SECONDARY RESPIRATORY INFECTIONS IN THE CONTEXT OF ACUTE AND CHRONIC PULMONARY DISEASES

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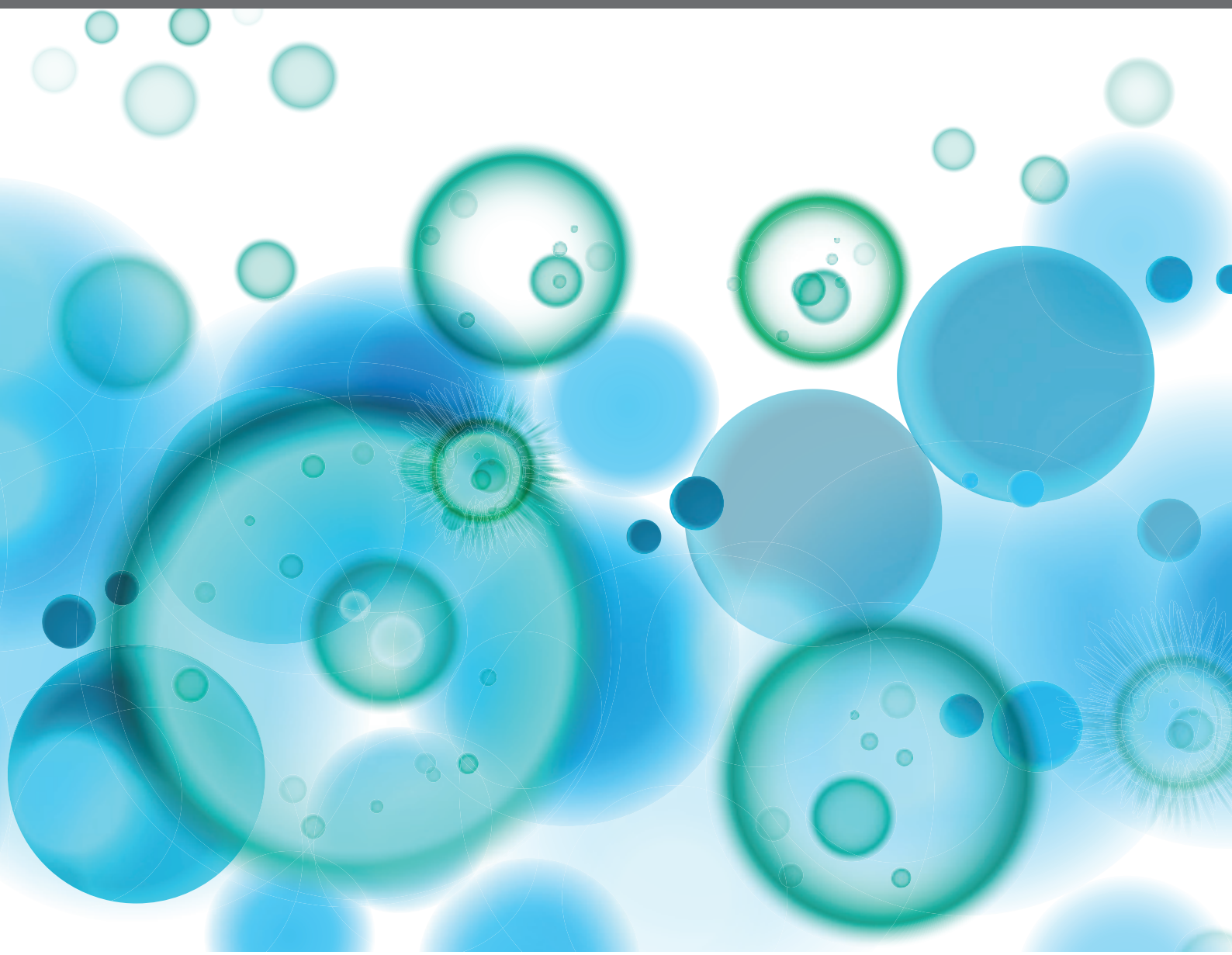

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SECONDARY RESPIRATORY INFECTIONS IN THE CONTEXT OF ACUTE AND CHRONIC PULMONARY DISEASES

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EDITORIAL
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Editorial: Secondary Respiratory Infections in the Context of Acute and Chronic Pulmonary Diseases

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Keywords: respiratory viruses, influenza, COPD, cystic fibrosis, sepsis, pneumonia, innate immune response, immunotherapy

Editorial on the Research Topic

Secondary Respiratory Infections in the Context of Acute and Chronic Pulmonary Diseases

Despite major advances in the identification of key pathophysiological mechanisms and in treatment, respiratory bacterial infections arising from pulmonary insults remain a major clinical issue today. Acute or chronic, sterile or infection-driven, pulmonary inflammation predisposes to secondary respiratory tract infections. Secondary bacterial infections often result in lethal synergy with primary infection in the lung or, in the case of sepsis, throughout the body. Different mechanisms are implicated in this enhanced susceptibility including loss of barrier integrity and impaired host defenses. Today's treatments of secondary bacterial infections are still not effective enough and antibiotic resistance is a major issue. Hence, there is an urgent need for novel therapies. A better understanding of the mechanisms of why secondary bacterial infections arise is of key importance in order to propose novel interventional strategies. In this Research Topic, a series of reviews and original articles provide a timely survey of mechanisms leading to respiratory tract bacterial infections that occur following pulmonary insult including viral (mostly influenza) infections, cystic fibrosis, chronic obstructive pulmonary disease (COPD), and sepsis.

Viral infections predispose patients to secondary bacterial infections, which often have a more severe clinical course. The mechanisms underlying post-viral bacterial infections are complex, and include multifactorial processes mediated by interactions between viruses, bacteria, and the host immune system. Significant advances have been made in recent decades as illustrated by several reviews in this Research Topic. Morgan et al. review the current knowledge about mechanical and immunological mechanisms leading to bacterial super-infection post-viral infections. The authors present the emerging literature describing the role of innate immune cell suppression in secondary bacterial complications. They provide an overview of the principal functions that these cells play in pulmonary immunity, highlighting their unique ability to sense environmental factors and promote protection against respiratory bacterial infections. In particular, the authors discuss mechanisms through which respiratory viruses alter the beneficial cross-talk between airway epithelial cells and macrophages. The role of apoptotic cell clearance (efferocytosis) and reduced responsiveness of pattern recognition receptors (innate imprinting) following viral infection in susceptibility to secondary bacterial infections is highlighted. The authors discuss the importance of extracellular matrix alterations (elevated production following severe acute viral infection) in bacterial attachment, colonization and infection. Finally, they propose different areas for potential new investigation. Paget and Trottein focus on unconventional T lymphocytes (NKT cells, γδ T cells, and MAIT cells) in pulmonary defense against bacterial infection and review mechanisms leading to their dysfunctions in the context of viral-bacterial super-infection.

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The impact of the antiviral response on unconventional T cell-mediated anti-bacterial host defense is detailed. Finally, they discuss recent advances and future therapeutic opportunities based on the targeting of these cells to prevent bacterial super-infection post-influenza. Hanada et al. discuss the effect of prior viral (influenza) infection on the intestinal and (upper and lower) respiratory tract microbiomes. In this review, the authors summarize the literature on the interactions between host microbial communities in the lung mucosa and host defense. Data generated from mouse models of influenza and from human studies are presented. They also discuss mechanisms through which respiratory viruses disrupt these interactions and contribute to the pathogenesis of secondary bacterial infections. Using examples drawn from current literature, the authors highlight the great complexity of the field and how far we still need to go before proposing microbiome-based therapy. Kiedrowski and Bomberger summarize the current knowledge about the interactions between viruses and bacteria in the cystic fibrosis upper and lower respiratory tract and how super-infections impact the health of individuals with cystic fibrosis. The authors discuss the altered immune and metabolic states in the cystic fibrosis lung due to persistent inflammation and how this may impact bacterial infections. The link between viral exacerbations of cystic fibrosis and bacterial acquisition and outgrowth are covered in detail. Similarly, Wang et al. focus their review on dysregulated inflammation and altered reactive oxygen species production as central causes for acute and chronic bacterial infections in the context of COPD. In the COPD lung, persistent impairment of lung phagocytes results in protease imbalance and tissue damage. The authors go on to focus on interactions between serum amyloid A and formyl peptide receptor 2 (FPR2), which promotes neutrophil chemotaxis and survival. FPR2 signaling in this fashion inhibits the function of resolvin D1, a fatty acid metabolite with anti-inflammatory properties. Potential targeting of resolvin D1 is speculated as a method to limit COPD exacerbations. Finally, two reviews focus on bacterial (nosocomial) super-infection in the context of sepsis which is also characterized by depressed host (pulmonary) defenses. First, Denstaedt et al. recapitulate the dynamics of the septic host response and the balance of inflammatory and anti-inflammatory cellular programs that occur. The authors summarize the epidemiology of nosocomial infections and characteristic immune responses associated with sepsis, as well as immunostimulatory therapies currently under clinical investigation. Sepsis inhibits host defense in distal organs through a variety of pathways including: decreased cytokine production, cytokine receptor antagonism, impaired pattern recognition receptor signaling, suppressor cell activation (myeloid-derived suppressor cells, regulatory T cells), induction of T cell exhaustion, and epigenetic reprogramming. The potential to target these immune deficiencies is discussed in detail. Complementary to this work, Bouras et al. focus their review on dendritic cell paralysis during sepsis and in their contribution to sepsis-induced immunosuppression. They describe the underlining mechanisms involved and propose a set of interventional strategies to overcome this process. Finally, Paolicelli et al. discuss the complexity of the epithelial barrier and introduce the use of lung organoids to better understand interleukin (IL)-17 signaling and host defense against bacterial infections. The relative strengths and weaknesses of air-liquid interface epithelial cell cultures, lung on a chip technology, and lung epithelial organoids are discussed. The use of these approaches will provide critical insight into cytokine, epithelial cross-talk in host defense against extracellular pathogens.

In original research articles, Shepardson et al. and Gopal et al. focus on type I interferons (IFN), a family of cytokines that regulate both anti-influenza immunity and host susceptibility to subsequent bacterial super-infections. The type 1 IFN/signal transducer and activator of transcription 1 (STAT1) axis was previously shown to inhibit type 17 immune response resulting in exacerbation of bacterial burden and mortality during influenza and bacterial (pneumococcal and staphylococcal) infections. Gopal et al. demonstrated that STAT2, which is required for type I and type III IFN signaling and virus clearance, participates in bacterial super-infection. In this setting, STAT2 regulates macrophage phenotype and suppresses bactericidal activity. Deletion of STAT2 during influenza results in increased numbers of dual M1/M2 marker expressing macrophages in the lung that demonstrate improved bacterial clearance. Despite impaired anti-viral host defense in STAT2-deficient mice, these mice are protected from super-infection induced mortality. Shepardson et al. investigate the role of type I IFN receptor (IFNAR2). While the impact of IFNAR1 signaling in influenza and super-infection contexts has been reported, little is known about the specific role or IFNAR2. The authors show that IFNAR2-deficient mice have significantly impaired anti-viral host defense and have increased morbidity and mortality following influenza challenge. In super-infection, IFNAR2 appears to play a divergent role compared to IFNAR1 depending upon timing of bacterial challenge. At day 3 post-influenza infection, IFNAR2-deficient mice do not display altered susceptibility like IFNAR1-deficient mice. However, when challenged with bacteria at day 7 post-influenza, IFNAR2-deficient mice are protected similar to IFNAR1-deficient mice. These two studies illuminate as of yet unknown details regarding interferon signaling and susceptibility to secondary infections. Using a novel influenza A virus/*Klebsiella oxytoca* super-infection model, Lee et al. investigate host resistance and host tolerance, two processes essential for host survival during infection. In this system, the authors show that combined dysfunctional tolerance and resistance mechanisms cause worsened outcomes for the host. The authors identify *K. oxytoca* as a component of the human microbiome and cause of secondary infection post-influenza. Several unique features of this model are described including, delayed bacterial clearance, increased M1 macrophage activation, and, despite a small impact on lung leak, increased mortality vs. influenza infection alone. Finally, Jubrail et al. investigate the response of macrophages to human rhinovirus, a virus frequently isolated from COPD patients during exacerbations. Little is known about mechanisms of secondary bacterial infections with regard to non-influenza viruses. In this study, prior viral exposure blunts the macrophage responses and cytokine production to *Haemophilus influenzae*. This paralyzed macrophage phenotype was not observed in response to

bacterial stimuli alone and likely indicates commonalities between influenza and other respiratory virus induced super-infections. Additional work is necessary to determine if the immune pathways of susceptibility implicated in influenza super-infection can be generally applied to the myriad of common respiratory viruses.

