HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2021 September 15.

Published in final edited form as:

J Immunol. 2020 September 15; 205(6): 1601–1607. doi:10.4049/jimmunol.2000094.

Influenza infection induces alveolar macrophage dysfunction and thereby enables noninvasive *Streptococcus pneumoniae* to cause deadly pneumonia

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Abstract

Secondary *S. pneumoniae* infection is a significant cause of morbidity and mortality during influenza epidemics and pandemics. Multiple pathogenic mechanisms such as lung epithelial damage, and dysregulation of neutrophils and alveolar macrophages (AMs), have been suggested to contribute to the severity of disease. However, the fundamental reasons for influenza-induced susceptibility to secondary bacterial pneumonia remain unclear. Here we revisited these controversies over key pathogenic mechanisms in a lethal model of secondary bacterial pneumonia, with a *S. pneumoniae* strain that is innocuous to mice in the absence of influenza infection. Using a series of *in vivo* models, we demonstrate that rather than a systemic suppression of immune responses or neutrophil function, influenza infection activates IFN-γ receptor (IFN-γR) signaling and abrogates alveolar macrophage (AM)-dependent bacteria clearance, and thereby causes extreme susceptibility to pneumococcal infection. Importantly, using mice carrying conditional knockout of *Ifngr1* gene in different myeloid cell subsets, we demonstrate that influenza-induced IFN-γR signaling in AMs impairs their antibacterial function, thereby enabling otherwise noninvasive *S. pneumoniae* to cause deadly pneumonia.

Introduction

Bacterial pneumonia after influenza is a leading cause of severe respiratory infections worldwide. In particular, secondary *S. pneumoniae* infection is a significant cause of morbidity and mortality during influenza epidemics and pandemics (1–4). However, the key mechanism underlying this devastating disease remains incompletely understood.

Multiple studies have suggested that lung epithelial damage, directly or indirectly caused by influenza infection, promotes bacterial invasion and systemic inflammation, thereby leading to excessive mortality after secondary bacterial infection (2, 5–8). To evaluate this possibility, we have recently developed a secondary bacterial infection model using *S. pneumoniae* TJO983, a serotype 14 strain that is innocuous to mice even after systemic challenge (9). Considering the high prevalence of “noninvasive” *S. pneumoniae* serotypes in

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human nasal carriage (10), we believe that this model is highly clinical-relevant. Importantly, this normally innocuous *S. pneumoniae* strain allows us to discern the local and systemic impact of influenza infection on innate antibacterial defense.

Defective bacterial control has recently been demonstrated as a primary cause of influenza-induced susceptibility to secondary bacterial infection. Many studies have suggested neutrophil dysregulation as a critical mechanism underlying defective antibacterial immunity (11–13). Conversely, we and others have shown that influenza inhibits alveolar macrophage (AM) responsiveness, by either functional impairment or direct depletion, thereby increasing host susceptibility to secondary bacterial pneumonia (14–19). Given these apparently disparate observations, it remains to be established the impact of influenza infection on neutrophil versus AM-mediated bacterial control, and their relative contribution to severe outcomes after secondary *S. pneumoniae* infection.

In this study, we revisited these controversies in our new mouse models of influenza/*S. pneumoniae* coinfection. We demonstrate that rather than a general suppression of innate immune response or neutrophil recruitment, preceding influenza infection disrupts AM-mediated bacterial clearance in the airway, and thereby enables otherwise innocuous *S. pneumoniae* to cause deadly pneumonia. Furthermore, using a series of conditional knockout mouse models, we show that this functional impairment is mediated, at least partially, through influenza-induced IFN-γ receptor signaling in AMs.

Materials and Methods

Murine model of viral and bacterial infection

Specific pathogen-free, C57BL/6 WT, *Csf2rb*$^{-/-}$ (20), *Ifngr1*$^{-/-}$, *Ifngr1*$^{fl/fl}$ (21), *LysM*^{Cre} (22), *Cd11c*^{Cre} (23), *Cx3cr1*^{Cre} (24), *Mrp8*^{Cre} (25), *Rag1*$^{-/-}$, *Ifng*$^{-/-}$ and mT/mG mice (26), as well as BALB/c WT, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, and *Csf2/Il3*$^{tm1.1(CSF2\ IL3)Flv}$ (referred to as *hIl3/Csf2* KI) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred at University of Nebraska Medical Center following IACUC guidelines. CD169-DTR mice were provided by the RIKEN BRC through the National Bio-Resource Project of the Mext, Japan, with approval from Drs. Kenji Kohno and Masato Tanaka. *LysM*^{Cre}, *Cd11c*^{Cre}, *Cx3cr1*^{Cre}, and *Mrp8*^{Cre} reporter mice were generated by crossing mT/mG mice with corresponding Cre^{+} mice. *LysM*^{Cre}*Ifngr1*$^{fl/fl}$, *Cd11c*^{Cre}*Ifngr1*$^{fl/fl}$, *Cx3cr1*^{Cre}*Ifngr1*$^{fl/fl}$, and *Mrp8*^{Cre}*Ifngr1*$^{fl/fl}$ mice were generated by crossing *Ifngr1*$^{fl/fl}$ mice with corresponding Cre^{+} mice. All animal experiments were approved by University of Nebraska Medical Center, and all experiments were carried out in accordance with University of Nebraska Medical Center Assurance of Compliance with PHS Policy on humane Care and Use of Laboratory Animals, which is on file with the Office of Protection from Research Risks, NIH.

Viral challenge was performed with a sublethal dose of PR8 (~10 PFU/mouse) administered i.n. to anesthetized, sex and age-matched adult mice in 50 μl of sterile PBS. Titers of virus stocks and viral levels in the bronchoalveolar lavage fluids (BALF) and lungs of infected mice were determined by plaque assays on MDCK cell monolayers.

To induce *S. pneumoniae* infection, anesthetized mice were inoculated i.n. or i.p. with 50~100 μl of PBS containing 2×10^4 CFU of serotype 14 strain TJO983 unless otherwise specified. Bacterial burdens in the BALF and lungs were measured by sacrificing infected mice 24 h after infection, and plating serial 10-fold dilutions of each sample onto blood agar plates.

Bronchoalveolar lavage (BAL) cell analysis

BALF samples were collected by making an incision in the trachea and lavaging the lung twice with 0.8 ml PBS, pH 7.4. Total leukocyte counts were determined using a hemacytometer.

For flow cytometry analysis, BALF were incubated with 2.4G2 mAb against FcγRII/III, and stained with APC conjugated anti-CD11c (Biolegend), BUV395-conjugated anti-CD11b (BD Biosciences), FITC-conjugated or PE-Cy7-conjugated anti-Ly6G (clone 1A8, Biolegend), PerCP-Cy5.5-conjugated (eBiosciences) or PE-conjugated anti-Ly6C (BD Biosciences), and BV421-conjugated or PE-conjugated anti-Siglec-F (BD Biosciences) mAbs. The stained cells were analyzed on a BD LSRII-green using BD FACSDiva and FlowJo software analysis.

Determination of cytokine/chemokine production by ELISA

BALF were harvested and assayed for TNF-α and IFN-γ by ELISA using commercially available kits from BD Biosciences.

