S. p. 2d). After another 24 h, in the same mouse groups substantially enhanced bacterial replication was observed. At both time points enhanced bacterial growth also correlated with induction of the interferon-inducible genes MX1 and ISG15 (Fig. 3C) and was different from the kinetics shown by KC after superinfection. Therefore, in influenza virus-infected hosts, the presence of type I IFNs correlated with the antimicrobial response, leading to increased susceptibility to secondary bacterial infection.

Enhanced bacterial replication does not occur in superinfected mice lacking IFN signaling. The coincident timing between the appearance of type I IFN and enhanced bacterial replication suggested that this cytokine profoundly alters the host antibacterial defense. To test this hypothesis, wild-type (WT) mice or mice lacking type I IFN receptors (IFNAR KO) or both type I and type III IFN receptors (IFNAR KO) were inoculated with S. pneumoniae 4 days after X31 infection. Type III IFN contributes to protection of mice infected with influenza A virus (18, 26, 40). Strikingly, in the absence of IFN receptors, no enhancement of bacterial growth was observed. At 24 h following S. pneumoniae infection, lungs of IFNAR KO mice coinfected with influenza virus and S. pneumoniae contained approximately 2 logs fewer CFU of S. pneumoniae than WT mice. Moreover, the bacterial titers were undetectable in the lungs of superinfected type I and III IFN KO mice. The bacterial clearance rates in animals given S. pneumoniae alone were not significantly different between WT and IFN receptor-deficient mice (Fig. 4A and B). Hence, the presence of type I and type III IFN signaling did not affect the bacterial clearance in naïve mice but markedly increased susceptibility to secondary bacterial infection following influenza virus infection.

IL-17 release and neutrophil migration to the lungs is reduced by IFN signaling. Neutrophil migration and associated cytokine levels in lungs from IFNAR KO and WT mice were measured after superinfection (Fig. 4C). Among the groups infected with both influenza virus and S. pneumoniae, IFN receptor-deficient mice showed increased neutrophil recruitment relative to their WT counterparts. Enhanced G-CSF and MIP-2 transcription was observed in superinfected WT mice and was partially dependent upon IFN signaling, whereas KC message levels were identical in both groups. Importantly, both IL-17A and IL-17F were negatively regulated by type I IFN signaling in the lungs (Fig. 4D). IL-17A and IL-17F genes showed significantly higher transcription levels in IFNAR KO mice infected with influenza virus and S. pneumoniae than in their WT counterparts. No difference was seen between IFNAR KO and WT mice infected with S. pneumoniae alone or influenza virus alone or mock infected. Thus, prior influenza virus infection induced type I IFNs that inhibited the recruitment of neutrophils and expression of IL-17, which correlated with the host’s increased susceptibility to secondary bacterial infection.

Adoptive transfer of IFNAR KO γδ T cells inhibits bacterial replication in superinfected WT mice. In some bacterial and viral infection models, IL-17R signaling had been reported to regulate neutrophil migration and be suppressed by type I IFNs (10, 19, 51). Furthermore, in multiple in vivo or in vitro models, IL-17 has been reported to come from CD4 cells, NK cells, γδ T cells, and neutrophils (36). We characterized the source of IL-17 in the superinfection model by isolating different cell populations from the lungs of mice following superinfection or infection with virus or bacteria alone. IL-17 gene transcription was measured from each cell population. The isolated γδ T cells from animals infected with S. pneumoniae showed the highest level of expression of both the
IL-17A and IL-17F genes, but these levels were significantly reduced in superinfected mice (Fig. 5A and B).

The primary source of the rapid production of IL-17 in our model is γδ T cells. Hypothetically, the adoptive transfer of IFNAR KO γδ T cells that are insensitive to type I IFN should restore antibacterial defense in WT mice infected with both influenza virus and S. pneumoniae. To address this, γδ T cells from IFNAR KO or WT mice were transferred intratracheally into WT mice coinfected with influenza virus and S. pneumoniae. Bacterial replication in the lungs was measured 20 h later (Fig. 5C). Animals receiving no cells or WT γδ T cells allowed rapid replication of bacteria. In contrast, superinfected animals receiving γδ T cells from IFNAR KO mice demonstrated an enhanced ability to clear the bacteria (Fig. 5D). These data argue that γδ T cells are crucial for clearing bacteria during superinfection and that type I IFN production impairs/alters their function.