To conclude, recent advances in the field have sparked interest in the role of pulmonary injury and inflammation in secondary bacterial pneumonia. By expanding this knowledge base and understanding, researchers and clinicians hope to pave the way toward devising strategies to positively modulate lung immune responses within diverse clinical scenarios to combat against opportunistic bacteria. It is clear that from a mechanistic and public health point of view, such studies will be important because of the constant aging of the population, antibiotic resistance, and limits of vaccine efficacy. Immunomodulatory therapy has become increasingly important in the treatment of cancer and auto-immune diseases. Its potential in the context of acute pulmonary illness is only now emerging. The majority of the mechanistic information that we now know regarding secondary bacterial infections has been demonstrated in the mouse model. While complex polymicrobial infections have been studied in chronic lung disease in humans, very little is known in the acute viral infection context. Translational studies are needed to determine the conservation of susceptibility mechanisms to bacterial super-infections between mice and humans in order to advance therapeutic options in support of improved clinical care.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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STAT2 Signaling Regulates Macrophage Phenotype During Influenza and Bacterial Super-Infection

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Influenza is a common respiratory virus that infects between 5 and 20% of the US population and results in 30,000 deaths annually. A primary cause of influenza-associated death is secondary bacterial pneumonia. We have previously shown that influenza induces type I interferon (IFN)-mediated inhibition of Type 17 immune responses, resulting in exacerbation of bacterial burden during influenza and *Staphylococcus aureus* super-infection. In this study, we investigated the role of STAT2 signaling during influenza and influenza-bacterial super-infection in mice. Influenza-infected *STAT2*$^{-/-}$ mice had increased morbidity, viral burden, and inflammation when compared to wild-type mice. Despite an exaggerated inflammatory response to influenza infection, we found increased bacterial control and survival in STAT2 deficient mice during influenza-MRSA super-infection compared to controls. Further, we found that increased bacterial clearance during influenza-MRSA super-infection is not due to rescue of Type 17 immunity. Absence of STAT2 was associated with increased accumulation of M1, M2 and M1/M2 co-expressing macrophages during influenza-bacterial super-infection. Neutralization of IFNγ (M1) and/or Arginase 1 (M2) impaired bacterial clearance in *Stat2*$^{-/-}$ mice during super-infection, demonstrating that pulmonary macrophages expressing a mixed M1/M2 phenotype promote bacterial control during influenza-bacterial super-infection. Together, these results suggest that the STAT2 signaling is involved in suppressing macrophage activation and bacterial control during influenza-bacterial super-infection. Further, these studies reveal novel mechanistic insight into the roles of macrophage subpopulations in pulmonary host defense.

Keywords: influenza, *Staphylococcus aureus*, super-infection, STAT2, macrophages, lung, pneumonia

INTRODUCTION

Influenza A virus infection causes significant mortality and morbidity worldwide. It is estimated that influenza infection causes 3 million hospitalizations and 250,000 deaths, globally each year (1). The innate immune system senses invading influenza viruses through a variety of pattern recognition receptors, inducing the production of inflammatory cytokines and chemokines, including type I (IFNα/β) and type III interferons (IFNλ) (2–4). Type I IFNs (IFNα/β) bind to the heterodimer of IFNaR1 and IFNaR2 receptors, associated with Jak1 and Tyk2 kinases, respectively, to produce signaling effects during influenza infection (5). Signaling through IFNaR1/2 leads to phosphorylation of STAT1 and STAT2, activating downstream complex formation with IRF9, and transcription of IFN stimulated genes (ISGs), thereby controlling viral burden. Type II IFN (IFNγ) activates only STAT1 signaling through IFNγR1 and IFNγR2 and plays a role in cell-mediated immunity (6, 7). Type III IFNs signal through IFNLR and IL10R2 to induce ISGs similar to those induced by type I IFN (7). Type I, type II and type III IFNs have been shown to have important roles during influenza infection (7–9). In the absence of type I or type II IFN during influenza infection, increased granulocytic or lymphocytic inflammation, respectively, has been reported (7). Absence of STAT1 signaling specifically resulted in an increased granulocytic and Th2-skewed response (7), and both STAT1 and STAT2 were crucial for viral control and survival in mice (9).

Secondary bacterial pneumonia during influenza infection is a common cause of influenza-associated hospitalization and mortality during both seasonal and pandemic outbreaks (10, 11). During the 1918 influenza pandemic, *Streptococcus pneumoniae* was the most common bacteria isolated from influenza-bacteria super-infected patients (10). However, recent reports have shown that *Staphylococcus aureus* is now the most frequent super-infecting bacteria (10, 12). We have shown that during influenza-bacterial super-infection, influenza-induced type I IFN inhibited *S. aureus*-induced Type 17 immunity and associated antimicrobial peptide (AMP) production (13, 14). Further, we and others have shown that mice lacking the type I IFN receptor cleared *S. aureus* and *S. pneumoniae* better than wild-type (WT) mice during super-infection (13, 15). While influenza infection alters host defense to *S. aureus*, the converse is also likely true; *S. aureus* increases influenza burden in the lung, possibly by affecting STAT1-STAT2 dimerization during super-infection (13, 16). These data suggest that influenza-induced STAT1 and STAT2 signaling is critical to mediating susceptibility to secondary bacterial pneumonia. We have recently shown that STAT1 is involved in increasing bacterial burden through suppression of the Type 17 immune response during influenza-bacterial super-infection (17). However, little is known regarding the specific role of STAT2 in super-infection.

Since type I and type III IFN signaling relies on STAT2, while type II IFN signals solely through STAT1, examination of STAT2 deficiency enables a more targeted evaluation of type I and type III IFN-mediated immune responses. In this study, we investigated the role of STAT2 signaling during influenza infection and influenza-bacterial super-infection by infecting WT and *Stat2*$^{-/-}$ mice with influenza A/PR/8/34 followed by MRSA (USA300) challenge, and evaluating subsequent survival, morbidity, viral and bacterial burden, and inflammatory responses. We then elucidated differential host responses in *Stat2*$^{-/-}$ mice using RNA expression, flow cytometry, immunohistochemistry and *in vitro* macrophage culture. Further, we investigated the role of hematopoietic and non-hematopoietic STAT2 signaling during influenza-bacterial super-infection. These studies are the first to define the role of STAT2 signaling in influenza, bacterial super-infection and identify a novel macrophage-dependent mechanism of susceptibility to secondary bacterial pneumonia.

MATERIALS AND METHODS

Mice

WT C57BL/6 (6 to 8-week-old) mice were purchased from Taconic Farms (Germantown, NY). *Stat2*$^{-/-}$ mice on C57BL/6 background were a kind gift from Dr. Christian Schindler, Columbia University, NY (18), and colonies were subsequently maintained under specific pathogen-free conditions. *In vivo* studies were performed on age matched adult male mice, unless otherwise indicated. All experiments were approved by the University of Pittsburgh IACUC (19).

Murine Infections

Influenza A/PR/8/34 (influenza H1N1) was propagated in chicken eggs as described (20). Mice were infected with 100 plaque-forming units (PFU) of influenza in 40 μl of sterile PBS, unless otherwise noted. MRSA, USA 300, was provided by Dr. Alice Prince, Columbia University, NY. MRSA stocks were grown overnight in casein hydrolysate yeast extract-containing modified broth medium at 37°C and diluted to an inoculum of 5×10^7 CFU in 50 μl of sterile PBS. MRSA dosing was calculated using OD_{660} measurement of overnight cultures and application of an extinction coefficient. For survival experiments, 2×10^8 CFU of MRSA were delivered. All infections were performed on isoflurane-anesthetized mice via oropharyngeal aspiration. For super-infection experiments, mice were challenged with influenza or vehicle and then infected with MRSA or vehicle on day 6 after influenza infection (13, 21, 22). Mouse tissues were collected 24 h after MRSA or vehicle challenge. To neutralize IFNγ, mice were treated with 300 μg anti-IFNγ (XMG1.2) antibody in 200 μl sterile PBS (BioXCell, West Lebanon, NH) or rat IgG isotype control via intraperitoneal (IP) injection on days 4 and 6 post-influenza infection. To neutralize arginase, mice were treated with 100 μg N-$^{-}$hydroxy-nor-L-arginine (nor-NOHA) via IP injection on days −1, 0, 3, and 6 post-influenza.

Measurement of Lung Inflammation

Lungs were perfused with 1 ml of sterile PBS and cell differential counts were performed on cytospin smears from bronchoalveolar lavage (BAL) fluid stained with Protocol Hema 3 staining (Fisher Scientific, Kalamazoo, MI). The cranial lobe of the right lung was homogenized in sterile PBS by mechanical grinding, for quantification of bacterial burden by plating serial dilutions and for cytokine production measurement

by Lincoplex (Millipore, Billerica, MA), Bio-plex (Bio-Rad, Hercules, CA), or ELISA (R&D Systems, Minneapolis, MN). The middle and caudal lobes of the right lung were snap-frozen and homogenized in liquid nitrogen for RNA isolation using the Absolutely RNA Miniprep Kit (Agilent Technologies, Santa Clara, CA). Gene expression was analyzed by RT-PCR utilizing commercially available Taqman primer and probe sets (Applied Biosystems, Foster City, CA). Fold changes in mRNA expression were calculated using the $\Delta\Delta$CT method, and were normalized to the endogenous housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). The left lobe of the lung was pressure inflated and fixed in 10% neutral-buffered formalin for histology, or collected in DMEM media for flow cytometry. Histology was scored by a sample blinded pathologist.

Flow Cytometry

Mouse lungs were aseptically dissected into small sections, digested for 30 min at 37°C in 1 mg/mL collagenase media, and passed through 70 μm filters (23). Single cell suspensions were stained with anti-CD3 (145-2C11), CD4 (RM4-5), CD11b (M1/70), Ly6C (HK1.4), CD80(16-10A1), and macrophage galactose lectin (MGL) (LOM-14). After the singlets were gated from total cells, macrophages were gated for $CD11b^+Ly6C^+$ cells. Further, these cells were gated for CD80 and MGL to determine macrophage phenotype as M1 ($CD80^+$) and/or M2 (MGL^+). The frequency of $CD11b^+Ly6C^+$, $CD11b^+Ly6C^+$ $CD80^+$, $CD11b^+$ $Ly6C^+$ $CD80^+$ MGL^+, and $CD11b^+$ $Ly6C^+$ MGL^+ cells were calculated from the frequency of total cells. For intracellular staining, cells were stimulated for 4 h with 50 ng/mL of phorbol myristate acetate (PMA) and 750 ng/mL ionomycin (Sigma-Aldrich, St Louis, MO) with Golgi plug (BD Pharmingen, San Diego, CA) added 1 h into stimulation. After stimulation, cells were surface stained, permeabilized with cytofix-cytoperm solution (BD Pharmingen, San Diego, CA), and stained with antibodies specific for IL-17 (TC11-18H10), IL-22 (Poly5164) for 30 min at 4°C. The percentage of IL-17^+ and IL-22^+ cells were determined from gating on $CD3^+CD4^+$ T cells. Cells were collected in a Becton Dickinson FACS Aria flow cytometer with FACS Diva software (BD, Franklin Lakes, NJ). Flow cytometric analysis was performed using FlowJo (Tree Star, Ashland, OR).

Immunohistochemistry

Left lung lobes were perfused and stored in 10% neutral buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and pulmonary inflammation was evaluated by microscopy. For immunofluorescent staining, formalin-fixed lung sections were incubated at 60°C, and quickly immersed in xylene to remove paraffin. Sections were then hydrated in 96% alcohol and PBS. Antigen retrieval was performed by incubating slides in boiling Dako Target Retrieval Solution (S1699, DAKO Cytomation), followed by blocking with 5% (v/v) normal donkey (017-000-121, Jackson ImmunoResearch Laboratories) serum and Fc block (BD Pharmingen, San Diego, CA). Sections were stained with antibodies against inducible NO synthase (iNOS) (goat anti-mouse iNOS, clone M-19; Santa Cruz Biotechnology Inc.), F4/80 (clone Cl:A3-1, MCA497GA, Bio-Rad), and arginase-1 (Arg1) (rabbit anti–arginase I, clone H-52; Santa Cruz Biotechnology Inc.). Primary antibodies were detected with Alexa Fluor 568-donkey anti-goat Ig G (H+L) cross adsorbed (A-11057; Invitrogen) to detect iNOS, donkey anti-rabbit Ig G (H+L) antibody conjugated to FITC (711-095-152, Jackson Immuno Research Laboratories) to visualize Arg1. Slides were incubated with biotin-$F(ab')_2$ donkey anti-rat (712-006-153 Jackson Immuno Research Laboratories), followed by Cy5-Streptavidin (405209, Biolegend) to reveal the location of $F4/80^+$ macrophages. Vectashield anti-fade mounting medium with DAPI (H-1200, Vector Laboratories) was used to counterstain tissues and to detect nuclei. Images were obtained with a Zeiss Axioplan 2 microscope and recorded with a Zeiss AxioCam digital camera. iNOS, F4/80, and Arg1 positive cells were enumerated in three random 200x fields per lung sample, and the average number of $iNOS^+$, $Arg1^+$ or double positive macrophages was calculated. Samples were analyzed in a blinded fashion.