AM reconstitution in Csf2rb$^{-/-}$ mice

For isolation of AMs, BALF cells harvested from naïve WT mice were pelleted and resuspended in PBS for adoptive transfer. *Csf2rb*$^{-/-}$ recipient mice were anesthetized and $2\sim3\times10^5$ AMs were administered i.n. in 100 μl PBS. Mice were used for *S. pneumoniae* infection more than six weeks after receiving AM transfer. Reconstitution of AMs in the BAL was confirmed by flow cytometry.

Adoptive cell transfer to Rag1$^{-/-}$ mice

C57BL/6 *Rag1*$^{-/-}$ mice were injected i.p. with splenocytes (2×10^7 cells/mouse) isolated from naïve IFN-γ$^{-/-}$ or WT mice and infected with PR8 10 weeks after cell transfer. Influenza-induced airway recruitment of adoptively transferred T cells was confirmed by flow cytometric analysis.

Quantitative reverse transcription (RT)-PCR

AMs were purified using a Mouse CD11c Positive Selection Kit (STEMCELL Technologies). Total RNA derived from enriched AMs was characterized by using iScript Reverse Transcription and iTaqUniversal SYBR Green Supermix (Bio-Rad) on a Bio-Rad CFXConnet Real-Time system. *Hprt* specific primer sets were used to normalize the expression value for the genes of interest.

Diphtheria toxin (DT) treatment

Mice were i.p. injected with 50μg/kg body weight of DT (Sigma) four days before *S. pneumoniae* infection. The efficiency of AM depletion in CD169-DTR mice was confirmed by flow cytometry.

Neutrophil depletion

Neutrophils were depleted using anti-Gr-1 mAb RB6–8C5 (BioXCell). Specifically, starting at one day before bacterial infection, mice were injected i.p. with anti-Gr-1 mAb RB6–8C5 (0.1 mg/day) to deplete neutrophils or with rat IgG as a control. The efficiency of neutrophil depletion in bacterial-infected mice was confirmed by flow cytometry (27).

Statistics

Significant differences between experimental groups were determined using a two-tailed Student *t*-test (to compare two samples), an ANOVA analysis followed by Tukey's multiple comparisons test (to compare multiple samples) or Mann Whitney test (nonparametric test) in GraphPad Prism 6 (La Jolla, CA). Survival analyses were performed using the log-rank test. For all analyses, a P value <0.05 was considered to be significant.

Results

AMs and neutrophils are critical for innate defense against airway and systemic *S. pneumoniae* infection, respectively

As airway-resident macrophages, AMs are crucial for immune defense against many respiratory infections (17, 27–29). However, neutrophils are commonly considered to play a more important role in killing of extracellular bacteria such as *S. pneumoniae* (30–32). Accordingly, an essential role of AMs in pneumococcal clearance remains uncertain. In this study, we evaluated the relative contribution of AMs and neutrophils to bacterial control during a low dose of *S. pneumoniae* serotype 14 strain TJO983 (*Spn*) infection alone.

It is known that AMs require GM-CSF (also known as CSF2) signaling for their development (29). Indeed, AMs ($CD11c^+Siglec\text{-}F^+$) were completely absent in CSF2 receptor β chain knockout ($Csf2rb^{-/-}$) mice (Fig. 1A). To determine the role of AMs in bacterial clearance, we infected WT and $Csf2rb^{-/-}$ mice intranasally (i.n.) with 10^4 CFU/mouse of *Spn*; and 24 h later, we detected ~10^4-fold increased bacterial burdens in $Csf2rb^{-/-}$ lungs as compared with WT controls (Fig. 1B). Of particular interest, AM reconstitution in $Csf2rb^{-/-}$ mice, by intranasal transfer of AMs from naïve WT mice, was sufficient to restore bacterial clearance (Fig. 1A-B). In fact, there was no significant difference in lung bacterial burdens between WT and $Csf2rb^{-/-}$ mice after AM reconstitution (Fig. 1B). Together, these results demonstrate that AMs are not only critical but also sufficient for *Spn* clearance in the lower respiratory tract.

$Rag2^{-/-}Il2rg^{-/-}$ (also known as $Rag2^{-/-}\gamma c^{-/-}$) mice are deficient in T, B and innate lymphoid cells (33). Due to knock-in (KI) replacement of mouse GM-CSF in $Rag2^{-/-}Il2rg^{-/-}$ mice (34), human *Il3*/*Csf2* KI mice are also devoid of AMs (Fig. 1C). Similar to $Csf2rb^{-/-}$ mice, *hIl3*/*Csf2* KI mice exhibited 1000-fold increased bacterial burdens 24 h after *Spn*

infection alone, as compared with both BALB/c WT and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ controls (Fig. 1D). These results further confirm that AMs are essential and sufficient for innate clearance of airway *Spn* infection. On the other hand, we have shown that antibody-mediated depletion of neutrophils in WT mice has no effect on lung bacterial control (27). All WT mice survived i.n. *Spn* infection despite of neutrophil depletion (Fig. 1E). In contrast, neutrophil-depleted WT mice were highly susceptible to intraperitoneal (i.p.) *Spn* infection (Fig. 1E). Taken together, these results suggest that although dispensable for airway bacterial control, neutrophils are essential for innate defense against systemic *Spn* infection.

Influenza induces lethal susceptibility to airway but not systemic *S. pneumoniae* super-infection

Based on the differential role of AMs and neutrophils in innate protection (Fig. 1), we next examined the impact of influenza infection on the local and systemic antibacterial defense. In agreement with our previous findings (15), on day seven after a low dose of PR8 virus challenge, WT mice become highly susceptible to *Spn* respiratory infection (Fig. 2A). Specifically, all day seven PR8-infected mice succumbed to i.n. *Spn* super-infection (Fig. 2B). In sharp contrast, all PR8-infected WT mice survived from i.p. *Spn* super-infection at the same time (Fig. 2B). This result suggests that neutrophil-dependent systemic antibacterial defense is relatively competent after influenza infection. Importantly, the distinct outcomes of local versus systemic *Spn* super-infection suggest that, rather than directly disrupting epithelial barrier to promote systemic dissemination, influenza infection impairs airway antibacterial immunity to enable otherwise noninvasive *S. pneumoniae* to cause lethal pneumonia.

Preceding influenza infection abrogates AM-mediated bacterial clearance in the airway

Considering that systemic antibacterial defense is relatively competent, we focused on airway antibacterial immune response to understand how influenza infection induces local susceptibility to pneumococcal infection. Using *Csf2rb*$^{-/-}$ mouse model, we first examined the impact of influenza infection on AM-associated immune responses. Due to the lack of AMs (Fig. 1A), *Csf2rb*$^{-/-}$ mice exhibited ~4-log-fold increases in lung CFU as compared with WT controls during *Spn* single infection (Fig. 3A). Conversely, compared with their corresponding *Spn* single-infected controls, PR8/*Spn* coinfection resulted in ~10-fold but >4-log-fold increased bacterial burdens in *Csf2rb*$^{-/-}$ and WT mice, respectively. Of particular interest, lung bacterial burdens were almost comparable between *Spn* single-infected *Csf2rb*$^{-/-}$ mice and PR8/*Spn* coinfected WT mice (Fig. 3A). These results indicate that similar to *Csf2rb*$^{-/-}$ mice devoid of AMs, AM-mediated bacterial clearance is completely absent in WT mice one week after influenza infection.

