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MINIREVIEW

Influenza and Bacterial Superinfection: Illuminating the Immunologic Mechanisms of Disease

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Seasonal influenza virus infection presents a major strain on the health care system. Influenza virus infection has pandemic potential, which was repeatedly observed during the last century. Severe disease may occur in the young, in the elderly, in those with preexisting lung disease, and in previously healthy individuals. A common cause of severe influenza pathogenesis is superinfection with bacterial pathogens, namely, *Staphylococcus aureus* and *Streptococcus pneumoniae*. A great deal of recent research has focused on the immune pathways involved in influenza-induced susceptibility to secondary bacterial pneumonia. Both innate and adaptive antibacterial host defenses are impaired in the context of preceding influenza virus infection. The goal of this minireview is to highlight these findings and synthesize these data into a shared central theme of pathogenesis.

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Seasonal infection with influenza viruses has occurred annually in the human population over the last several centuries. Periodically, due to the antigenic shift in circulating strains, influenza virus infections reach pandemic levels, with global spread of disease affecting up to 1/4 to 1/3 of the world population. Despite significant vaccination efforts, antigenic drift and shift of the virus can result in large-scale susceptibility to disease. While most influenza virus infections result in mild to moderate pulmonary infection, severe, life-threatening disease can occur. Often, the worst disease outcomes are associated with secondary bacterial pneumonia caused primarily by *Staphylococcus aureus* or *Streptococcus pneumoniae*. Influenza with bacterial superinfection can result in the hospitalization and/or death of both patients with preexisting lung disease and previously healthy individuals. While much progress has been made in this area over the last decade, there are still many controversies and apparent inconsistencies in the published literature. The goal of this minireview is to discuss current findings and attempt to place these data in a larger context of disease pathogenesis, perhaps resolving existing conflicts.

INFLUENZA AND BACTERIAL SUPERINFECTION EPIDEMIOLOGY

During the last 100 years, four well-documented influenza pandemics have impacted the United States of America and the world. In 1918, influenza A H1N1 virus infected 500 million people worldwide and resulted in the deaths of more than 50 million individuals (1, 2). Only a small percentage (<5%) of those who died succumbed early during infection, and most deaths occurred between days 7 and 14 postinfection (3). Two distinct clinical-pathological syndromes have been described by Morens and Fauci, with the first (10 to 15% fatal cases) being similar to a severe acute respiratory distress-like syndrome and the second (85 to 90% fatal cases) manifesting as acute bronchopneumonia, with pathogenic bacteria cultured on autopsy (4). Among the causative pneumopathogenic bacterial species, *S. pneumoniae* was the bacterial pathogen most commonly identified (1). These data strongly implicate influenza virus infection combined with bacterial superinfection as the primary cause of mortality during that influenza pandemic.

The next two pandemics occurred in 1957 and 1968 and were caused by influenza virus descendants of the 1918 virus (H2N2 and H3N2, respectively) in which new gene segments had been acquired by reassortment. Attenuated pathogenicity and decreased mortality were seen in both 1957 and 1968 compared to the 1918 pandemic. Noteworthy changes had occurred in medicine between the 1918 and 1957 pandemics, with the introduction of antibiotics and influenza vaccines and the use of public health services to collect and report data on influenza. Bacterial pneumonia, caused predominantly by *S. aureus*, still accounted for 44% of deaths in 1957, but the death rate was substantially lower than the 1918 rate (5). Although the incidence and distribution data representing pneumonia-associated mortality were similar between 1957 and 1968, *S. pneumoniae* was the primary pathogen of bacterial superinfection in the 1968 pandemic (6).

The most recent influenza pandemic occurred in 2009 and was caused by a triple-reassortment influenza A H1N1 virus. Although it remains difficult to accurately assess the global mortality associated with this pandemic, modeling studies estimate that the 2009 H1N1 influenza virus caused approximately 200,000 respiratory deaths. The global mortality rate was similar to prepandemic seasonal influenza estimates, but the burden of mortality shifted to persons less than 65 years of age (7, 8). Bacterial pneumonia complicated between 25% and 50% of severe infections in both children and adults (9–14). *S. aureus* and *S. pneumoniae* were the most common complicating organisms, although *Streptococcus pyogenes*, *Haemophilus influenzae*, and Gram-negative rods were also found in biologic specimens of critically ill patients. Regardless of the organism, bacterial superinfection was associated with higher morbidity and mortality during the 2009 influenza pandemic.