Arginase-1 Activity Determination

Arginase-1 activity was quantified in lung BAL using the QuantiChrom Arginase Assay Kit as per the manufacturer's instructions (BioAssay Systems). In BAL samples, total protein was measured by using BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL). Protein concentration was adjusted to 100 μg/ml per sample. Arg-1 activity is expressed as U/l of sample.

Nitrite Quantitation

Nitrite was quantified in BAL samples by using Griess reagent as per the manufacturer's instructions (Promega Corporation).

RNAseq Analysis

RNA was isolated from C57BL/6 and $Stat2^{-/-}$ mouse lungs using the Agilent RNA miniprep kit. RNA integrity was determined using an Agilent 2100 bio analyzer. mRNA was purified by using Sera-Mag Oligo(dT) Beads, fragmented with magnesium-catalyzed hydrolysis, and reverse transcribed into cDNA using random primers (Superscript II; Invitrogen). Then, cDNA underwent end repair with T4 DNA polymerase and Klenow DNA polymerase, followed by the addition of "A" bases to the 3′ end and ligation to adaptor oligonucleotides. Products from the ligation were run on a 2% agarose gel. A gel slice consisting of the 200 bp region (±25 bp) was excised and used as a template for PCR amplification. The final PCR product was purified, denatured with 2 N NaOH, and diluted to 10–12 pM prior to cluster amplification on a single-read flow cell v4, as outlined in the Single-Read Cluster Generation Kit v4 (Illumina). The flow cell was sequenced on an Illumina Genome Analyzer II. The data were analyzed as previously described (24). Full sequencing data has been uploaded to the National Center for Biotechnology Information Gene Expression Omnibus, GSE119029.

Bone Marrow-Derived Macrophage (BMDM) and Dendritic Cell (BMDC) Generation

Bone marrow cells were isolated from the femurs and tibias of mice, and grown for 7 days in complete DMEM media supplemented with 20 ng/mL GM-CSF (PeproTech, NJ), as previously described (25). On day 7, non-adherent cells (BMDCs) were collected, and adherent cells (BMDMs) were recovered using cell scraper (Genemate, Kaysville, UT) or by gentle mechanical scraping.

Bone Marrow-Derived Macrophage (BMDM) and Dendritic Cell (BMDC) Stimulation

Bone marrow derived cells were plated to 1×10^6 cells/mL and cells in 1 mL of media in 24 well tissue culture-treated plates, rested overnight at 37°C in 5% CO_2, then treated with IFN-β (10 units/mL) or IFN-γ (10 ng/mL) (R&D Systems, MN). Twenty-four hours later, cell culture supernatants were harvested and cells were lysed in RLT buffer and frozen at −80°C for RNA extraction using the RNAeasy mini kit (QIAGEN, Germantown, MD. Gene expression was analyzed as described above.

BMDM Phagocytosis and Killing Assay

BMDMs were incubated with fluorescein isothiocyanate or Alexa Fluor-647 (Molecular Probes) —labeled MRSA (10 MOI) for 30 min. After washing, flow cytometry was performed to determine bacterial uptake. To determine bacterial killing by macrophages, BMDMs were incubated with MRSA for 1 h. Cells were washed with PBS and the remaining macrophages were incubated with gentamycin (300 μl/ml) for 15 min to kill extracellular bacteria. Cells were washed and incubated for an additional hour to determine killing of intracellular bacteria. Cells were washed and lysed with 0.5 ml of 0.02% Triton X-100 in PBS, and plated to determine the percentage of intracellular bacterial killing.

Bone Marrow Chimera (BMC) Mice

To generate BMC mice, C57BL/6 (Thy1.1), C57BL/6 (Thy1.2), and *Stat2*$^{-/-}$ (Thy 1.2) mice were fed with Sulfa-Trimm diet containing 1.2% sulfamethoxazole and 0.2% trimethoprim for 2 weeks before irradiation (26, 27). Mice were sub-lethally irradiated twice with two doses of 600 rad delivered 4 h apart. Mice were reconstituted with 1×10^7 bone marrow cells from either C57BL/6 (Thy1.1), C57BL/6 (Thy1.2), or *Stat2*$^{-/-}$ (Thy 1.2) mice as previously described (26). Mice were allowed to reconstitute for 9 weeks.

Statistical Analysis

Data were analyzed using GraphPad Prism software. Experiments were repeated 2 to 5 times. All data are presented as mean ± SEM, unless otherwise noted. Significance was determined by unpaired Student's *t* test or one-way ANOVA followed by *post-hoc* Bonferroni or Tukey's test for multiple comparisons. Mortality data was analyzed by Log-rank (Mantel-Cox) test.

RESULTS

Influenza Severity Is Increased in *Stat2*$^{-/-}$ Mice Compared to WT

To determine the role of STAT2 signaling during influenza infection, WT and *Stat2*$^{-/-}$ mice were infected with influenza. By day 6 of influenza infection, influenza-challenged *Stat2*$^{-/-}$ mice showed significantly more weight loss than WT mice and increased lung viral burden (**Figures 1A,B**). In males, infection was sub-lethal in both *Stat2*$^{-/-}$ and WT mice, but *Stat2*$^{-/-}$ mice showed markedly delayed recovery, requiring 35 days to return to baseline weight, versus 14 days for WT mice (**Figure 1C**). In females, *Stat2*$^{-/-}$ mice showed increased mortality compared to WT during influenza infection (**Figure 1D**). Next, we determined the cellular inflammatory response to influenza infection in WT and *Stat2*$^{-/-}$ mice. There was a significantly greater number of polymorphonuclear cells (PMN, neutrophils) observed in bronchoalveolar lavage (BAL) from *Stat2*$^{-/-}$ mice when compared to WT (**Figure 1E**). Interestingly, *Stat2*$^{-/-}$ mice had significantly fewer numbers of macrophages and lymphocytes in BAL compared to WT mice (**Figure 1E**).

Next, we determined whether type I IFN levels were altered during influenza infection. We found a trend toward increased levels of IFNβ in *Stat2*$^{-/-}$ mice when compared to WT mice during primary influenza infection (**Figure 1F**). Influenza infection induced a variety of inflammatory cytokines such as IL-1β, IL-6, TNFα, IL-8, CCL2, CCL5, and CXCL10 from epithelial and immune cells (2). We found an increase in the levels of the inflammatory cytokines IL-1α, IL-1β, TNFα, IL-6, IL-12p40, IL-17, and IFNγ, and increased levels of the Type 2 cytokines IL-4 and IL-5, in influenza-infected *Stat2*$^{-/-}$ mice when compared to WT mice (**Figures 1G,H**).

It has been shown that IL-17 signaling induces the chemokines CXCL1, CXCL2, CXCL5, and CXCL8 to mediate granulopoiesis and increased neutrophil recruitment to mucosal sites (28, 29). Consistent with the increase in PMNs, we observed an increase in CXCL1 and the granulopoietic factor, G-CSF in *Stat2*$^{-/-}$ when compared to WT mice (**Figure 1I**). Further, scoring of pulmonary inflammation indicated an increase in perivascular infiltration of cells in the lungs of *Stat2*$^{-/-}$ mice when compared to WT mice (**Figures 1J,K**). Together, these data demonstrate that influenza-infected *Stat2*$^{-/-}$ mice have more severe influenza infection, which is associated with an increased inflammatory response, compared to WT mice.

Stat2$^{-/-}$ Mice Are Rescued From Impaired Bacterial Clearance During Influenza/MRSA Super-Infection

We have previously shown that influenza-associated IFNβ attenuated host bacterial defense due to suppression of Type 17 immunity (13, 14). Also, we recently identified that Type 17 immunity is rescued in the absence of STAT1 signaling during influenza-bacterial super-infection (17). In this study, we examined the effects of MRSA challenge on day 6 of influenza infection in WT and *Stat2*$^{-/-}$ mice. One day following

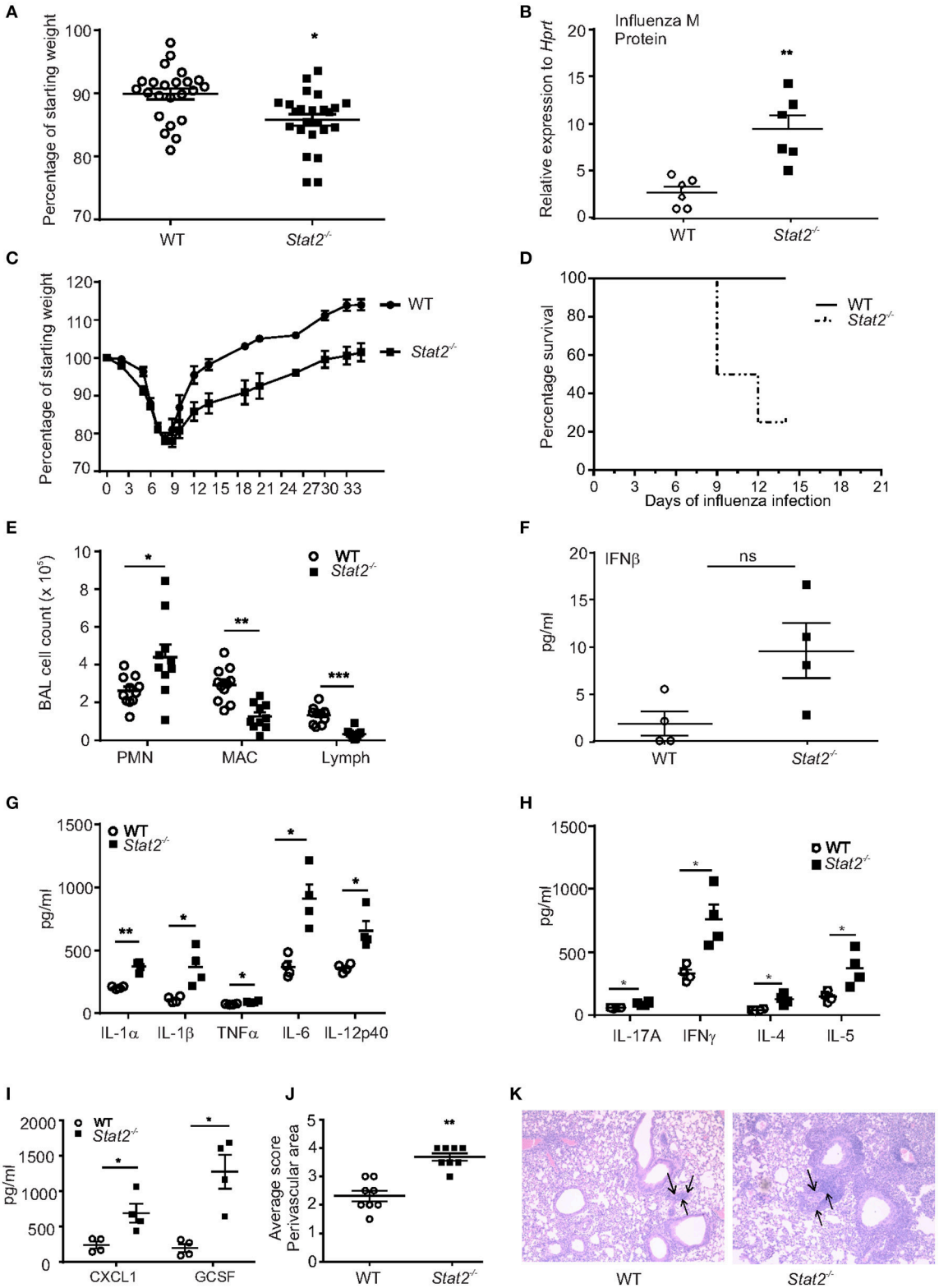

FIGURE 1 | *Stat2*$^{-/-}$ mice have increased susceptibility to influenza infection. **(A)** WT, *Stat2*$^{-/-}$ male 6–8 weeks mice were infected with 100 PFU of influenza, percentage weight loss on day 6 of influenza infection, $N = 22$–24 per group. **(B)** Viral burden was measured by influenza M protein expression 6 days following influenza infection in whole lung, $N = 6$ per group. **(C)** Weight loss during 35 days following influenza infection, $N = 8$ per group. **(D)** Percentage survival following

(Continued)

FIGURE 1 | influenza infection in females, $N = 8$ per group. **(E)** BAL samples collected on day 6 following influenza infection from mice lungs, cytospin the cells and differential counts were made $N = 10–12$ per group. Right upper lung lobes were homogenized in PBS, **(F)** IFNβ levels were measured in BAL samples by using ELISA, $N = 4$ per group. **(G)** IL-1α, IL-1β, TNFα, IL-6, and IL-12p40, **(H)** IL-17A, IFNγ, IL-4, and IL-5 **(I)** CXCL1, and GCSF levels were measured by Luminex assay, $N = 4$ per group. Representative data shown from 3 or more experiments are shown **(J)** On day 6 post infection, lungs were fixed in 10% formalin, embedded in paraffin, stained with H&E, lung perivascular areas (arrows) were scored. **(K)** Representative histology pictures are shown. Original magnification for H&E sections × 100. Data are represented as mean±SEM, two tailed Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, not significant.