DISCUSSION

Secondary bacterial infection is the most common complication that occurs during influenza pandemics. Studies of the 2009 H1N1 pandemic have shown the presence of secondary S. pneumoniae infections in more than 20% of the fatal cases (9). The observation that prior influenza virus infection increased susceptibility to S. pneumoniae was reported many years ago, and there have been several models proposed to explain the associations between primary viral infection and secondary bacterial infection (3). One theory proposes that the respiratory epithelial surface is damaged during influenza virus infection and that the exposure of new binding partners, such as platelet-activating factor receptor, enhanced bacterial adherence and growth in the respiratory tract (2, 22, 34, 50). Another theory suggested that the production of a variety of different cellular mediators during influenza virus infection affected the recruitment and function of effector leukocytes, such as neutrophils, in response to a secondary bacterial infection (20, 23, 39, 41–44).

Recent work from our laboratory utilized an aerosol model of influenza virus infection to describe the timing in the initiation of the lung inflammatory response. Influenza virus effectively blocks innate immunity for approximately 48 h, after which an inflammatory breakthrough occurs, culminating in a massive release of cytokines in the lungs and into the blood (25). The systemic release of cytokines impacts cells throughout the body, including the bone marrow, which quickly display an “interferon signature” indicating distal type I interferon signaling (17). In the current study, we used aerosol infection with influenza virus (A/X31) at a nonlethal dose and attempted to determine the earliest time point for increased susceptibility to bacterial superinfection. Our results shown in Fig. 3 demonstrated that only following the inflammatory burst at day 2 did the animals become susceptible to enhanced bacterial replication. Mice inoculated with S. pneumoniae at day 1...
after influenza virus infection cleared bacteria as efficiently as animals given *S. pneumoniae* alone, but after an additional 24 h mice became fully susceptible to superinfection. These data argued that the inflammatory burst in response to influenza virus infection, characterized by the dominant antiviral cytokine, type I IFN, was responsible for altering innate immunity in such a way that bacterial replication was enhanced. The period of susceptibility to superinfection has been described to be broad, ranging from a few days after influenza virus infection up to several weeks. Our data are consistent with previous reports that showed enhanced superinfection as early as 3 days after influenza virus infection (20, 23, 39, 42, 44, 50). Additionally, when we infected mice with *S. pneumoniae* at day 7 after influenza virus infection, we found that mice became more susceptible to bacteria and survived less well than the mice infected with *S. pneumoniae* at day 4 and had higher levels of type I IFN (our unpublished data). In light of the other study showing the inhibition of antibacterial defense by IFN-γ at day 7 after viral infection (44), further studies are required to determine the relationship between type I IFN and IFN-γ and whether they play nonredundant roles in the pathogenesis of superinfection.

Other studies arguing for type I IFN playing an important role in superinfection suggested a mechanism that contrasted with our observations (28, 39). Nakamura et al. argued that type I IFNs from mice coincidently infected with influenza virus and *S. pneumoniae* decreased the production of the chemokine CCL2 and impaired the recruitment of macrophages, leading to the bacterial colonization in the upper respiratory tract (28). In the other study, Shahangian et al. reported that type I IFNs functioned in part through selective impairment of KC and MIP-2 production, attenuating the neutrophil responses following secondary bacterial challenge *in vivo* (39). In our study, neither KC nor MIP-2 production correlated with exacerbated infection, nor did we observe any alterations in macrophage numbers or recruitment (data not shown).

A central and synergistic role for type I and type III IFNs was confirmed in our studies. Mice deficient in both type I and III IFN receptors cleared bacteria as well as animals not infected with virus, whereas IFNAR KO mice had an intermediate phenotype (Fig. 4). Studies current in our lab are investigating the synergy between type III and type I IFNs in superinfection resistance in mice.

Cytokine profiling identified a number of differences in lungs of animals infected with influenza virus or *S. pneumoniae* or superinfected. In our study, type I interferon production was exclusively triggered by virus infection, and *S. pneumoniae* was incapable of inducing this cytokine to detectable levels. Strikingly, in contrast to the high levels of other cytokines such as IL-1β, IL-6, TNF-α, KC, MIP-2, and G-CSF observed in superinfected animals, the significant cytokine triggered by bacterial infection alone was IL-17, which was reduced in superinfected mice (Fig. 2). Recently another study (30) reported a similar observation in mice infected with vesicular stomatitis virus (VSV) and the intracellular bacteria *L. monocytogenes*. The coinfectcd animals had significantly lower expression of IL-17A mRNA than mice infected with *L. monocytogenes* alone. Using this system, the authors argue that the RIG-I-like receptor signaling during superinfection interfered with Toll-like receptor (TLR)-induced IL-17 production. In our model, compared to WT mice, IL-17 production was increased in superinfected IFNAR KO mice. The decreasing production of MIP-2/G-CSF in superinfected IFNAR KO mice suggested that these cytokines were correlated with bacterial replication rather than neutrophil recruitment to the lungs (Fig. 4).