Notably, in contrast to AM-deficient *Csf2rb*$^{-/-}$ mice, the actual numbers of AMs in WT mice were not significantly affected at this early stage of coinfection (Fig. 3B), even though their percentages in the bronchoalveolar lavage fluid (BALF) decreased due to intensive inflammatory cell infiltration (Supplemental Fig. S1). Consistent with increased airway Ly6C^{+} monocytes and Ly6G^{+} neutrophils (Fig. 3C), we detected significantly increased inflammatory cytokines, particularly TNF-α and IFN-γ production after PR8/*Spn* coinfection (Fig. 3D). Furthermore, *Csf2rb*$^{-/-}$ mice exhibited increased TNF-α production

and neutrophil recruitment after *Spn* single- or super-infection, as compared with corresponding WT controls (Fig. 3C-D). In line with other reported studies (29), we did not detect significant differences in viral burden between coinfected WT and *Csf2rb*$^{-/-}$ mice (Fig. 3E). Taken together, these data demonstrate that, rather than a general suppression of airway inflammatory response, influenza infection diminishes the antibacterial function of AMs, thereby leading to defective bacterial control during secondary pneumococcal infection.

A modest impairment of AM-independent bacterial clearance during influenza/*Spn* coinfection

Notably, compared with *Spn* single-infection, *Csf2rb*$^{-/-}$ mice exhibited 10-fold further increased bacterial burdens after PR8/*Spn* coinfection (Fig. 3A). This increased susceptibility suggests that influenza also impairs AM-independent bacterial clearance during secondary pneumococcal infection. However, considering that a similar difference (10-fold) in lung CFU was detected between coinfected WT and *Csf2rb*$^{-/-}$ mice, it is also possible that this increased susceptibility is due to other immune defects in *Csf2rb*$^{-/-}$ mice, such as dendritic cell (DC) development. To exclude these inherent deficits, we temporarily depleted CD169^{+} macrophages in CD169-diphtheria toxin receptor (DTR) transgenic mice (35, 36). Similar to the key findings in *Csf2rb*$^{-/-}$ mice, diphtheria toxin (DT)-induced AM depletion in CD169-DTR mice resulted in ~1000-fold bacterial outgrowth after *Spn* single infection (Supplemental Fig. S2A & Fig. 4A-B).

To minimize the impact on antiviral immune response (37), we next administrated DT to induce AM depletion three days after PR8 infection (Supplemental Fig. S2B&Fig. 4C). As expected, PR8/*Spn* coinfection led to bacterial outgrowth in both WT and CD169-DTR mice (Fig. 4D). Furthermore, DT-induced AM depletion in CD169-DTR mice led to a modest but significant increase in their lung bacterial burden, as compared with both DT-treated WT and PBS-treated CD169-DTR controls (Fig. 4D). Interestingly, the numbers of inflammatory monocytes and neutrophils also significantly decreased in CD169-DTR mice after AM depletion (Fig. 4E), even though their TNF-α and IFN-γ levels remained unaffected (Supplemental Fig. S2C). We have shown that neutrophils are not required for airway bacterial clearance during *Spn* infection alone (27). However, as suggested by other reported studies (11, 12, 19), neutrophils play a compensatory protective role in bacterial clearance during influenza/*S. pneumoniae* coinfection. Thus, the modestly (~4-fold) increased bacterial burden in AM-depleted CD169-DTR mice is consistent with their decreased airway neutrophils during PR8/S*pn* coinfection, as compared with PBS-treated CD169-DTR controls (Fig. 4E). These results, together with findings in *Csf2rb*$^{-/-}$ mice (Fig. 3), suggest that in addition to the prime defect in AM-mediated direct bacterial clearance, influenza infection further suppresses compensatory protective mechanisms to exacerbate influenza/*Spn* coinfection.

Influenza-induced IFN-γ signaling enables noninvasive *S. pneumoniae* to cause lethal pneumonia

We next investigated the regulatory pathways underlying influenza-suppressed airway antibacterial immunity. Multiple animal studies have shown that influenza-induced cytokines

suppress lung bacterial clearance (11–13, 38, 39). In particular, we have reported that influenza-induced IFN-γ increases susceptibility to secondary infection by highly virulent *S. pneumoniae*, *i.e.*, serotype 2 strain D39 and serotype 3 strain A66.1 (15). In agreement, we found that influenza-induced IFN-γ signaling was responsible for suppressing innate clearance of *S. pneumoniae* TJO983 (Fig. 5A). Notably, in contrast to 100% mortality in WT mice, all IFN-γ receptor 1 knockout (*Ifngr1*$^{-/-}$) survived PR8/*Spn* coinfection (Fig. 5B). These results indicate that activation of IFN-γ receptor signaling after influenza infection is sufficient to enable otherwise innocuous *S. pneumoniae* to cause lethal bacterial pneumonia.

Influenza-induced IFN-γ signaling in alveolar macrophages directly impairs their antibacterial capability

To further understand the cell-specific effect of IFN-γ receptor signaling on airway antibacterial immunity, we developed mouse models deleted of the *Ifngr1* gene in myeloid cells (*LysM*^{Cre}), AMs/DCs (*Cd11c*^{Cre}), macrophages/monocytes (*Cx3cr1*^{Cre}), or neutrophils (*Mrp8*^{Cre}) (Supplemental Fig. S3). Compared with *Ifngr1*$^{fl/fl}$ controls, mice deficient in IFN-γ-responsive myeloid cells (*LysM*^{Cre}*Ifngr1*$^{fl/fl}$, referred to as *Ifngr1*$^{\Delta Myelo}$) exhibited significantly increased resistance to PR8/*Spn* coinfection (Fig. 6A). Similarly, mice carrying *Ifngr1* deficiency in macrophages/monocytes (*Cx3cr1*^{Cre}*Ifngr1*$^{fl/fl}$, referred to as *Ifngr1*$^{\Delta AM/Mo}$) exhibited significantly improved lung bacterial clearance. In contrast, neutrophil-specific *Ifngr1* deletion (*Mrp8*^{Cre}*Ifngr1*$^{fl/fl}$, referred to as *Ifngr1*$^{\Delta PMN}$) had no beneficial effect on lung bacterial clearance during coinfection, as indicated by comparable bacterial burdens between *Ifngr1*$^{\Delta PMN}$ and *Ifngr1*$^{fl/fl}$ mice. These results establish that rather than regulation of neutrophil function, mononuclear phagocytes are responsive to the inhibitory effect of IFN-γ on airway antibacterial defense. Importantly, a selective deletion of *Ifngr1* gene in CD11c^{+} cells (*Cd11c*^{Cre}*Ifngr1*$^{fl/fl}$, referred to as *Ifngr1*$^{\Delta AM/DC}$), mainly CD11c^{+}Siglec-F^{+} AMs in the airway, resulted in significantly improved bacterial clearance after PR8/*Spn* coinfection (Fig. 6A). Taken together, these results indicate that IFN-γ signaling in AMs directly impairs their antibacterial function during influenza and *S. pneumoniae* coinfection.