MRSA challenge, super-infected WT mice had significantly increased lung bacterial burden compared to mice infected with MRSA alone, demonstrating impaired bacterial clearance from the lung caused by preceding influenza infection (**Figure 2A**). However, *Stat2*$^{-/-}$ mice were rescued from this clearance defect (**Figure 2A**). To assess survival in the context of super-infection, adult female mice were subjected to influenza/MRSA super-infection using a dose of MRSA previously determined to be lethal to WT mice. Given the increased severity of influenza infection seen in females, mice were infected with a lower dose of influenza to minimize the confounding effects of lethal influenza infection. Under these conditions, *Stat2*$^{-/-}$ mice showed significantly delayed mortality (**Figure 2B**). Next, we found that BAL fluid from both *Stat2*$^{-/-}$ and WT mice had significantly greater numbers of neutrophils compared to macrophages or lymphocytes (**Figure 2C**). However, we observed no significant differences in neutrophils, macrophages, or lymphocytes between super-infected WT and *Stat2*$^{-/-}$ mice (**Figure 2C**). Further, we found no differences in the levels of cytokines IL-1α, IL-1β, IL-6, TNFα, IL-12p40, IL-17A, IL-10, CXCL1, and GCSF between WT and *Stat2*$^{-/-}$ mice (**Figures 2D–F**). However, the levels of IFNγ, IL-4 and IL-5 were elevated in *Stat2*$^{-/-}$ mice during super-infection (**Figure 2E**). In agreement with similar cellularity and local production of pro-inflammatory cytokines, histological scoring showed no differences in perivascular inflammation (**Figures 2G,H**). These findings demonstrate that *Stat2*$^{-/-}$ mice are rescued from impaired bacterial clearance in the lung during super-infection, which translated into a detectable survival advantage, but this was independent of any noticeable differences in lung inflammation.

We next addressed whether the increased bacterial clearance observed in *Stat2*$^{-/-}$ mice was due to altered Type 17 immunity. Interestingly, numbers of IL-17 and IL-22 positive CD4 cells, and the levels of Type 17 cytokines (IL-17, IL-22, and IL-23) were not altered in *Stat2*$^{-/-}$ mice compared to WT mice (**Figures S1A–E**). In further support of this finding, no differences in expression levels of the Th17 transcription factors *Rorc* and *Rora*, and Th17 immune mediated antimicrobial peptide (AMP) such as *Lcn2* and *Reg3b* production in the lungs were detected between *Stat2*$^{-/-}$ and WT mice (data not shown). These data suggest that protection in *Stat2*$^{-/-}$ mice is not mediated by rescue of Type 17 immunity.

Rescue of Bacterial Clearance in *Stat2*$^{-/-}$ Mice Is Associated With M1 and M2 Signature in Infected Lungs

Next, to determine the pathways associated with this increased bacterial clearance in *Stat2*$^{-/-}$ mice, we performed RNA sequencing analyses. We found increased expression of Type 1 (IFNγ) and Type 2 (IL-4 and IL-13) cytokines in *Stat2*$^{-/-}$ mice compared to WT (**Figures 2E**, **3A,B**). In response to IFNγ stimulation, macrophages increase their production of nitric oxide and reactive oxygen intermediates to become M1 type macrophages, which exhibit proinflammatory and antibacterial activity (30, 31) Accordingly, we found increased RNA abundance of genes associated with the M1 phenotype including *Nos2*, *Tnfa*, *Tnfsf13b*, *Batf2,Ido1,* and chemokines such as *Cxcl9*, *Ccl5,* and *Ccl8* in the lungs of *Stat2*$^{-/-}$ mice compared to WT during super-infection (**Figure 3A**).

In response to IL-4, IL-13, and glucocorticoids, macrophages express markers of the M2 phenotype, which is characterized by induction of Arg1, chitinase 3-like 3 (Ym), found in inflammatory zone-1 (Fizz1), resistin-like molecule (Relmα/Retnla), chemokines such as CCL17, CCL20, and CCL22, scavenger receptors (CD36, Macrophage receptor with collagenous structure or MARCO), and c-type lectin receptors or CLRs (CD209, Macrophage Galactose Lectin or MGL) (30, 32). Accordingly, we found increased RNA abundance of *Arg1, Socs1, Sra1, Saa1, Ciita, Marco, Mgl2, Cd209d, Cd209e, Cd209f, Ccl1, Ccl11, Ccl17, Ccl19, Ccl20, Ccl22, and Ccl24* genes in *Stat2*$^{-/-}$ mice when compared to WT mice during super-infection (**Figure 3B**).

Next, we confirmed the observed RNA abundance of the classical M1 and M2 macrophage markers. We found increased gene expression of *Nos2* and Nitrite in *Stat2*$^{-/-}$ mice compared to WT with super-infection (**Figures 3C,D**). IFNγ-inducible chemokines CXCL9, CXCL10, and CXCL11 are ligands for the receptor CXCR3, which is essential in the attraction of effector lymphocytes to inflammatory sites (33, 34). Accordingly, we found increased mRNA expression of *Cxcl9* in influenza/MRSA infected *Stat2*$^{-/-}$ mice compared to WT mice (**Figure 3E**). Further, we found increased expression of M1 macrophage markers *Ccl8, Ido1,* and *Tnfsf13b in Stat2*$^{-/-}$ mice during super-infection when compared to WT mice (**Figure 3F**). These data confirm the increased expression of genes associated with M1 macrophages in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection.

Next, we found increased mRNA expression and activity levels of Arg1, mRNA expression of *Fizz*, *Chi3l3*, *Cd209d*, *Cd209e, Mrc2* (*Cd206*), *Marco*, *Il13*, *Saa1* (Serum amyloid A1 protein), *Ccl17, Ccl19, Ccl20, Ccl22*, and *Ccl24* in *Stat2*$^{-/-}$ mice compared to WT mice during super-infection (**Figures 3G–K**). Together, these data show that in the absence of STAT2 signaling, both the M1 and M2 macrophage phenotypes are enhanced during super-infection.

IFNγ signals through the IFNGR1/ IFNGR2 complex and activates the transcription factor STAT1, thereby inducing ISGs.

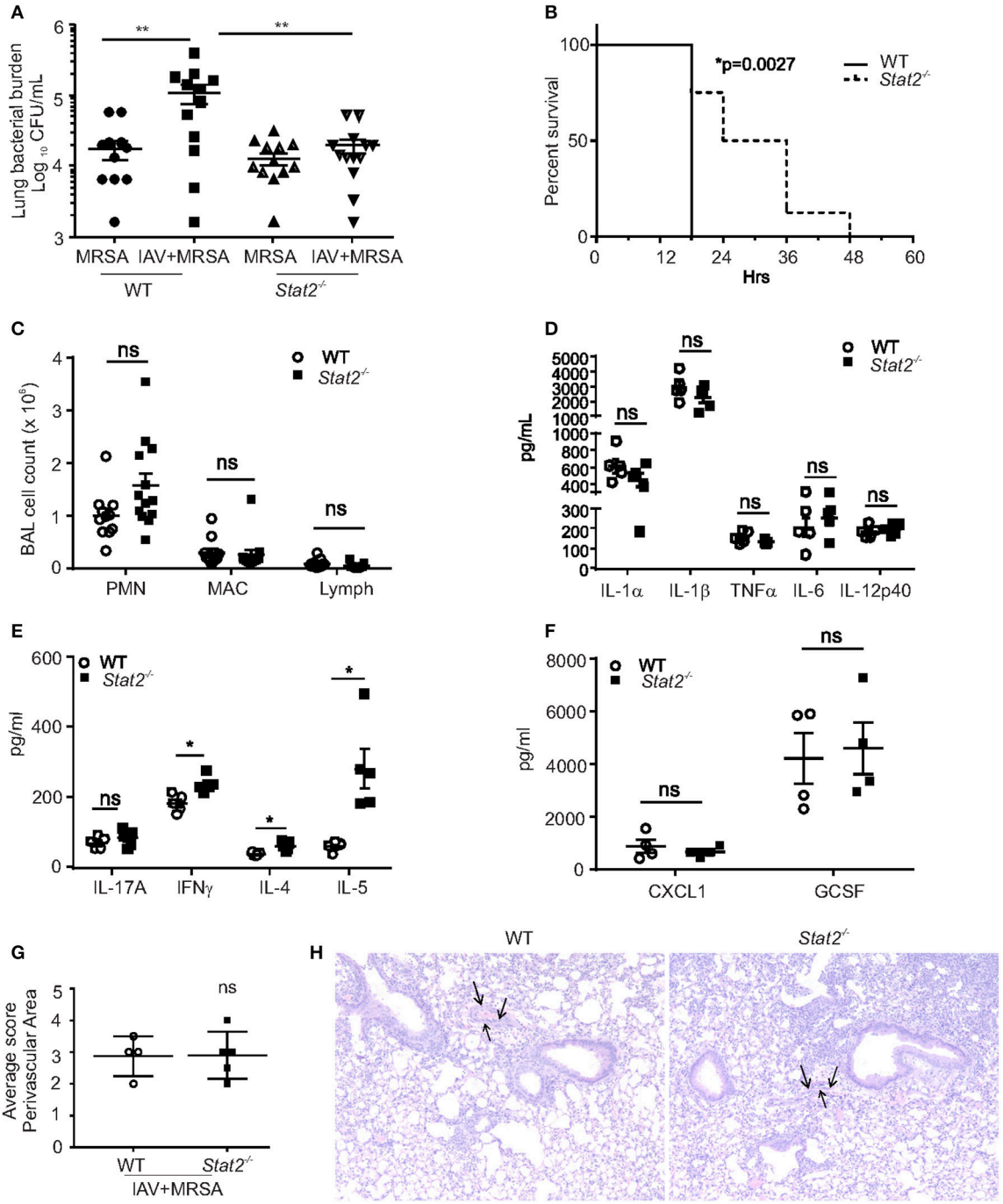

FIGURE 2 | *Stat2*$^{-/-}$ are rescued from impaired bacterial clearance from the lung following influenza infection. **(A)** WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Right upper lung lobes were homogenized in PBS and bacterial burden was determined. $N = 12$–13 per group. **(B)** Adult female mice were infected with 66 PFU influenza A PR/8/H1N1 followed by challenge with 2×10^8 CFU MRSA and the survival was determined, $N = 7$–8 per group. **(C)** BAL samples collected from co-infected B6 and *Stat2*$^{-/-}$ mice, cytospin the cells and differential counts were made, $N = 11$–13 per group. Right Upper lung lobes were homogenized in PBS, and **(D)** IL-1α, IL-1β, TNFα, IL-6, IL-12p40, **(E)** IL-17A, IFNγ, IL-4, IL-5 **(F)** CXCL1, and GCSF levels were measured by luminex assay, $N = 4$ per group. Representative data shown from three or more experiments. Lungs were fixed in 10% formalin, embedded in paraffin, **(G)** perivascular areas (arrows) were scored in formalin fixed lungs by staining with H&E **(H)** representative figures are shown, $N = 4$ per group. Original magnification for H&E sections × 100. Data are represented as mean±SEM. Data analyzed using 2-tailed Student's *t*-test or One way ANOVA followed by Bonferroni test for multiple comparisons, $^*p < 0.05$, $^{**}p < 0.01$, ns, not significant.