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ROLE OF PHAGOCYTES IN HOST SUSCEPTIBILITY TO SUPERINFECTION

(i) Role of neutrophils in susceptibility to superinfections. Primary phagocytes, namely, macrophages and neutrophils, are of pivotal importance for an effective immune response to both viral and bacterial infections in the lung. Influenza-induced alterations of the recruitment and/or function of these cells have been implicated in susceptibility to postinfluenza bacterial superinfections at approximately day 7 postinfection. Influenza with bacterial superinfection results in increased neutrophil recruitment to the lung compared to single-infection controls. A number of reports have linked the development of increased inflammatory neutrophilia with an increased susceptibility of mice to superinfection at days 6 and 7 after influenza virus infection (15–17). Increased numbers of neutrophils in the lungs of superinfected animals compared to those in mice infected with either *S. pneumoniae* or *S. aureus* alone correlated with increased bacterial load and increased mortality; however, depletion of neutrophils did not improve or worsen the outcome of superinfection (15, 18). Other work has demonstrated significant neutrophil accumulation in murine lungs in response to *S. pneumoniae* infection in mice infected with influenza virus for either 3 or 6 days as well as in mice not infected with influenza virus (19). However, only mice that were infected with influenza virus for 6 days showed increased susceptibility to secondary streptococcal infection. Depletion of neutrophils resulted in increased susceptibility to pneumococcal pneumonia in the mice challenged with bacteria at 3 days after influenza virus infection but not in those challenged with bacteria at 6 days after influenza virus infection.

Reduced susceptibility to *S. aureus* superinfection in mice infected with influenza virus for 2 to 3 days compared to mice infected only with bacteria is dependent on both increased levels of interleukin-13 (IL-13) (20) and the presence of neutrophils in the lung (unpublished observation). These data suggest that enhanced neutrophil-mediated responses, perhaps mediated by an IL-13-dependent mechanism, may contribute to improved resolution of superinfection early after influenza virus infection (day 3) (20–22). As in prior experiments with pneumococcal pneumonia, depletion of neutrophils (with anti-Ly6G antibodies) in mice infected with influenza virus for 3 days significantly increased their susceptibility to *S. aureus* superinfection compared to mice infected only with bacteria (A. Rynda-Apple, unpublished observation). Thus, neutrophils are important contributors to reduced susceptibility to superinfection early after influenza virus infection.

Interestingly, enhanced recruitment of neutrophils in response to superinfection at day 7 after influenza virus infection may contribute to tissue pathology by increasing lung tissue damage, perhaps by formation of neutrophil extracellular traps (NETs) (23, 24). Furthermore, NET-forming neutrophils may be an additional inducer of type I interferons (IFNs) (whose role in susceptibility to superinfection is discussed below) during influenza and bacterial superinfection (25).

Cumulatively, these data indicate that although properly functioning neutrophils are important to bacterial clearance during superinfection early after influenza virus infection, it is unclear what the role of neutrophil dysfunction may be during the time period of enhanced susceptibility to superinfection at days 6 and 7 after influenza virus infection. It is possible that an increased accumulation of dysfunctional neutrophils in the lung at days 6 and 7 after influenza virus infection may contribute to increased susceptibility to superinfection via both impaired bacterial clearance and damage to the lung tissue.

(ii) Impairment of bacterial killing by mononuclear cells. Alveolar macrophages (AMs) constitute the predominant, highly phagocytic, CD11c integrin-expressing cell population and play a critical role in homeostasis and host defense against numerous pulmonary infections, including infections by influenza virus, *S. aureus*, and *S. pneumoniae*, as well as against superinfections (26–32). Insufficient numbers of AMs in influenza virus-infected mice due to either depletion of clodronate liposomes or the lack of granulocyte-macrophage colony-stimulating factor (GM-CSF) seen in *Csf2*$^{-/-}$ mice resulted in impaired gas exchange, fatal hypoxia, and severe morbidity but affected viral clearance only moderately (29). Nearly complete (90%) ablation of a CD11b^{-} subset of resident AMs (defined as CD11c^{hi} F4/80^{hi} CD11b^{dim} cells), but not of inflammatory monocytes (IMs; CD11c^{hi} F4/80^{hi} CD11b$^{lo\text{-}int}$ cells), by day 7 after influenza virus infection severely impaired early pneumococcal clearance (up to 3 h after superinfection) (31). Treatment of mice with exogenous GM-CSF, which enhances proliferation and regulates differentiation and activation of lung-resident macrophages, at approximately the same time as influenza challenge significantly expanded the pool of IMs, partially restored influenza-mediated depletion of AMs, improved early pneumococcal clearance, and reduced the number of influenza virus-infected mice developing secondary pneumococcal pneumonia (31, 33). These results suggest that insufficient numbers of AMs at day 7 after influenza virus infection may contribute to the host's susceptibility to superinfection. Further, in another study, influenza virus infection did not affect either binding or uptake of *S. aureus* by either AMs or neutrophils at day 6 postinfection; in fact, macrophages from influenza virus-infected lungs were able to bind and take up significantly more *S. aureus* than naive macrophages *in vitro* (18). This suggests that insufficient numbers of AMs rather than their inability to take up bacteria contributed to the host's susceptibility to superinfection at days 6 and 7 after influenza virus infection.