In accordance with their improved bacterial control, *Ifngr1*$^{\Delta AM/DC}$ and *Ifngr1*$^{\Delta AM/Mo}$ mice exhibited significantly increased survival from PR8/*Spn* coinfection, as compared with either *Ifngr1*$^{\Delta PMN}$ animals or *Ifngr1*$^{fl/fl}$ controls (Fig. 6B). These *in vivo* findings further verify that IFN-γ signaling in AMs impairs their antibacterial function and thereby contributes to lethal pneumococcal pneumonia after influenza.

Discussion

In this study, we revisited the controversies over critical determinants of severe outcome in influenza and *S. pneumoniae* coinfection. We show that influenza infection enables otherwise noninvasive serotype 14 *S. pneumoniae* to cause lethal pneumonia in mice. Rather than disrupted mucosal integrity or general immune suppression, we found that this influenza-induced extreme susceptibility is attributable to IFN-γ signaling and abrogation of AM antibacterial function. Furthermore, by comparative analyses of mice with selective

deletion of *Ifngr1* gene in myeloid cells, AMs/DCs, macrophages/monocytes, or neutrophils, we demonstrate that influenza-induced IFN-γ signaling in AMs directly impairs their capability for bacterial control, thereby resulting in lethal bacterial outgrowth during secondary pneumococcal infection. Collectively, our current study contributes critical insights to the fundamental pathogenic mechanism of influenza and *S. pneumoniae* coinfection.

Multiple coinfection studies have found that, depending on the virulence of influenza virus and bacteria, the timing of bacterial super-infection, and the route of infection, a variety of outcomes are possible. Influenza virus replicates preferably in epithelial cells, and multiple studies have demonstrated that influenza-infected epithelial cells provide increased attachment sites for bacteria. These direct influenza-bacterial interactions can promote bacterial colonization in the upper respiratory tract (40), increase the risk of bacteria transmission into the normally sterile middle ear and lower respiratory tract, resulting in invasive diseases such as otitis media and pneumonia. On the other hand, many recent studies have demonstrated that dysregulation of host immune defenses critically contributes to the coinfection pathogenesis. For example, in a model of secondary systemic bacterial infection with *L. monocytogenes*, it has been shown that prior influenza infection leads to an increased inflammatory response in the lung but a suppression of systemic immune response (41, 42). Furthermore, it is proposed that the increased susceptibility to secondary bacterial infection is due to impaired ability to tolerate lung tissue damage (41). However, *L. monocytogenes* is not commonly associated with bacterial infections after influenza in humans.

The current understanding of influenza and *S. pneumoniae* coinfection in the lower respiratory tract is primarily built on animal models induced by highly virulent *S. pneumoniae*, such as serotype 2 and serotype 3 strains. These invasive bacterial strains alone can cause 100% mortality in mice after a high dose of infection, even in the absence of influenza infection. Accordingly, it has been suggested that influenza-induced epithelial damage promotes bacterial systemic dissemination, and thereby contributes to excessive mortality during secondary bacterial infection (8, 38, 43, 44). Using comparative mouse models, here we demonstrate that influenza-infected mice were more susceptible to i.n. than i.p. *Spn* super-infection (Fig. 2B). These surprising findings indicate that systemic antibacterial immunity is largely intact during influenza infection. Importantly, these results suggest that the acute disruption of lung integrity and therefore systemic bacterial spread is not the prime driver of lethal synergy between influenza and *S. pneumoniae*.

Using clodronate liposomes for AM depletion in naïve mice, we have previously demonstrated that AMs play a critical role in clearance of *S. pneumoniae* infection alone (27). However, clodronate liposomes also deplete other phagocytic cells, and therefore this approach is inapplicable for investigating AM-specific effect after influenza infection. In the current study, we employed both *Csf2rb*$^{-/-}$ and CD169-DTR mouse models to study the impact of influenza infection on AM-dependent and -independent bacterial control. We show in both models that in the absence of influenza infection, AMs are essential and sufficient for innate pneumococcal clearance; this antibacterial capability of AMs, however,

diminishes after influenza infection, thereby resulting in extensive bacterial outgrowth during secondary pneumococcal infection.

It has been shown that influenza infection depletes AMs in BALB/c mice, especially after a high dose of viral infection (14). However, after a low dose of PR8 infection, the numbers of AMs were largely unaltered in B6 WT mice within 24 h after *Spn* super-infection (Fig. 3B). Thus, we conclude that influenza infection primarily impairs AM function to abrogate initial bacterial clearance. It should be noted that during PR8/*Spn* coinfection, the heightened bacterial burden is associated with an intensive inflammatory response in the lung. This progressive worsening of condition may eventually lead to AM depletion and lethal lung damage at the later stage of influenza and pneumococcal coinfection.

As airway sentinel cells, AMs produce pro-inflammatory cytokines that recruit and activate neutrophils to help control infection. This innate signaling function likely becomes critical when AM-mediated direct bacterial control diminishes after influenza infection. In line with that, it has been shown that influenza infection inhibits AM TLR signaling, and thereby reduces neutrophil recruitment and enhances susceptibility to secondary bacterial infection. Here we show that AM-depleted CD169-DTR mice have a reduced neutrophil response after PR8/*Spn* coinfection, in agreement with their increased bacterial burden. Therefore, we speculate that AMs are still capable of innate sensing during influenza/*Spn* coinfection, in an effort to facilitate neutrophil recruitment for compensatory bacterial clearance.

In the absence of influenza infection, airway clearance of a low dose of *Spn* does not require the presence of neutrophils (27), indicating that neutrophil dysregulation is not a prime deficit for bacterial control after influenza. On the other hand, neutrophils are critically involved in pneumococcal clearance after influenza infection (27), likely to compensate for AM dysfunction. Accordingly, some studies have suggested that suppression of lung inflammatory responses, *i.e.*, inadequate acute cytokine/chemokine response and neutrophils, enhances influenza-induced susceptibility to secondary bacterial infection. In the current study, we show that compared with *Spn* single-infection, AM-deficient *Csf2rb*$^{-/-}$ mice exhibited 10-fold further increased bacterial burdens during PR8/*Spn* coinfection (Fig. 3A). This influenza-induced, AM-independent defect in bacterial control is probably due to neutrophil dysregulation. Nonetheless, this compensatory protective mechanism is too subtle to change the course and outcome of coinfection, as evidenced by the extensive neutrophil recruitment but heightened bacterial burden in both WT and *Csf2rb*$^{-/-}$ mice.

We have previously shown that T cell IFN-γ production suppresses acute (*i.e.*, 4 h) bacterial clearance in the lung (15). In agreement, *Ifng*$^{-/-}$ mice showed ~10-fold reduced bacterial burdens as compared with WT controls at 24 h after TJO983 super-infection. Interestingly, *Rag1*$^{-/-}$ mice deficient in T and B cells exhibited 100-fold further reductions in bacterial CFU as compared with *Ifng*$^{-/-}$ mice (Supplemental Fig. S4A). In line with this, adoptive transfer of WT splenocytes into *Rag1*$^{-/-}$ mice led to significantly increased bacterial outgrowth during coinfection, as compared with *Rag1*$^{-/-}$ animals received *Ifng*$^{-/-}$ splenocytes (Supplemental Fig. S4B-C). These findings suggest that influenza infection induces both IFN-γ-dependent and -independent suppression of antibacterial immunity.