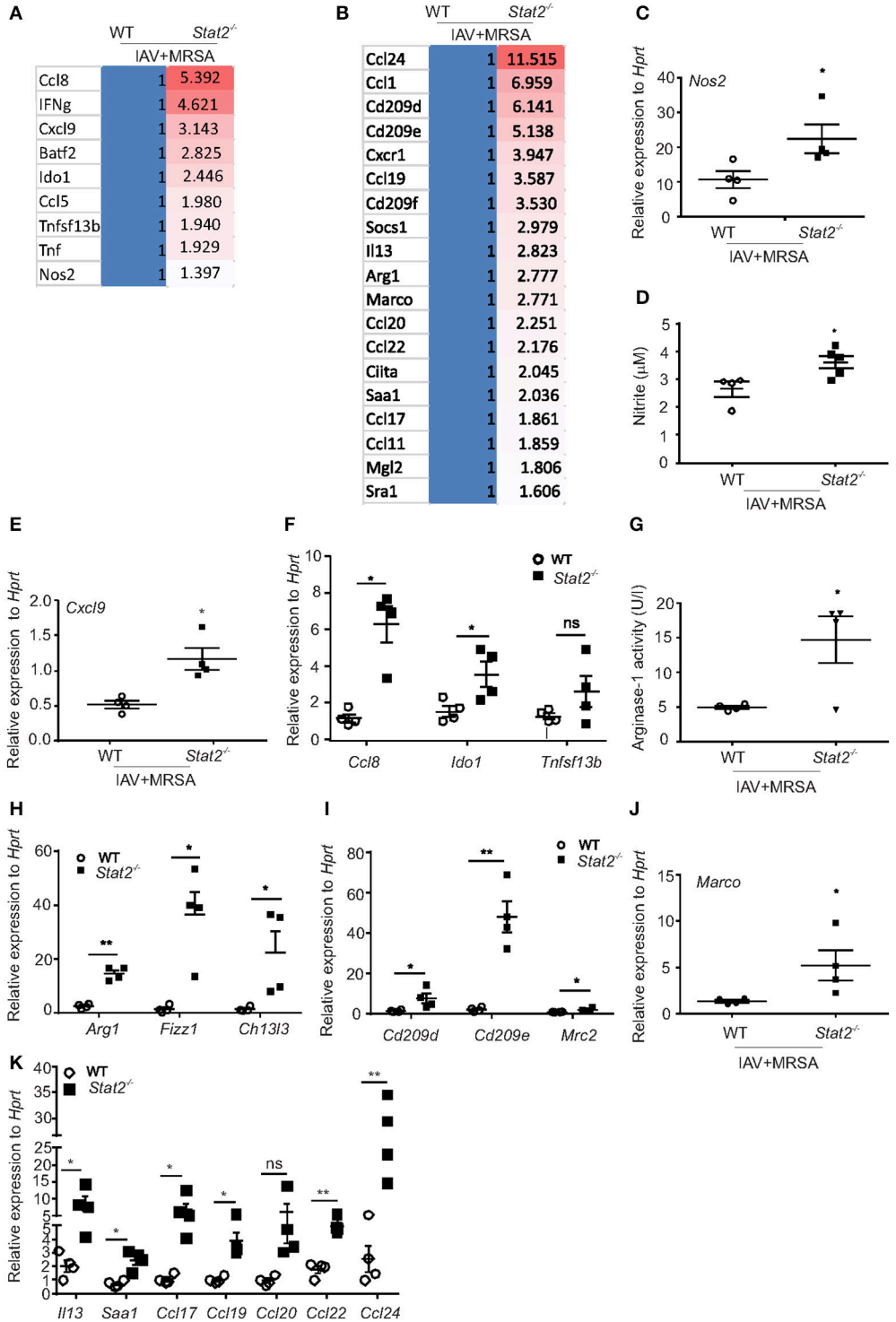

FIGURE 3 | M1 and M2 macrophages are associated with increased bacterial clearance in *Stat2*$^{-/-}$ during influenza-MRSA super-infection. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Gene expression analyses were measured in lung by RNAseq analysis. Heat-map representing RNA abundance associated with M1 **(A)** and M2 **(B)** macrophages from WT and *Stat2*$^{-/-}$ mice, $N = 4$ per group. **(C)** *Nos2*, **(D)** Nitrite, **(E)** *Cxcl9*, **(F)** *Ccl8, Ido1, Tnfsf13b* were analyzed by RT-PCR, $N = 4$ per group. **(G)** Arginase-1 activity was determined from BAL samples from both WT and *Stat2*$^{-/-}$ mice infected with influenza and MRSA super-infection, $N = 4$ per group. **(H)** *Arg1, Fizz1*, and *Chi3l3*, **(I)** *Cd209d*, *Cd209e* and *Mrc2* **(J)** *Marco* mRNA expression levels were determined by RT-PCR, $N = 3$–4 per group. **(K)** *Il13* and *Saa1, Ccl17, Ccl19, Ccl20, Ccl22*, and *Ccl24* mRNA expression levels were determined by RT-PCR, $N = 4$ per group. Representative data shown from three or more experiments. Data are represented as mean±SEM. Data analyzed using 2-tailed Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, ns, not significant.

We found increased levels of IFNγ (**Figure 2E**) and IFNγ-induced ISGs (**Figure 3E**) in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. Next, we determined whether STAT1 is altered due to increased levels of IFNγ in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. As expected, we found increased STAT1 expression in *Stat2*$^{-/-}$ mice when compared to WT mice during influenza-bacterial super-infection (**Figure S2A**).

IL-4 activates the STAT6 signaling pathway through activation of transcription factors PPARγ and PPARδ and drives M2 polarization (31, 35). Here, we tested whether STAT6 signaling is altered due to increased IL-4 levels in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. However, we found no differences in STAT6 expression levels in between WT and *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection (**Figure S2B**). Further, we measured the RNA expression of *Pparg* from WT and *Stat2*$^{-/-}$ mice during influenza-MRSA super-infection. Interestingly, we found that *Pparg* expression was suppressed in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection (**Figure S2C**). Together, these data suggest that in the absence of STAT2, there is alteration of other associated signaling pathways during super-infection.

Next, we determined the frequency of M1 and M2 macrophages by using flow cytometry. CD80 and MGL (macrophage galactose lectin) have been shown to be markers for M1 and M2 macrophages, respectively (36, 37). We found a significant increase in the percentage of CD11b^{+}Ly6C^{+} cells in influenza/MRSA infected *Stat2*$^{-/-}$ lungs when compared to WT mice by flow cytometry (**Figure 4A**). Further, we found increased frequency of M1 (CD80^{+}) and M1/M2 (CD80^{+}MGL^{+}) co-expressing macrophages in *Stat2*$^{-/-}$ mice when compared to WT during influenza-bacterial super-infection (**Figures 4B,C**). Interestingly, we found no differences in M2 (MGL^{+}) macrophages in between WT and *Stat2*$^{-/-}$ mice in super-infection (**Figure 4D**). Further, we found higher frequency of M1/M2 co-expressing macrophages when compared to M1 or M2 populations (**Figures 4B–E**).

To then determine the specific macrophages involved in production of Arg1 and Nos2, we sorted M1, M2, and M1/M2 cells by using specific antibodies, and determined the RNA expression levels of *Nos2* and *Arg1* (**Figures 4F,G**). As expected, we found increased expression of *Nos2* in M1 and M1/M2 macrophages. Next, we found no differences in induction of *Arg1* in M1 and M2 macrophages. However, we found increased expression of *Arg1* in dual M1/M2 macrophages. These data suggest that these M1/M2 macrophages induce both Arg1 and Nos2 in influenza-bacterial super-infection. To further characterize specific Arg1^{+} and iNOS^{+} macrophage localization in the lung, we used an IHC technique using iNOS and Arg1 antibodies.

Increased Accumulation of M1 and M2 Macrophages in *Stat2*$^{-/-}$ Mice Is Dependent on Preceding Influenza Infection

To determine whether the increase in M1 and M2 macrophages is influenza or MRSA dependent, we infected WT or *Stat2*$^{-/-}$ mice with influenza or MRSA or super-infection, and determined the number of iNOS^{+}F4/80^{+} and Arg1^{+}F4/80^{+} macrophages in the lung by IHC. We found increased numbers of iNOS^{+}F4/80^{+} and Arg1^{+}F4/80^{+} cells during influenza infection and super-infection in *Stat2*$^{-/-}$ mice compared to WT mice (**Figure 5A**). In WT mice, we observed a higher number of iNOS^{+}F4/80^{+} cells during MRSA and super-infection compared to influenza infection alone. In contrast, we observed a decrease in the number of Arg1^{+}F4/80^{+} cells in MRSA and super-infection compared to WT mice infected with influenza alone (**Figure 5B**). Further, no differences were observed between WT and *Stat2*$^{-/-}$ mice in the number of iNOS^{+}F4/80^{+} and Arg1^{+}F4/80^{+} cells during MRSA infection alone (**Figures 5A,B**). Next, we found increased gene expression of *Nos2* and *Arg1* in *Stat2*$^{-/-}$ mice when compared to WT mice during influenza, but not MRSA infection (**Figures 5C,D**). These data indicate that influenza infection, not MRSA infection, is likely the cause of the increased number of Arg1^{+} macrophages seen in *Stat2*$^{-/-}$ mice during super-infection.

The M1 and M2 macrophage phenotypes are thought to antagonize each other to establish inflammatory or anti-inflammatory responses (38). M1 macrophages induce L-hydroxy-arginine and citrulline through NOS2 induction, which is a potent inhibitor of arginase (39), to promote inflammation. Conversely, M2 macrophage induction of arginase antagonizes M1 markers to promote repair and resolution of infection (39). As we seen in flow cytometry data, we found an increased number of "dual function" iNOS^{+}Arg1^{+}F4/80^{+} cells during and super-infection in *Stat2*$^{-/-}$ mice compared to WT mice (**Figures 5E,F**). However, no significant differences were observed in the number of dual function iNOS^{+}Arg1^{+}F4/80^{+} cells between WT and *Stat2*$^{-/-}$ mice during MRSA infection (**Figures 5E,F**). Together, these data show that in the absence of STAT2 signaling, the increase in Type 1 and Type 2 cytokines during influenza infection creates a pulmonary environment that supports both M1 and M2 macrophage differentiation after super-infection. Unexpectedly and contrary to the idea that there is antagonism between M1 and M2 macrophages, we found increased accumulation of M1, M2 and M1/M2 co-expressing cells in the lungs of *Stat2*$^{-/-}$ mice after super-infection.

Dual Function M1/M2 Macrophages Are Required for Control of Bacterial Burden in *Stat2*$^{-/-}$ Mice

We next examined whether M1 and/or M2 macrophages are required for the improved bacterial clearance in *Stat2*$^{-/-}$ mice during super infection. We treated both influenza-infected WT and *Stat2*$^{-/-}$ mice with anti-IFNγ, followed by super-infection with MRSA, and measured subsequent bacterial burden. We found that blocking IFNγ decreased bacterial clearance in *Stat2*$^{-/-}$ mice (**Figure 6A**). IFNγ neutralization showed a trend toward decreased bacterial clearance in WT mice as well. Next, we found fewer iNOS^{+}F4/80^{+} cells in anti-IFNγ-treated WT and *Stat2*$^{-/-}$ mice compared to mice treated with isotype controls (**Figure 6B**). Further, we found a decreased number of dual function iNOS^{+}Arg1^{+}F4/80^{+} in mice treated with anti-IFNγ antibody (**Figures 6C,D**). However, consistent with the role of