Somewhat in contrast to those results, Sun and Metzger showed that type II interferon signaling induced by day 7 after influenza virus infection inhibited macrophage expression of the scavenger receptor MARCO, which the authors attributed to inhibition of *S. pneumoniae* uptake and killing during superinfection (34). In another study, influenza-altered production of reactive oxygen species (ROS) by an intracellular NADPH-dependent mechanism was shown to contribute to reduced bacterial killing by both monocytes and neutrophils in a model of *S. aureus* superinfection 7 days after influenza virus infection (32). Consistent with this report, overexpression of GM-CSF improved the resolution of secondary *S. aureus* pneumonia via stimulation of ROS production by AMs and enhancement of neutrophil functions (35). Furthermore, attenuated production of antimicrobial peptides (AMPs) was observed in response to superinfection at day 6 after influenza virus infection, providing another potential mechanism for both impaired bacterial killing and increased susceptibility to superinfection (18). Thus, these results suggest that the impairment of the ability of macrophages to clear superinfection may occur via impaired processes of bacterial killing, such as ROS-dependent killing or impaired production of AMPs.

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The role of dendritic cells (DCs) in influenza virus infection has been extensively studied, but their role in modulating susceptibility to superinfection requires further evaluation (36, 37). Plasmacytoid DCs are an important source of type I IFN in established influenza virus infection, suggesting their potential role in the host's susceptibility to superinfection. A CD11c$^+$ cell population was shown to be a primary source of IL-23 in response to *S. aureus*, and a preceding influenza virus infection attenuated this response markedly (16).

ROLE OF TYPE I IFN SIGNALING IN THE HOST'S SUSCEPTIBILITY TO SUPERINFECTION

Type I IFN signaling can be elicited by both viral and bacterial infections. During influenza virus infection, type I IFN signaling is a part of an initial antiviral response; however, the role for type I IFN signaling induced by bacterial infection is more enigmatic. As such, bacteria can take advantage of type I IFN signaling, as was previously demonstrated in the context of *S. aureus*-induced pneumonia (38, 39). Type I IFN signaling was shown to promote colonization of mice with *S. pneumoniae* during influenza virus-pneumococcus superinfection (40). Interestingly, mice deficient in type I IFN signaling (IFNAR1$^{-/-}$ mice) were less susceptible than wild-type (WT) mice to *S. pneumoniae* superinfection at day 7 after influenza virus infection, suggesting a negative effect of type I IFN signaling on superinfection (41). In contrast to those reports, administration of alpha IFN (IFN-α) (expressed in an adenoviral vector) prior to respiratory infection with *S. pneumoniae* improved the outcome of pneumococcal infection (42). Interestingly, IL-13 production at day 3 after influenza virus infection was dependent on type I IFN signaling, suggesting that type I IFN signaling may also play a beneficial role in the outcome of superinfection at day 3 after influenza virus infection (20; unpublished observation). Since the cellular source of type I IFNs is dynamic during respiratory virus infection, it is important to consider whether these specific type I IFN cytokines may play nonredundant roles in regulation of susceptibility to superinfection. Given the complexity and kinetics of influenza-induced type I IFN responses, it remains to be determined whether type I IFN signaling that is detrimental to the host during superinfection at day 7 is elicited early (after influenza virus infection) or late (in response to superinfection).