Recent evidence indicates that Type I IFN (IFN-I) also mediates host susceptibility to secondary bacterial infection after influenza. Furthermore, it has been shown that IFN-I inhibits bacterial phagocytosis by human macrophages (45, 46). It is noteworthy that influenza virus induces peak production of IFN-I and IFN-γ at the acute (~ 4 dpi) and recovery (~7 dpi) phase of viral infection, respectively (15, 47). Thus, the peak susceptibility to pneumococcal super-infection, *i.e.*, 7 days after influenza infection (Fig. 2A), coincides with the production of IFN-γ but not IFN-I. In line with that, it has been shown that IFN-I suppresses neutrophil recruitment and thereby mediates susceptibility to secondary bacterial pneumonia, when mice were super-infected with *S. pneumoniae* on day 5 after influenza infection (11). Conversely, it has been shown that IFN-I protects AMs from viral replication in influenza-infected mice (48, 49). Taken together, these findings suggest that at least in our model, IFN-γ plays a dominant role in suppression of AM antibacterial function, as compared with influenza-induced IFN-I.

It is well established that scavenger receptors are critical for AM phagocytosis of unopsonized pneumococci (50, 51). We have measured mRNA levels of the macrophage mannose receptor (*Mr*, also called *Cd206*) and macrophage receptor with collagenous structure (*Marco*) (52), in AMs isolated from WT and *Ifng*$^{-/-}$ mice. Compared with AMs obtained from *Ifng*$^{-/-}$ mice, WT AMs exhibit significantly decreased *Mr* and *Marco* expression after influenza infection (Supplemental Fig. S5A). A similar effect of IFN-γ on AM scavenger receptor expression was observed *in vitro* (Supplemental Fig. S5B). Thus, it is likely that through down-regulation of phagocytic receptors, IFN-γ impairs the capability of AM for bacterial phagocytosis, thereby leading to increased susceptibility to *S. pneumoniae* infection.

Even though there are likely additional contributing factors in impairing AM antibacterial function after influenza, our current studies have demonstrated a fundamental role for IFN-γ signaling in promoting lethal susceptibility to secondary pneumococcal infection. Furthermore, using a series of conditional knockout mouse lines, we demonstrate that IFN-γ receptor signaling in macrophages/monocytes impairs their antibacterial function. Taken together, we conclude that influenza-induced IFN-γ signaling impairs direct bacterial clearance by AMs, thereby enabling otherwise noninvasive *S. pneumoniae* to cause deadly pneumonia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

1. The authors thank University of Nebraska Medical Center (UNMC) Flow Cytometry Research Facility for assistance with FACS analysis. The UNMC Flow Cytometry Research Facility is supported by state funds from the Nebraska Research Initiative (NRI) and The Fred and Pamela Buffett Cancer Center's National Cancer Institute Cancer Support Grant.

Footnote

This work was supported by National Institutes of Health R21 AI128527 and R01 HL118408 to K.S.

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Key points

- Influenza enables noninvasive *Streptococcus pneumoniae* to cause deadly pneumonia
- Influenza mainly predisposes hosts to airway but not systemic bacterial infection
- Influenza temporally abolishes innate bacterial clearance by alveolar macrophages

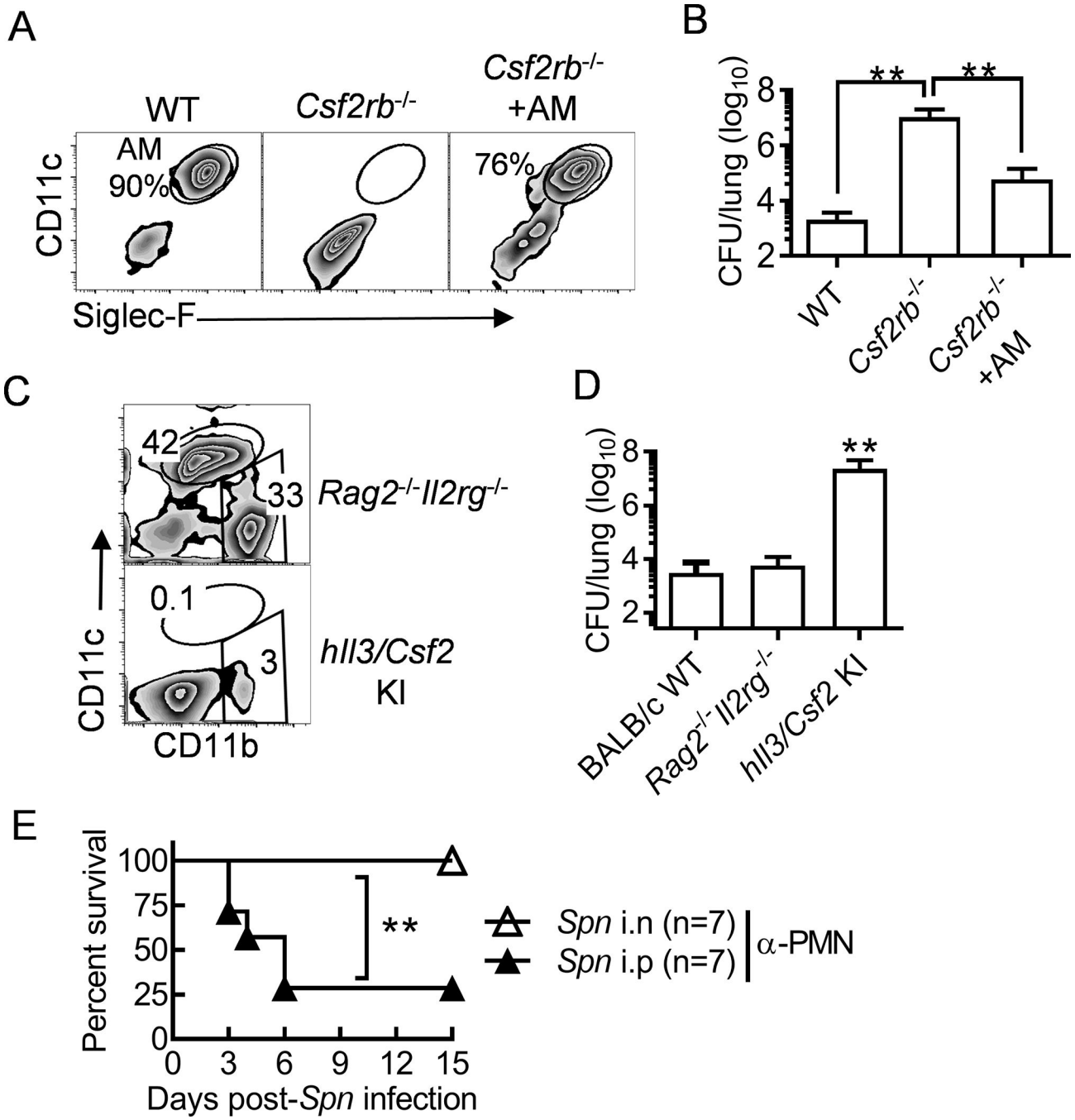

Figure 1. AMs are essential and sufficient for airway clearance of *S. pneumoniae*.