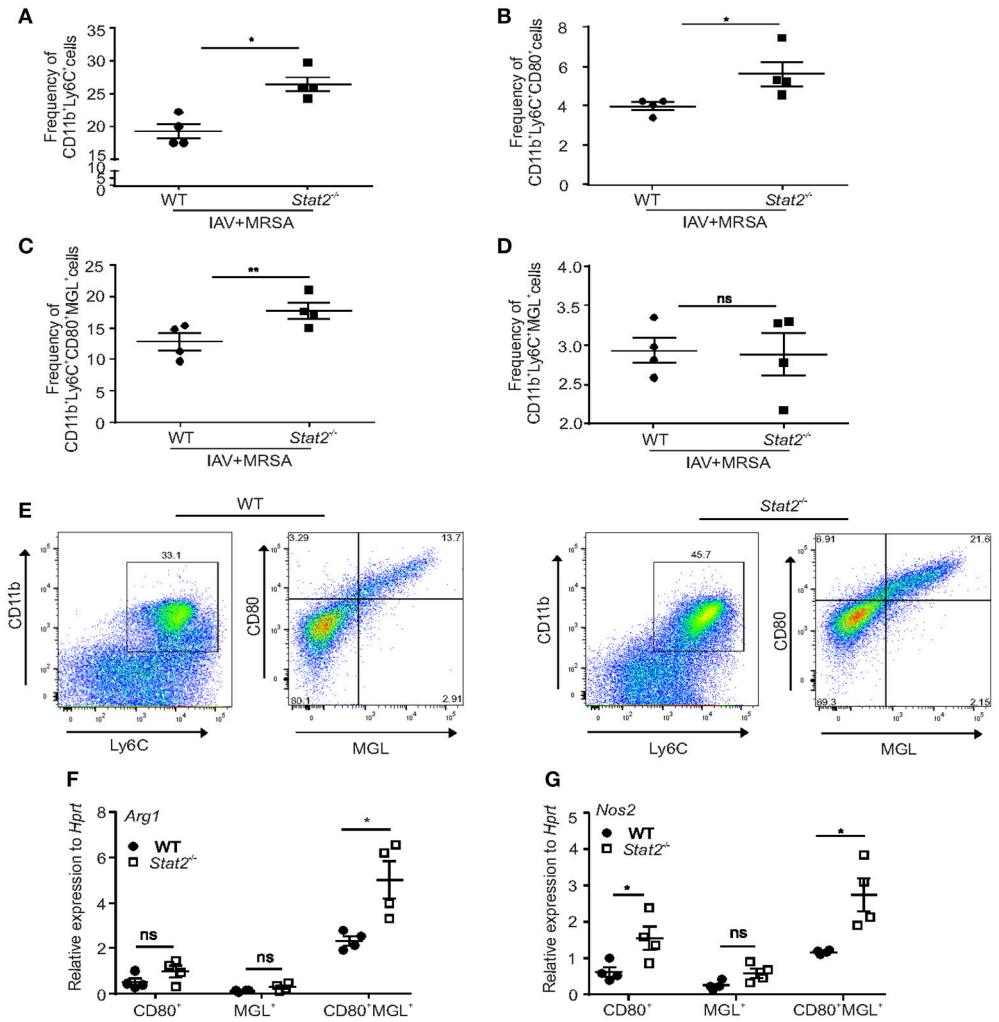

FIGURE 4 | Increased frequency of M1/M2 macrophages in *Stat2*$^{-/-}$ during influenza-MRSA super-infection. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. **(A)** Frequency of CD11b$^+$Ly6C$^+$ cells, **(B)** CD11b$^+$Ly6C$^+$CD80$^+$ cells, **(C)** CD11b$^+$Ly6C$^+$MGL$^+$ cells **(D)** CD11b$^+$Ly6C$^+$CD80$^+$MGL$^+$ cells from lungs were determined from influenza and MRSA super infection by flow cytometry, $N = 4$ per group. Double positive CD11b$^+$Ly6C$^+$ cells were gated for CD80 and MGL to identify the CD11b$^+$Ly6C$^+$CD80$^+$, CD11b$^+$Ly6C$^+$MGL$^+$, CD11b$^+$Ly6C$^+$CD80$^+$MGL$^+$ cells. **(E)** The representative figures were shown. CD80$^+$, MGL$^+$, CD80$^+$MGL$^+$ cells were sorted from the lung using FACS and Arg1 **(F)** NOS2 **(G)** RNA expression was analyzed using RT-PCR. $N = 4$ per group. Data analyzed using 2-tailed Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, ns, not significant.

IFNγ as an inducer of the M1 phenotype, we found no significant differences in the number of total Arg1$^+$F4/80$^+$ cells with IFNγ neutralization (**Figure S3A**).

Next, we determined whether M2 macrophage activation was required to control MRSA during super-infection in *Stat2*$^{-/-}$ super-infected mice. Arg1 was neutralized using nor-NOHA in both WT and *Stat2*$^{-/-}$ mice subjected to influenza/MRSA super-infection. Inhibition of Arg1 during super-infection increased bacterial burden in *Stat2*$^{-/-}$, but not WT mice (**Figure 6E**). Further, we observed a decrease in the number of Arg1$^+$F4/80$^+$ macrophages and dual function iNOS$^+$Arg1$^+$F4/80$^+$ macrophages in *Stat2*$^{-/-}$ mice treated with nor-NOHA when compared to *Stat2*$^{-/-}$ mice treated with vehicle (**Figures 6F,G**). Interestingly, we found increased numbers of iNOS$^+$F4/80$^+$ macrophages in both WT and *Stat2*$^{-/-}$ mice upon nor-NOHA treatment during influenza-bacterial infection (**Figure S3B**). Despite the presence of M1 macrophages during Arg1/M2 blockade and M2 macrophages during IFNγ/M1 blockade, bacterial control was suppressed in *Stat2*$^{-/-}$ mice during super-infection. These data suggest that neither M1 nor M2 macrophages alone are as proficient at MRSA clearance. We then inhibited both IFNγ and Arg1 simultaneously during super-infection in WT and *Stat2*$^{-/-}$ mice (**Figure 6H**). Inhibition of both M1 and M2 pathways increased bacterial burden in both WT and *Stat2*$^{-/-}$ mice. These data together suggest that activation of M1/M2 co-expressing macrophages is required for increased bacterial clearance during super-infection in *Stat2*$^{-/-}$ mice.

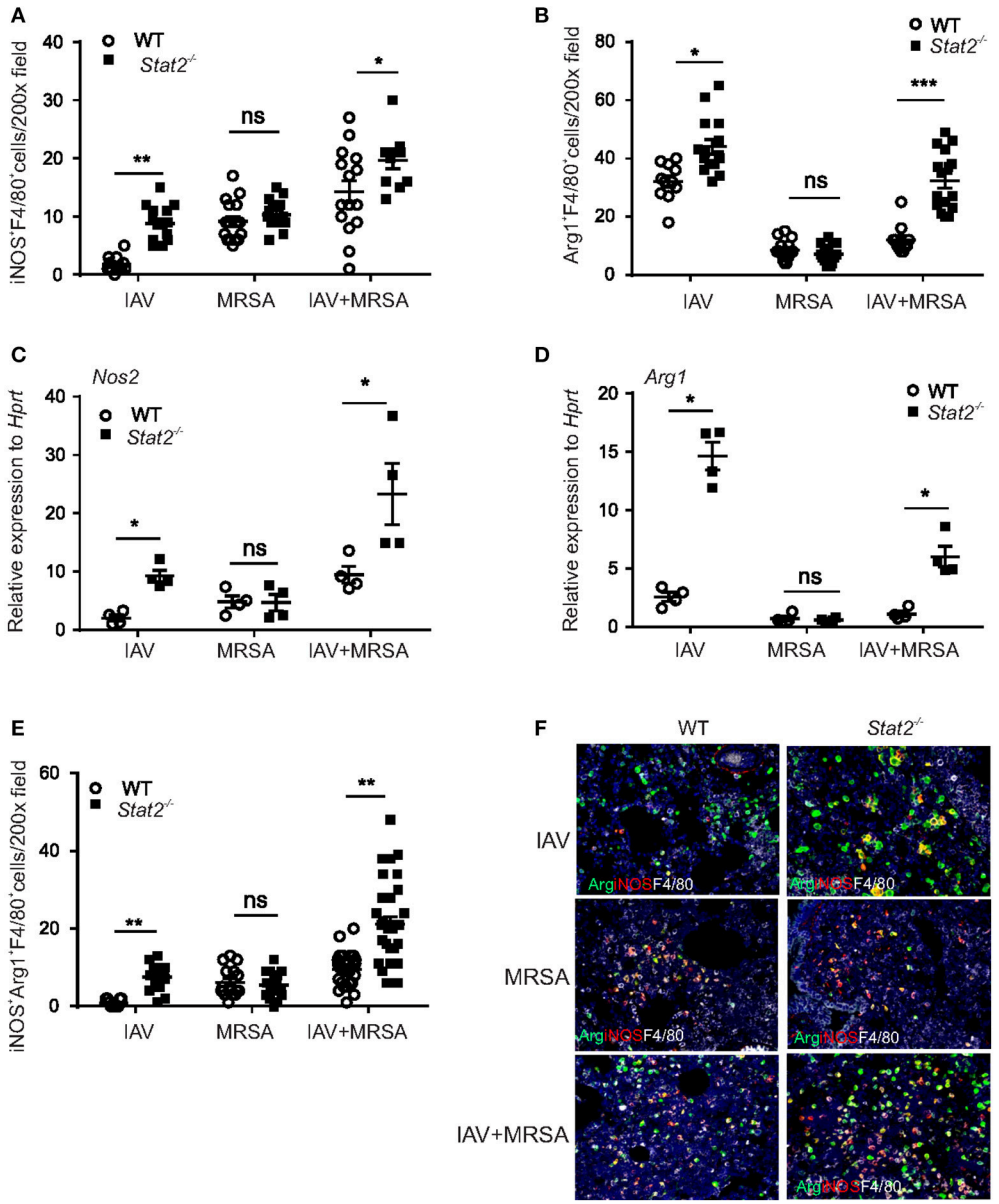

FIGURE 5 | Increased M1 and M2 macrophage expression in *Stat2*$^{-/-}$ is dependent on influenza but not MRSA infection. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days or 5 × 10^7 cfu of MRSA for one day or super-infection as described in methods. **(A)** iNOS$^+$F4/80$^+$, $N = 11$–16 **(B)** Arg1$^+$F4/80$^+$ producing cells were determined from lungs by IHC, $N = 11$–16. **(C)** *Nos2*, **(D)** *Arg1* gene expression was analyzed from lungs by RT-PCR, $N = 4$ per group. **(E)** iNOS$^+$Arg1$^+$F4/80$^+$ producing cells were determined from lung by immunohistochemistry, $N = 11$–32 per group. **(F)** Representative figures, ×200 magnification fields are shown. Data are represented as mean±SEM. Data analyzed using two-tailed Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, not significant.

STAT2 Signaling Negatively Regulates Macrophage Bacterial Uptake and Killing

We then examined bacterial uptake and killing by macrophages from WT and *Stat2*$^{-/-}$ mice. BMDMs isolated from WT and *Stat2*$^{-/-}$ mice were incubated with fluorescent-labeled MRSA for 30 min. Bacterial uptake percentage was determined by the number of FITC$^+$ BMDMs by flow cytometry. It has been shown that the FITC dye can be quenched in response to pH changes in endosomes (40, 41). Therefore, we compared phagocytosis by using FITC or Alexa Fluor-647-labeled bacteria. We found increased bacterial uptake in *Stat2*$^{-/-}$ mice compared to WT mice in both FITC or Alexa Fluor-647-labeled MRSA (**Figures 7A,B**). These data show that macrophages from *Stat2*$^{-/-}$ mice can bind and take up more bacteria than WT mice. Further, under basal conditions we found increased mRNA expression of *Mrc2* and *Cd209e* in *Stat2*$^{-/-}$ BMDMs compared to WT BMDMs (**Figures 7C,D**). However, no significant differences in the expression levels of *Arg1, Chi3l3*, and