Influenza-induced type I IFN signaling at day 5 after influenza virus infection was shown to attenuate the production of neutrophil chemoattractants Cxcl1 and Cxcl2, impairing neutrophil responses and resulting in the inability to efficiently resolve *S. pneumoniae* superinfection (41). Consequently, mice deficient in the type I IFN receptor had increased neutrophil recruitment and improved pneumococcal clearance. Type I IFN-driven expression of the histone methyltransferase *Setdb2* gene has been linked to downregulation of Cxcl1 production, reduced neutrophil recruitment, and increased lung bacterial burden in mice superinfected with *S. pneumoniae* (43). These findings suggest that type I IFN signaling may suppress recruitment of neutrophils and/or impair their bactericidal capacity during superinfection at days 5 to 7 after influenza virus infection.

In addition to its detrimental effect on the function of innate phagocytes, influenza-induced type I IFN signaling also affects anti-influenza virus T cell-mediated immune responses. As such, type I IFN signaling inhibited production of IL-23 and IL-1β, critical type 17 immunoregulatory cytokines (16, 44). Further, type I IFN receptor-deficient mice had elevated type 17 immune responses (whose importance in the context of superinfection is discussed below) and were protected against postinfluenza staphylococcal pneumonia. Cumulatively, these results suggest a detrimental role of type I IFN signaling during superinfection at days 6 and 7 after influenza virus infection. Importantly, it appears that this negative effect of type I IFN signaling affects both innate and adaptive immune responses during established influenza virus infection.

EFFECTS OF INFLUENZA ON T CELL-MEDIATED IMMUNITY

Host defense against *S. aureus* or *S. pneumoniae* requires activation of T cell-dependent pathways. Recent studies in mouse models of postinfluenza bacterial superinfections have implicated multiple cell types with a role in susceptibility to disease. T cell responses can be grouped into three major subsets corresponding to the helper T cell lineages Th1/type 1, Th2/type 2, and Th17/type 17. Each of these subsets has been implicated in the pathogenesis of superinfection.

(i) Altered production of type 1 cytokines late after influenza virus infection. The antiviral host response is generally thought of as being type 1 dominant, with production of IL-12 and activation of IFN-γ-producing cells. Natural killer cells, CD4$^+$ T cells, and CD8$^+$ T cells are critical to viral clearance during influenza virus infection. However, Sun and Metzger demonstrated that influenza virus infection of either CD8a$^{-/-}$ mice depleted of CD4$^+$ cells or Rag2$^{-/-}$ mice improved initial clearance of *S. pneumoniae* from bronchoalveolar lavage fluid (BALF) (4 to 8 h postchallenge) compared to the results seen with WT mice (34). This improved initial bacterial clearance compared to WT mouse results was related to a loss of IFN-γ production in the lung. These data suggest that antiviral Th1 cells may directly induce susceptibility to secondary bacterial pneumonia via increased production of IFN-γ. In addition, influenza-induced suppression of another type 1 cytokine, TNF-α (produced in this case by NK cells), was proposed as another potential mechanism by which mice were more susceptible to *S. aureus* infection (45). TNF-α was shown to activate macrophage uptake and killing of *S. aureus*. These data demonstrate a role for production of type 1 cytokines such as IFN-γ or TNF-α in susceptibility to secondary bacterial pathogens.

(ii) Dual role for type 2 cytokines in susceptibility to superinfection. Type 2 immune responses and related regulatory T cell cytokine production have been implicated in susceptibility to secondary infection. The canonical type 2 cytokine IL-13 was shown to be beneficial in host defense against *S. aureus* superinfection early after influenza virus infection (20). Although IL-13 may be derived from a non-T-cell source early during the course of influenza virus infection, it was shown to attenuate IFN-γ production when mice were challenged with *S. aureus* 3 days after influenza virus infection. By 7 days after influenza virus infection, IL-13 levels were no longer elevated, at least in part due to the increased production of soluble IL-13 decoy receptor, and IFN-γ production was high. In contrast to the beneficial role of IL-13 produced early after influenza virus infection, levels of another anti-inflammatory cytokine, IL-10, were shown to be elevated during bacterial superinfection at later times after influenza virus infection. Indeed, neutralization of IL-10 was beneficial in the context of *S. pneumoniae* superinfection (19, 46). Further, IL-10$^{-/-}$ mice were protected against secondary *S. aureus* infection, although this response was not observed in a pneumococcal model (34, 47). The influenza-induced IL-27 cytokine was shown to regulate IL-10