(**A**) The mean frequency of BALF AMs ($CD11c^+Siglec\text{-}F^+$), and (**B**) lung bacterial burdens (mean±SD) 24 h after *Spn* infection of B6 WT and *Csf2rb*$^{-/-}$ mice (n ≥ 5 mice/group). *Csf2rb*$^{-/-}$+AM: *Csf2rb*$^{-/-}$ mice received adoptive transfer of WT AMs. **, *P*<0.01, Mann Whitney test. (**C**) The mean frequency of BALF $CD11c^+$ and $CD11b^+$ myeloid cells, and (**D**) lung bacterial burdens (mean±SD) 24 h after *Spn* infection of BALB/c WT, *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ and *hIl3*/*Csf2* KI mice (n ≥ 5 mice/group). **, *P*<0.01, Mann Whitney test. (**E**) Animal survival after i.n. or i.p. challenge of B6 WT mice with 10^5 CFU *Spn*. Mice

were treated anti-Gr1 antibodies (α-PMN) to deplete PMNs. ** $P < 0.01$, log-rank test. Data shown are representative of at least two independent experiments.

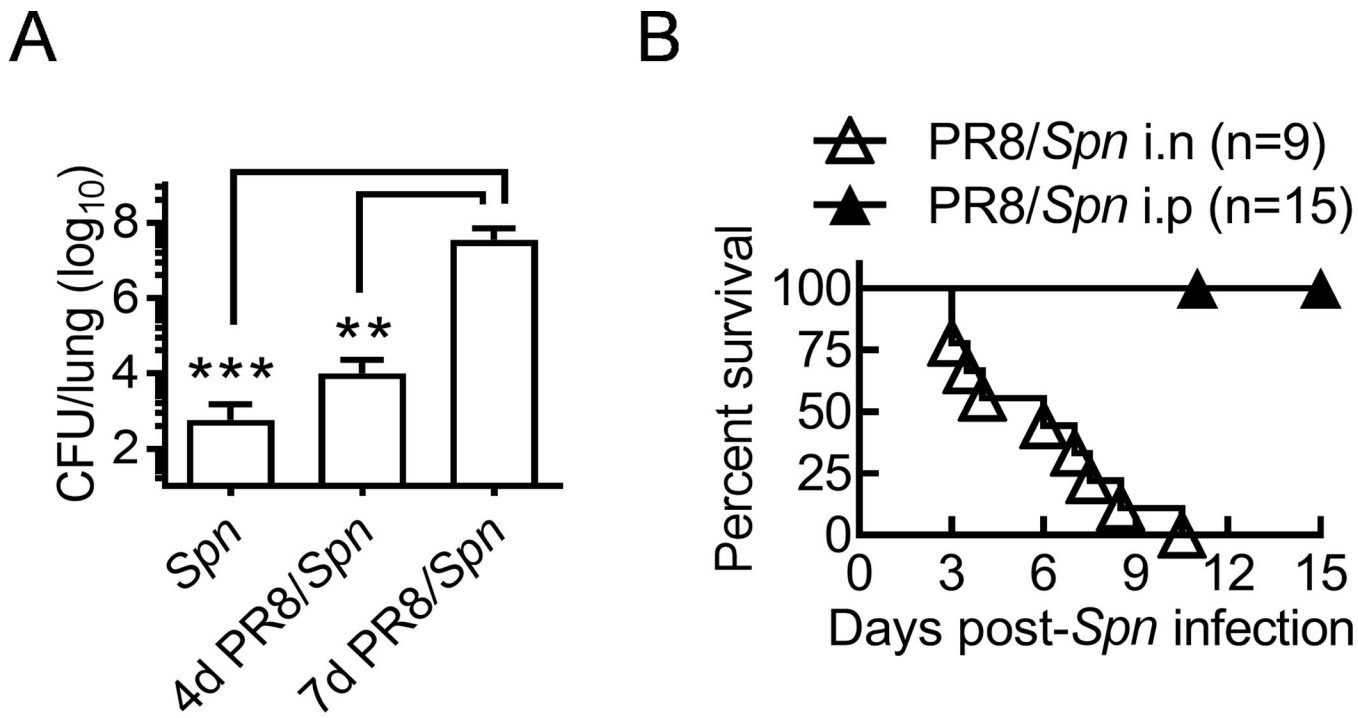

Figure 2. Influenza induces lethal susceptibility to local but not systemic *S. pneumoniae* super-infection.

(A) Lung bacterial burdens (mean±SD) 24 h after i.n. challenge naïve (*Spn*), days 4 and 7 PR8-infected (PR8/*Spn*) B6 WT mice (n ≥ 4 mice/group) with *Spn*. (**B**) Survival of B6 WT mice after i.n. or i.p. super-infection with 2×10^4 *Spn* on day seven after PR8 infection. $^{**}P<0.01$, $^{***}P<0.001$. Mann Whitney test. Data shown are representative of two independent experiments.