It was previously reported that type I IFNs inhibit Th17-mediated host defense against bacterial pneumonia, but at that time the cell type producing this cytokine was not identified (19). IL-17A plays a central role in autoimmune inflammation and in innate immunity to bacterial pathogens, such as cutaneous *Staphylococcus aureus*, *Francisella tularensis*, *Mycobacterium bovis* BCG, *L.
monocytes, and Salmonella enterica infection (10, 51). Many cell populations including CD4 T cells, γδ T cells, NK cells, and neutrophils have been shown to produce IL-17 (36). IL-17 production occurs rapidly after bacterial inoculation but is suppressed in the presence of IFN. CD4 T cells specific for bacterial proteins are unlikely to be present at the early time points after inoculation analyzed in this study. Although NK cells also showed suppressed production of IL-17 in superinfected mice, the levels of suppression were much lower than observed with the equivalent numbers γδ T cells. Our results showed that there was higher IL-17 gene transcription detected in γδ T cells than in the other cell populations during Streptococcus pneumoniae infection. Indeed, γδ T cells were identified as the major cell subset producing IL-17 in vivo in response to several bacterial infections (e.g., epidermal Vγ5 γδ T cells in S. aureus infection) (10). Further, RORγt was shown as a key transcription regulator in CD27− γδ T cells to modulate IL-17A/IL-17F production, and it would not be surprising that its activity was affected by type I IFN (16). We report here that type I IFNs produced during viral infection lead to a decrease in IL-17 expression by γδ T cells and a concomitant reduction in neutrophil recruitment to the lungs. Type I IFNs induced by prior influenza virus infection inhibited the IL-17 production of γδ T cells in locally infected sites, and IL-17 was required to regulate the recruitment of neutrophils from bone marrow. The γδ T cell number did not vary in superinfected type I IFNR KO mice and their WT counterparts (data not shown), which implied that survival of γδ T cells was not compromised but that their function as IL-17 producers was strictly regulated by IFN signaling. The adoptive transfer of γδ T cells from IFNAR KO mice to influenza virus-infected WT mice helped the infected host clear the bacteria particles from the body although the number of adoptive γδ T cells was higher than that of resident γδ T cells (4 × 10^3 to 7 × 10^3 cells/lung) in the lung. These observations confirm a critical role for γδ T cells in promoting immunity against Streptococcus pneumoniae but leaves open the possibility that other IL-17-producing cell populations may also contribute to bacterial clearance. Although several mechanisms might contribute in aggregate to virus-induced impairment in antibacterial immune responses, our data identify a novel mechanism for the enhancement of bacterial growth mediated by type I IFN suppression of IL-17 production in animals with sublethal virus infections. Administration of recombinant IL-17 (rIL-17) to superinfected mice improved bacterial growth mediated by type I IFN suppression of IL-17 production in animals with sublethal virus infections. Administration of recombinant IL-17 (rIL-17) to superinfected mice improved bacterial clearance in mouse lungs, demonstrating that IL-17 was sufficient to restore the attenuated antibacterial defense in the host (unpublished data). These findings suggest that immunomodulatory or vaccine therapy aimed at inducing IL-17 might provide a novel preventive and therapeutic strategy for superinfected patients.

Collectively, our results shed new light on the role of γδ T cells during secondary bacterial infection after influenza virus infection. The regulation of the IL-17-producing γδ T cell population is partially responsible for the increased susceptibility to secondary bacterial infection. We utilized type I IFNR KO mice and type I and III IFNR KO mice to determine that antiviral IFNs were the upstream modulators that suppress the production of IL-17 from γδ T cells and inhibit the recruitment of neutrophils to the lungs. In addition, we delineated the earliest susceptible day for secondary bacterial infection in influenza virus-infected mice. A better understanding of how this regulation works will lead to the development of useful treatments for severe influenza virus infection with suspected bacterial superinfection.

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