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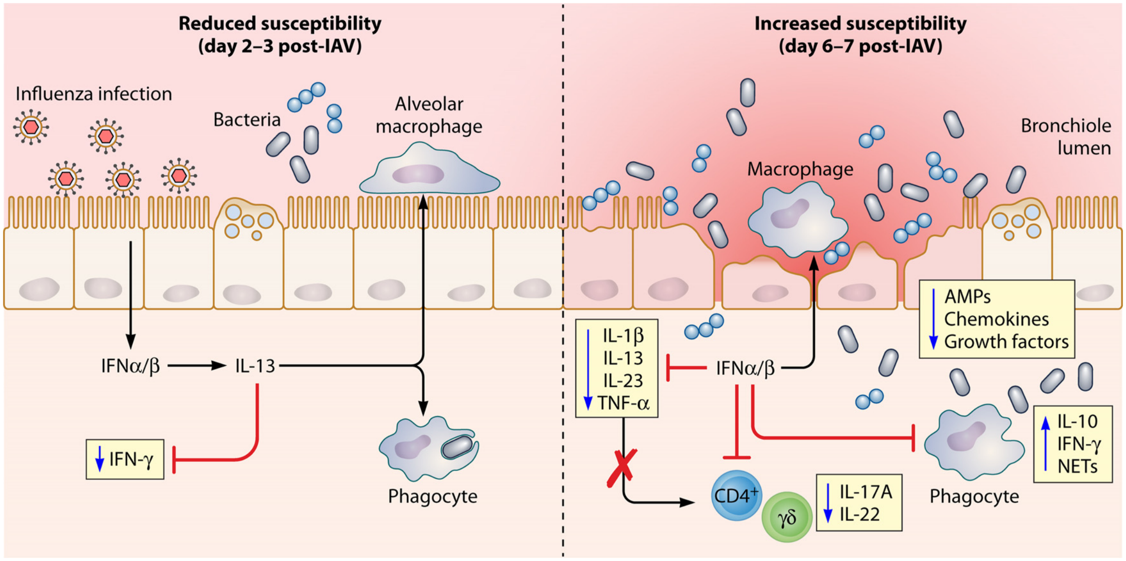

FIG 1 Common pathways of susceptibility to postinfluenza bacterial superinfections. Early after influenza virus infection, mice show reduced susceptibility to superinfection that is at least in part due to increased production of IL-13. This IL-13-rich environment does not permit IFN-γ production, allowing unaltered phagocytosis and clearance of bacteria. The role for either neutrophils or macrophages (phagocytes) in bacterial clearance early during influenza virus infection has not been fully investigated. Progression of influenza virus infection results in increased susceptibility to secondary infection. Type I IFN (or IL-27) signaling initiated in response to influenza virus infection results in downregulated production of IL-1β and Il-23 and impaired type 17 immune responses. Inhibition of IL-17 and IL-22 reduces production of antimicrobial peptides. Type I IFN signaling also reduces levels of neutrophil chemoattractants Cxcl1 and Cxcl2 and can induce formation of NETs. IL-27 induced during influenza virus infection further suppresses IL-17 production but stimulates production of regulatory cytokine IL-10, which contributes to increased susceptibility to superinfection, presumably by alteration of the anti-influenza inflammatory response. IAV, influenza A virus infection.

production during influenza virus-*S. aureus* superinfection, and IL-27R$^{-/-}$ mice had lower IL-10 levels and were protected in this model. Finally, a role for type 2 innate lymphoid cells (ILC2) in superinfection has recently been demonstrated. ILC2 cells express the IL-33 receptor ST2 (interleukin-1 receptor-like 1), and ST2$^{-/-}$ mice had higher *S. pneumoniae* burden and increased inflammation compared to WT animals during superinfection (48). These data suggest that ILC2 may play a protective role, perhaps by producing IL-13, during postinfluenza bacterial superinfection.