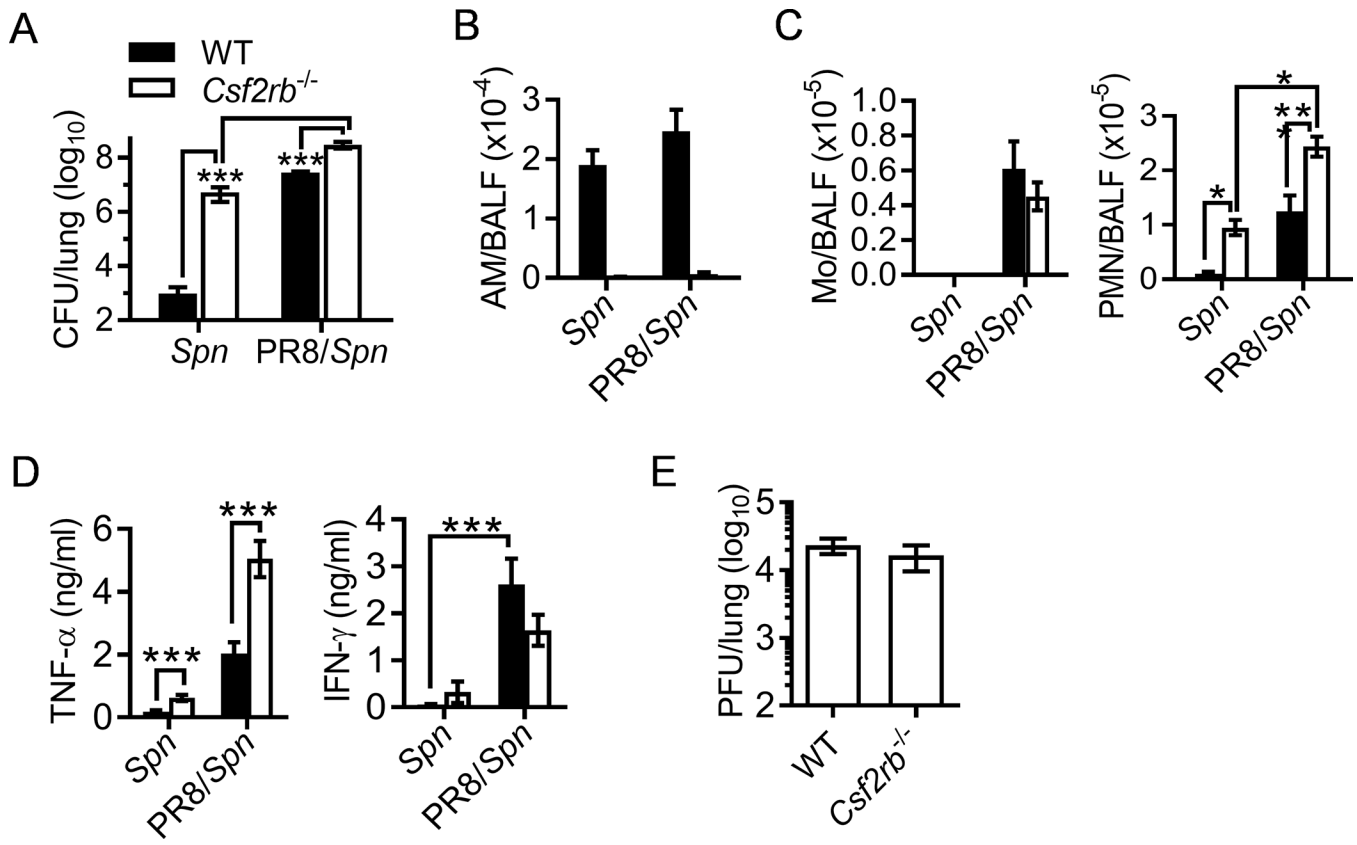

Figure 3. Influenza infection abolishes AM-mediated bacterial clearance during secondary pneumococcal infection.

(A) Lung bacterial burdens (mean±SD), (**B**) BALF $CD11c^{+}Siglec\text{-}F^{+}$ AM numbers (mean ±SEM), (**C**) the numbers of $CD11b^{+}Ly6C^{+}$ monocytes (Mo) and $CD11b^{+}Ly6G^{+}$ neutrophils (PMN), (**D**) the levels of TNF-α and IFN-γ (mean±SEM), and (**E**) lung viral burdens (mean ±SEM) 24 h after i.n. challenge of naïve (*Spn*) or day seven PR8-infected (PR8/*Spn*) B6 WT and *Csf2rb*$^{-/-}$ mice (n ≥ 5 mice/group) with *Spn*. ***P<0.001, Tukey's multiple comparisons test. Data shown are representative of two independent experiments.

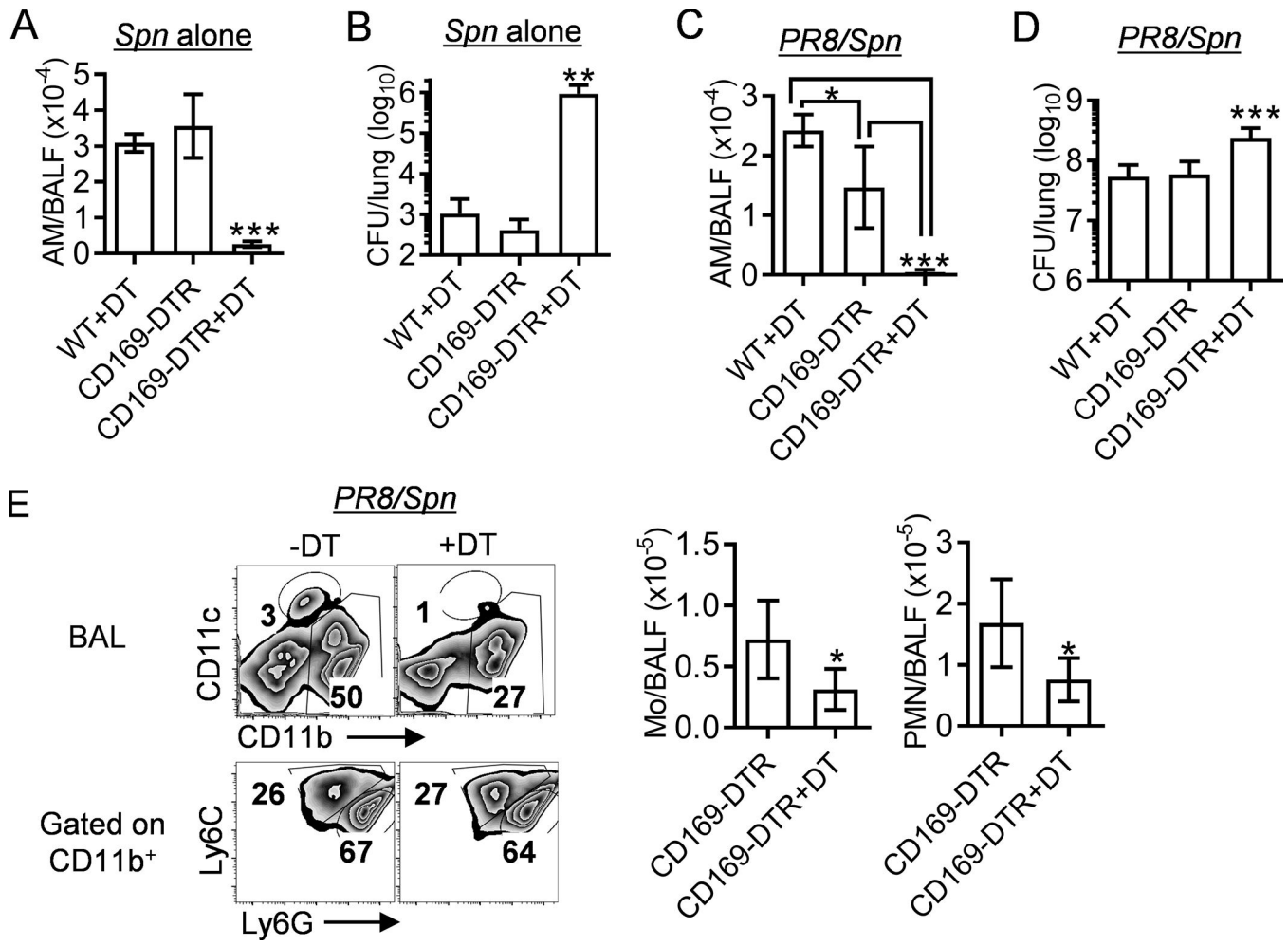

Figure 4. Temporary AM depletion decreases neutrophil recruitment and promotes bacterial outgrowth in CD169-DTR mice during influenza/*S. pneumoniae* coinfection.

(**A**) The numbers of BALF AMs ($CD11c^+Siglec\text{-}F^+$), and (**B**) lung bacterial burdens 24 h after i.n. infection of B6 WT and CD169-DTR mice (mean±SD, n ≥ 4 mice/group) with *Spn*. (**C**) The numbers of BALF AMs, (**D**) lung bacterial burdens, and (**E**) the numbers of $CD11b^+Ly6C^+$ monocytes (Mo) and $CD11b^+Ly6G^+$ neutrophils (PMN) 24 h after i.n. challenge of day seven PR8-infected B6 WT and CD169-DTR mice (mean±SD, n ≥ 5 mice/group) with *Spn*. Naïve (**A-B**) or PR8-infected (**C-E**) mice were treated i.p. with DT (+DT) or PBS control four days before *Spn* infection. **P*<0.05, ***P*<0.01****P*<0.001, Tukey's multiple comparisons test or *t*-test (**E**). Data shown are representative of two independent experiments.