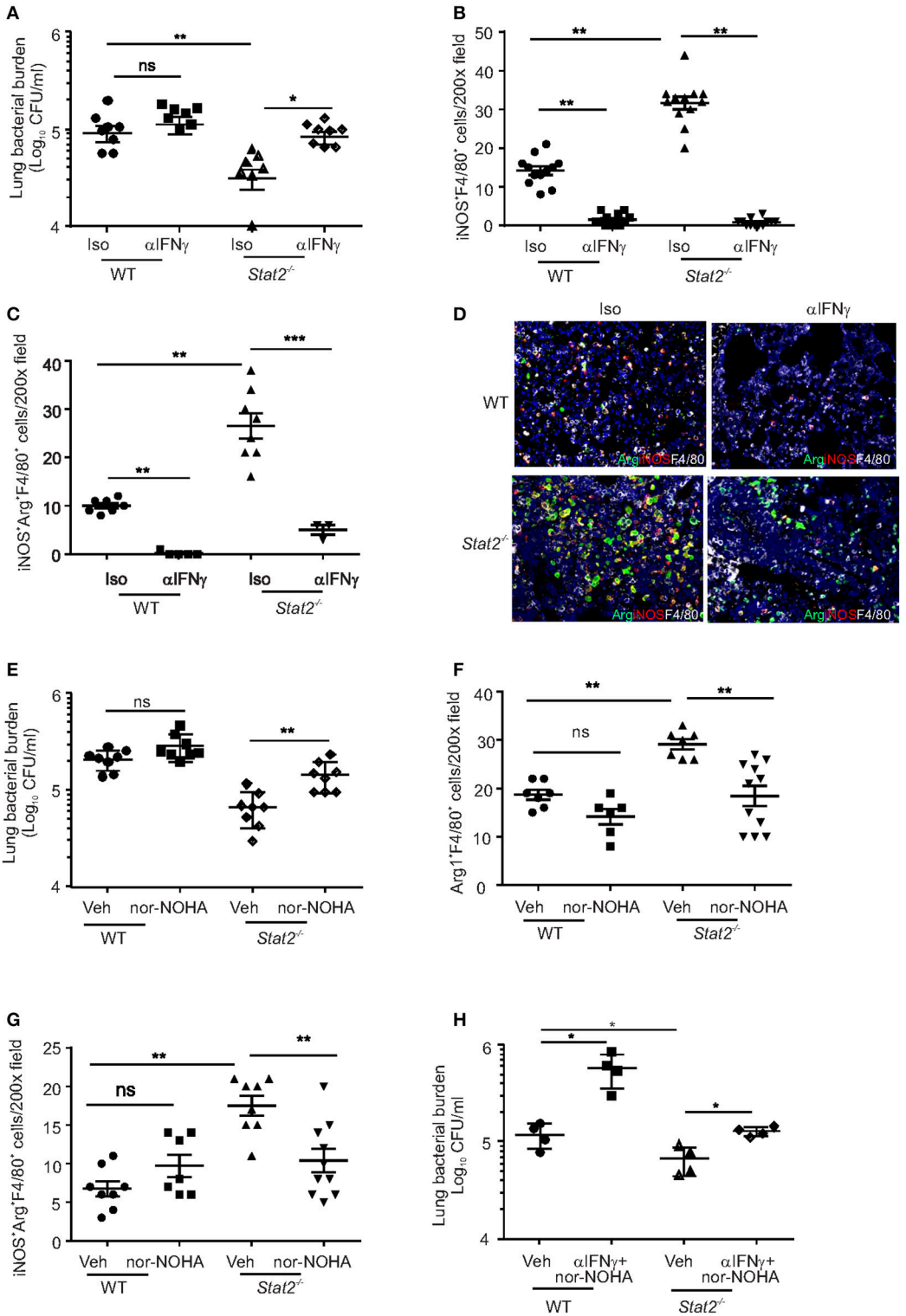

FIGURE 6 | M1/M2 co-expressing macrophages are required for increased bacterial clearance in *Stat2*$^{-/-}$ during influenza-MRSA super-infection. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Mice were treated with 300 μg of anti-IFNγ antibodies or 300 μg of rat IgG isotype controls as described in methods. **(A)** Bacterial burden was measured, $N = 7$–8 per group, **(B)** iNOS$^+$F4/80$^+$ producing cells, $N = 12$ per group **(C)** iNOS$^+$Arg1$^+$F4/80$^+$ producing cells, $N = 3$–7 per group **(D)** were determined by IHC, representative figures x200 magnification fields are shown. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Mice were treated

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FIGURE 6 | with Vehicle (DMSO) or N-$^{-}$hydroxy-nor-L-arginine (nor-NOHA) as described in methods **(E)** Bacterial burden, $N = 8$ per group, **(F)** $Arg^{+}F4/80^{+}$ cells, $N = 6$–10 per group **(G)** $iNOS^{+}Arg^{+}F4/80^{+}$ producing cells were determined, $N = 7$–10 per group. Representative pictures from two experiments are shown. **(G)** WT or $Stat2^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^{7} cfu of MRSA for one additional day. Mice were treated with 300 μg of anti-IFNγ antibodies and N-$^{-}$hydroxy-nor-L-arginine (nor-NOHA) or vehicle as described in methods **(H)** Bacterial burden was measured, $N = 4$ per group, Data are represented as mean±SEM. Data analyzed using 2-tailed Student's *t*-test or One way ANOVA followed by Bonferroni test for multiple comparisons, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns, not significant.

Marco were observed (**Figures 7E–G**). These results suggest that increased expression of the M2 phenotype-associated receptors MRC2 and CD209 is likely to be a mechanism involved in increased binding ability in BMDMs from $Stat2^{-/-}$ mice.

To test the ability of these BMDMs to kill MRSA, we performed a bacterial killing assay using BMDMs treated with mouse IFNγ. We found that $Stat2^{-/-}$ BMDMs have increased killing ability at baseline and following IFNγ treatment when compared to WT BMDMs (**Figure 7H**). However, in BMDMs from WT mice, IFNγ treatment had no effect on bacterial killing (**Figure 7H**). Next, we found that macrophages from $Stat2^{-/-}$ mice had increased expression of *Nos2* and *Cxcl9* compared to WT mice (**Figures 7I,J**). These data suggest that macrophages from $Stat2^{-/-}$ mice have an enhanced ability to kill bacteria due to increased activation and nitric oxide production, and have the potential to attract $CXCR3^{+}$ effector lymphocytes that can amplify M1 activation. These results together show that STAT2 modulates the expression of CLRs and iNOS production by macrophages, and thus negatively impacts their protective functions during super-infection.

Contribution of Hematopoietic or Stromal Cell Compartments in $Stat2^{-/-}$ Mediates Improves MRSA Clearance in Super-Infection

To further confirm that increased bacterial control in $Stat2^{-/-}$ mice during influenza-bacterial super infection is due to M1/M2 co-expressing macrophage activation, we generated bone marrow chimeric (BMC) mice. We transferred hematopoietic cells from WT or $Stat2^{-/-}$ mice to irradiated WT or STAT2 mice and vice versa. We used WT C57BL/6 mice with Thy1.1 or Thy 1.2 markers and $Stat2^{-/-}$ mice bred on a Thy1.2 background. After 9 weeks of bone marrow reconstitution, we infected mice with both influenza and MRSA. Consistent with our previous data, we found higher lung bacterial burden in WT BMC mice (WT host/WT BM) than $Stat2^{-/-}$ mice ($Stat2^{-/-}$ host/$Stat2^{-/-}$ BM) during influenza-bacterial super-infection (**Figure 8A**). As expected, we found decreased bacterial burden in hematopoietic $Stat2^{-/-}$ BMC mice (WT host/$Stat2^{-/-}$ BM) compared to WT BMC mice (WT host/ WT BM) during super-infection (**Figure 8A**). Interestingly, we also found decreased bacterial burden in non-hematopoietic $Stat2^{-/-}$ BMC ($Stat2^{-/-}$ host/WT BM) compared to WT BMC mice (WT host/ WT BM) during super-infection (**Figure 8A**).

Next, we found increased expression of $iNOS^{+}Arg1^{+}$ macrophages in hematopoietic $Stat2^{-/-}$ BMC (WT host/$Stat2^{-/-}$ BM) and $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/$Stat2^{-/-}$ BM), suggesting that hematopoietic cells are involved in bacterial control in $Stat2^{-/-}$ mice during influenza-MRSA super-infection (**Figures 8B,C**). Also, no significant differences were observed in the expression of $iNOS^{+}Arg1^{+}$ macrophages between WT BMC mice (WT host/WT BM) and non-hematopoietic $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/WT BM). These data suggest that $iNOS^{+}Arg1^{+}$ macrophages mediate bacterial control in hematopoietic $Stat2^{-/-}$ BMC (WT host/ $Stat2^{-/-}$ BM) and $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/$Stat2^{-/-}$ BM) during influenza-MRSA super-infection. However, we found increased bacterial clearance in non-hematopoietic $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/WT BM) during influenza-MRSA super-infection.

Type I and type II interferons induce interferon stimulated genes (ISGs) to establish anti-viral response responses (42–44). Based on RNAseq data and RT-PCR analysis, we identified that expression of type I IFN-induced genes such as *Mx1, Lhx2,* and *CCL12* was suppressed in $Stat2^{-/-}$ mice during influenza-MRSA super-infection (**Figures S4A–D**). However, the type II IFN-induced chemokine *CXCL9* was not suppressed during influenza-MRSA super-infection (**Figure S4E**). Further, *Mx1* and *Ccl12* gene expression was suppressed in bone marrow dendritic cells (BMDCs) from $Stat2^{-/-}$ mice stimulated with IFNβ compared to BMDCs from WT mice (**Figures S4F,G**). These data confirm that type I IFN, but not type II IFN responses are significantly attenuated in the lungs of $Stat2^{-/-}$ mice during influenza infection.

To determine if ISG expression in $Stat2^{-/-}$ BMC mice was suppressed, we measured the expression of *Mx1* and *Ccl12*. We found decreased expression of *Mx1* in $Stat2^{-/-}$ BMC ($Stat2^{-/-}$ host/$Stat2^{-/-}$ BM), hematopoietic $Stat2^{-/-}$ BMC (WT host/ $Stat2^{-/-}$ BM), and non-hematopoietic $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/WT BM) compared to WT BMC mice (WT host/WT BM) (**Figure 8D**). The expression of *Mx1* was further suppressed in $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/$Stat2^{-/-}$ BM) when compared to hematopoietic $Stat2^{-/-}$ BMC (WT host/ $Stat2^{-/-}$ BM) and non-hematopoietic $Stat2^{-/-}$ BMC mice ($Stat2^{-/-}$ host/WT BM) (**Figure 8D**). These data suggest that both hematopoietic and non-hematopoietic cells are involved in the expression of *Mx1* in response to influenza-bacterial super-infection.

Next, we determined the expression of *Ccl12* in the BMC mice. We found decreased expression of *Ccl12* in $Stat2^{-/-}$ BMC ($Stat2^{-/-}$ host/$Stat2^{-/-}$ BM) and hematopoietic $Stat2^{-/-}$ BMC mice (WT host/ $Stat2^{-/-}$ BM) as compared to WT BMC (WT host/WT BM) (**Figure 8E**). However, the expression of *Ccl12* was not suppressed in $Stat2^{-/-}$ non-hematopoietic BMC mice ($Stat2^{-/-}$ host/WT BM) (**Figure 8E**). These data suggest that STAT2 signaling in hematopoietic cells is crucial in induction of CCL12 during influenza-bacterial super-infection. Further, these