(iii) Critical role for type 17 cytokines for protection from superinfection. A critical role for immunity mediated by type 17 cytokines against secondary infections with either *S. aureus* or *S. pneumoniae* has been previously demonstrated (16, 18, 44, 49). In addition, preceding influenza virus infection was shown to suppress type 17 immune activation against secondary infection with the Gram-negative pathogens *Escherichia coli* and *Pseudomonas aeruginosa* (50). IL-17 and IL-22 were both shown to promote clearance of bacteria through the recruitment of phagocytes and induction of antimicrobial peptides (AMPs). Preceding influenza virus infection was shown to attenuate subsequent production of IL-17 by CD4$^+$ and γδ T cells in response to infection with *S. aureus* and *S. pneumoniae* (16, 49). In addition, IL-27R$^{-/-}$ mice had increased IL-17-producing γδ T cell responses and were protected versus controls during postinfluenza superinfection with either *S. pneumoniae* or *S. aureus* (47, 51). Treatment with exogenous IL-23 or IL-1β rescued type 17 immune responses during postinfluenza staphylococcal infection, resulting in improved bacterial clearance (16, 44). IL-22 was also shown to be protective in postinfluenza *S. pneumoniae* superinfection, as IL-22$^{-/-}$ mice had increased bacterial burden and mortality (52). Finally, both *S. pneumoniae* and IL-23 have independently been shown to induce ILC3 production of IL-22 in the lung, suggesting a role for these ILC3 in host responses during superinfection (53). Thus, these collective data suggest a critical pathway by which preceding influenza virus infection attenuates type 17 immunity against bacterial pathogens.

INFLUENZA-INDUCED CHANGES TO THE LUNG ENVIRONMENT AND SUSCEPTIBILITY TO SUPERINFECTION

Earlier work on host susceptibility to postinfluenza superinfection dealt with the role of lung environment, specifically, influenza-induced changes to respiratory epithelium, in this process. Aside from influenza-mediated damage to respiratory epithelium, it was shown that one of the influenza virus surface glycoproteins, neuraminidase (NA), strips sialic acid from the lung surface; this was previously suggested to be a mechanism for exposure of adherence receptors facilitating infection by pneumococci (54). Increased bacterial adherence to the epithelium during influenza virus infection has been suggested to be a susceptibility mechanism.

In addition to effects on epithelial integrity, desensitization of macrophages to Toll-like receptor (TLR) ligands at between 2 and 6 weeks after influenza virus (or respiratory syncytial virus [RSV]) infection was proposed as another mechanism of increased susceptibility to superinfection (55). In that study, reduced expression of TLR4 coincided with increased lung pneumococcal burden, reduced production of neutrophil and macrophage chemoattractants and growth factors, and reduced neutrophil recruitment. For a more comprehensive description of how lung environment and lung homeostasis are affected by respiratory virus infections and how these changes may influence the host's

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susceptibility to superinfections, please refer to recently published review articles (56, 57).

COMMON PATHWAYS INVOLVED IN SUSCEPTIBILITY TO SECONDARY PNEUMONIA

A large body of data generated in the last decade by various laboratories has significantly improved our grasp of the mechanisms underlying the susceptibility to postinfluenza superinfections. Although a number of common pathways involved in susceptibility to superinfections have been identified (Fig. 1), there are still a number of issues that remain. For instance, although investigators agree that influenza-induced type I and II IFNs contribute to pathogenesis of superinfections, how the IFNs determine pathogenesis of superinfections requires further investigation. Temporal regulation of type I interferon versus II interferon during primary influenza virus infection may explain this irregularity. In our opinion, many of the discrepancies with regard to the roles for both type I and type II IFNs can be explained by the timing of secondary bacterial infection, the different species or strain of a bacterium used for the challenge, and, finally, the different doses of primary (viral) and/or secondary (bacterial) inoculum. Regardless of the precise mechanism of interaction between the IFNs, both classes of these cytokines seem to impair the bacterial clearance capacity of phagocytes, as well as the function of T cells. A number of mechanisms, ranging from cellular depletion and suppression of cell recruitment to impaired bacterial killing due to altered production of ROS, have been proposed for phagocyte impairment. In addition, attenuation of AMP production by lung epithelial cells may play a role in susceptibility to secondary bacterial pneumonia. The involvement of T cell immunity during superinfection is likely temporally regulated. IL-13 production early during influenza virus infection is beneficial due to its inhibition of IFN-γ. At day 7 after influenza virus infection, the lack of IL-13 (and resulting production of IFN-γ) and type I IFN-mediated impairment of type 17 immune responses directly contribute to impairment of innate antibacterial responses. Although researchers have identified critical cytokines and cells that determine pathogenesis of superinfections, we have not yet identified how these cytokines interact with each other or with target cells in the lung. Current research has focused upon and already defined many of the immune deficiencies present during postinfluenza bacterial superinfection. However, the greater challenge is to identify interventional opportunities that would form the basis for preclinical testing. Although we know a lot of the players involved in severe disease pathogenesis at this time, our current challenge is to translate this information into improved clinical approaches for treatment of critically ill patients.

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