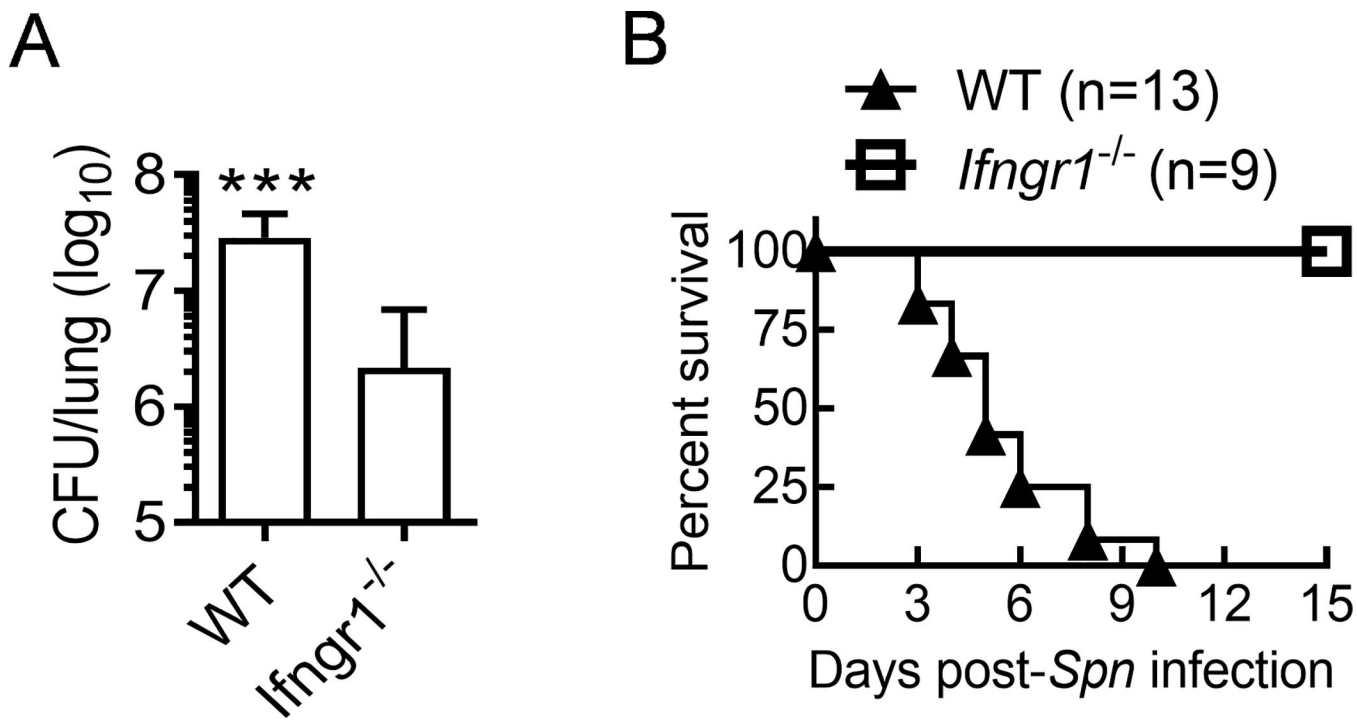

Figure 5. Influenza-induced IFN-γ receptor signaling causes lethal susceptibility to secondary pneumococcal infection.

(A) Lung bacterial burdens (mean±SD, n ≥ 10 mice/group) at 24 h and (**B**) animal survival of B6 WT and *Ifngr1*$^{-/-}$ mice after i.n. challenge with *Spn* on day seven after PR8 infection. ***P<0.001, t-test. Data shown combined from two independent experiments.

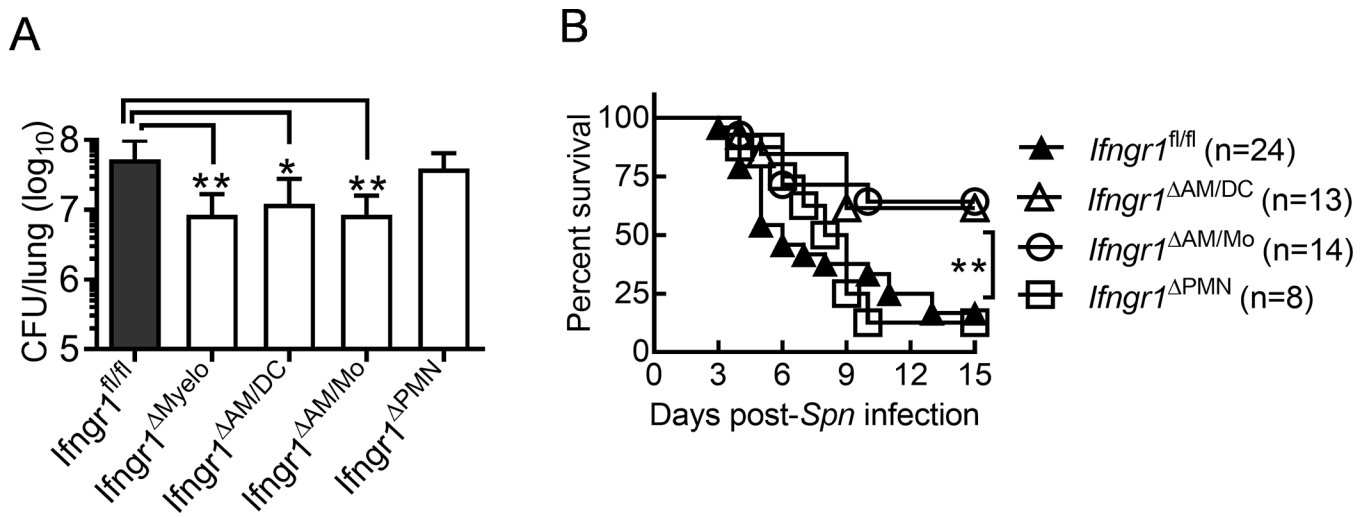

Figure 6. Influenza-induced IFN-γ signaling in AMs directly impairs their antibacterial function during secondary pneumococcal infection.

(A) Lung bacterial burdens (mean±SD, n ≥ 8 mice/group) at 24 h and (**B**) animal survival of B6 *Ifngr1*$^{fl/fl}$, *LysM*^{Cre}*Ifngr1*$^{fl/fl}$ (*Ifngr1*$^{\Delta Myelo}$), *Cd11c*^{Cre}*Ifngr1*$^{fl/fl}$ (*Ifngr1*$^{\Delta AM/DC}$), *Cx3cr1*^{Cre}*Ifngr1*$^{fl/fl}$ (*Ifngr1*$^{\Delta AM/Mo}$), and *Mrp8*^{Cre}*Ifngr1*$^{fl/fl}$ (*Ifngr1*$^{\Delta PMN}$) mice after i.n. challenge with *Spn* on day seven after PR8 infection. **P*<0.05, ***P*<0.01, Tukey's multiple comparisons test (**A**) or log-rank test (**B**). Data shown were combined from more than two independent experiments.