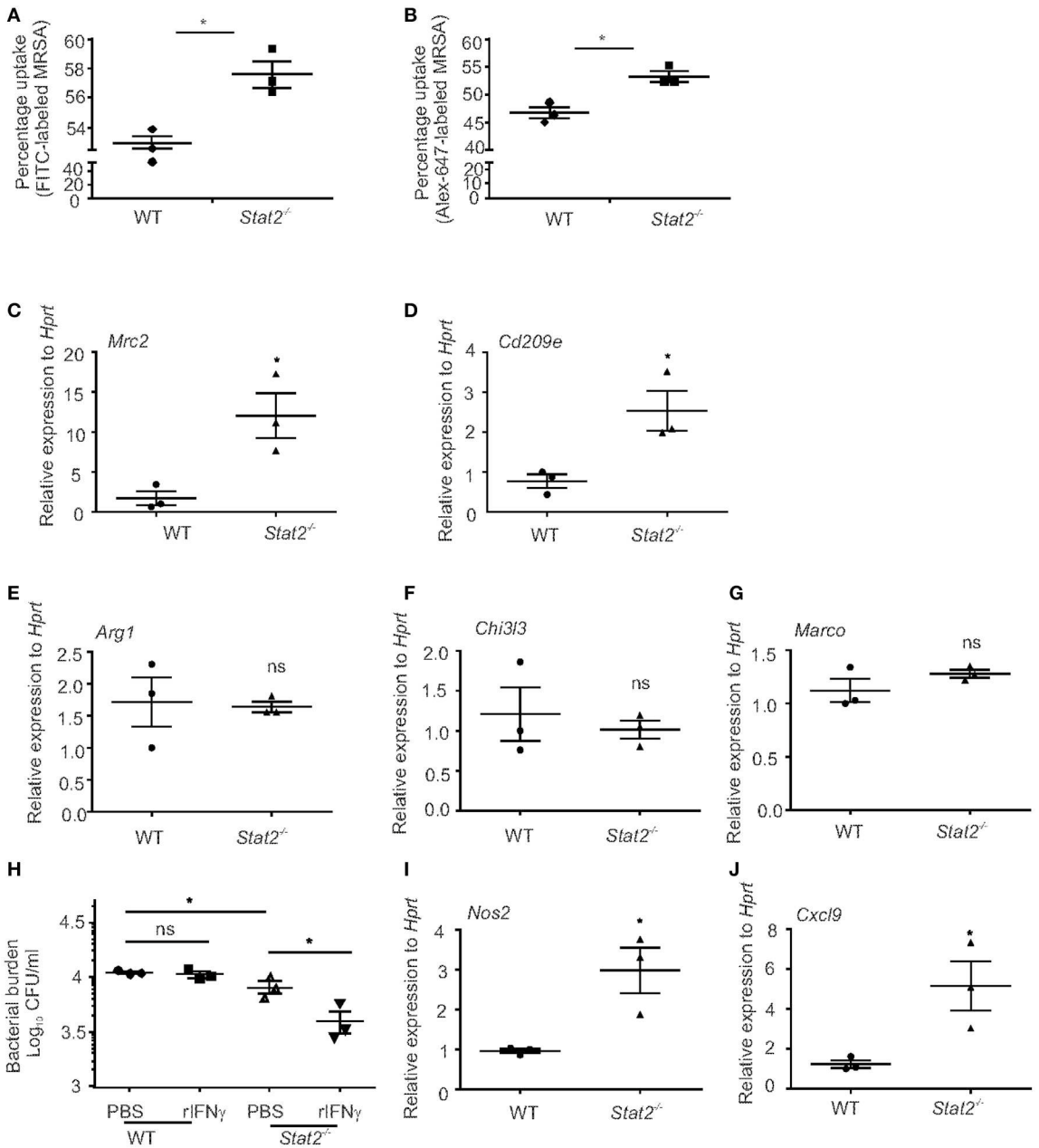

FIGURE 7 | Increased bacterial uptake and killing efficiency in BMDMs from *Stat2*$^{-/-}$ mice BMDMs were generated and infected with FITC or Alex-647-labeled MRSA (10 MOI) for 30 min, washed, fixed with 1% formaldehyde and the number of FITC^{+} **(A)** or Alex-647^{+} **(B)** BMDMs were determined by Flow cytometry. **(C)** *Mrc2* and **(D)** *Cd209e*, **(E)** *Arg1*, **(F)** *Chi3l3*, **(G)** *Marco* gene expression levels were measured by RT-PCR. **(H)** BMDMs were generated, treated with recombinant IFNγ (10 μg/ml) for 24 h, and infected with MRSA (10 MOI) and bacterial killing was determined. **(I)** *Nos2* and **(J)** *Cxcl9* gene expression levels were measured from naïve BMDMs, *N* = triplicates per treatment. Representative pictures from two experiments are shown. Data are represented as mean±SEM. Data analyzed using 2-tailed Student's *t*-test, $^{*}p < 0.05$, ns, not significant.

data suggest that the absence of stromal cell STAT2 resulted in decreased ISG expression and improved bacterial control in *Stat2*$^{-/-}$ non-hematopoietic BMC mice (*Stat2*$^{-/-}$ host/WT BM) during influenza-bacterial super-infection.

Next, we determined the influenza viral burden in the BMC mice. We found higher viral burden in *Stat2*$^{-/-}$ BMC (*Stat2*$^{-/-}$ host/*Stat2*$^{-/-}$ BM) and non-hematopoietic *Stat2*$^{-/-}$ BMC mice (*Stat2*$^{-/-}$ host/WT BM) when compared to WT BMC (WT host/WT BM) and hematopoietic *Stat2*$^{-/-}$ BMC (WT host/ *Stat2*$^{-/-}$ BM) (**Figure S5**). These data suggest that non-hematopoietic cells are involved in STAT2-mediated viral control during influenza-MRSA super-infection.

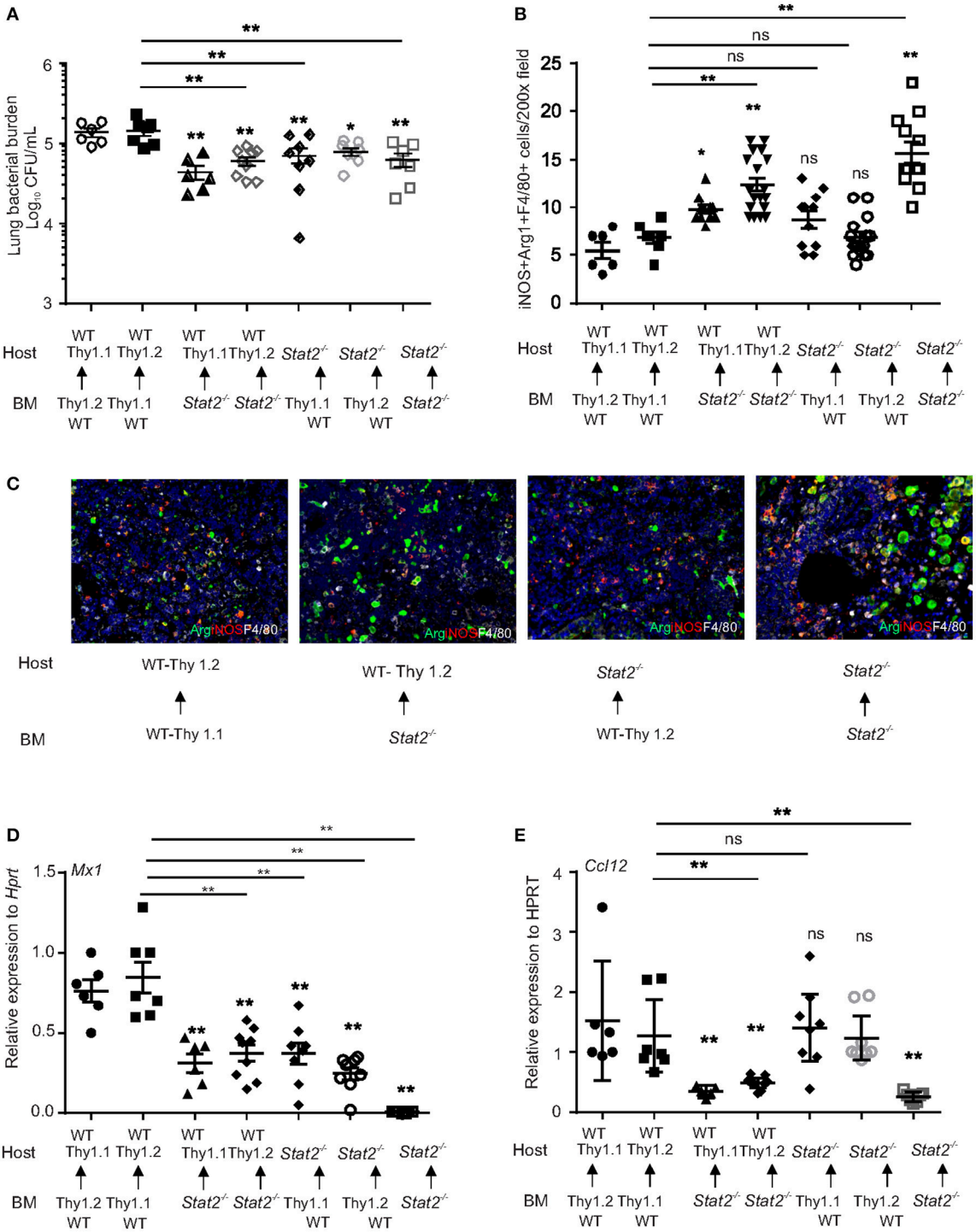

FIGURE 8 | Increased bacterial control in cells from hematopoietic or non-hematopoietic compartments of *Stat2*$^{-/-}$ mice. WT BMC (Thy1.1 host, Thy 1.2 BM or Thy 1.2 host, Thy 1.1 BM), *Stat2*$^{-/-}$ BMC (*Stat2*$^{-/-}$ host, *Stat2*$^{-/-}$ BM), Hematopoietic *Stat2*$^{-/-}$ BMC mice (Thy 1.1 or Thy 1.2 host, *Stat2*$^{-/-}$ BM), non-hematopoietic *Stat2*$^{-/-}$ BMC (*Stat2*$^{-/-}$ host, Thy 1.1 or Thy 1.2 BM) were generated as described in methods. These mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. **(A)** Mice were sacrificed and right upper lung lobes were homogenized in PBS and bacterial burden was measured. **(B)** iNOS$^+$Arg1$^+$F4/80$^+$ producing cells were determined from lung by immunohistochemistry, $N = 6$–19 per group. **(C)** Representative figures, ×200 magnification fields are shown. **(D)** *Mx1*, **(E)** *Ccl12* mRNA expression was measured, $N = 6$–9 per group. Data are represented as mean±SEM. Data analyzed using One way ANOVA followed by Bonferroni test for multiple comparisons, $^*p < 0.05$, $^{**}p < 0.01$, ns, not significant.

DISCUSSION

During influenza infection, epithelial cells, macrophages and dendritic cells all induce type I and type III IFNs, proinflammatory cytokines and chemokines (2). Type I IFN signaling mediates lung pathology and infiltration of granulocytes during influenza infection (7). Absence of STAT1 or STAT2 signaling compromises viral control and survival in mice (9). In the current study, we have demonstrated that in the absence of STAT2 signaling the type I IFN response is impaired. This was associated with increased viral burden, inflammation, and pathology in the lungs during influenza infection. These results show that STAT2-dependent signaling is crucial in controlling influenza burden and inflammatory immune responses during primary influenza infection. Consistent with these findings, elevated levels of type I IFNs during influenza infection correlates with disease severity in outbred mice (45).

Influenza-associated secondary bacterial pneumonia is a serious complication of influenza infection (10, 11, 46). Reducing morbidity and mortality requires insight into the immune mechanisms that alter susceptibility to secondary bacterial infection. In this study, we show that STAT2 deficiency improves survival and rescues the impairment of bacterial clearance from the lung otherwise observed during influenza-bacterial super-infection. Further, we have identified increased accumulation of M1, M2 and M1/M2 co-expressing macrophages by influenza-MRSA super-infection in the setting of STAT2 deficiency as a novel mechanism that mediates this protection.

We have previously demonstrated that influenza-induced type I IFN-mediated suppression of Type 17 responses to both Gram (+) (*S. aureus*) and Gram (−) bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*) during influenza-bacterial super-infection (13, 14). In these studies, loss of Type 17 immune responses was associated with exacerbation of secondary bacterial challenge during influenza infection. Further, we have also shown increased bacterial control in *Ifnar*$^{-/-}$ mice during super-infection, suggesting that influenza-induced type I IFN is a critical mediator of antibacterial immune suppression (13). Also, we have recently shown increased Type 17 immunity in STAT1$^{-/-}$ mice during super-infection (17). Therefore, we hypothesized that STAT2 deficiency would rescue suppression of Type 17 responses during influenza infection. Surprisingly, we found that the increased bacterial clearance we observed in the absence of STAT2 was not due to rescue of the Type 17 immune response. These data suggest that Type 17 immunity is predominantly regulated by STAT1 and not STAT2 during super-infection. This finding prompted further investigation into the mechanism of protection in *Stat2*$^{-/-}$ mice.

Since Type 17 responses were not associated with the observed phenotype, we next explored other possible mechanisms involved in bacterial control. In our study, we found no differences in neutrophils, macrophages and lymphocyte numbers in BAL of WT and *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. A possible role for neutrophils in bacterial killing exists, as shown by a trend toward increased neutrophil numbers in BAL from *Stat2*$^{-/-}$ mice. However, based on RNAseq data analysis, we found increased RNA abundance of M1 and M2 macrophage markers, and further characterized the RNA expression, frequency and immunolocalization of these cells. We then determined the role of these macrophages in bacterial control in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection.

M1 macrophages are known to be involved in pathogen defense and inflammation, whereas M2 macrophages are thought to have a suppressive or regulatory role during inflammation (30, 47). In the current study, we found the majority of CD11b^{+}Ly6C^{+} macrophages are CD80^{+}/MGL^{+} (M1/M2) positive. Also, we found increased frequency of M1 (CD80^{+}), but not M2 (MGL^{+}) single positive cells in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. Further, these M1/M2 cells had increased expression levels of both *Arg1* and *Nos2* in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. We then confirmed these flow cytometry findings by IHC and observed an increase in Arg1^{+}iNOS^{+}F4/80^{+} cells in *Stat2*$^{-/-}$ mice. These data confirm the ability of STAT2 to regulate macrophage phenotype during pneumonia, and identify an M1/M2 dual phenotype macrophage population in the context of influenza-associated secondary bacterial infection.

Further, in accordance with increased levels of IFNγ and ISGs, we found increased *Stat1* expression levels in *Stat2*$^{-/-}$ mice when compared to WT mice. This suggests that in the absence of STAT2 signaling, other pathways are activated as a compensatory effect. However, *Stat6* expression is not altered in the absence of STAT2 in super-infection. IFNγ is known to suppress expression of the nuclear receptor PPARγ (48, 49). We also found suppression of *Pparg* expression in *Stat2*$^{-/-}$ mice during influenza-bacterial super-infection. Alterations in IFNγ and PPARγ signaling may be a possible mechanism by which M1/M2 macrophage populations are altered in *Stat2*$^{-/-}$ mice in super-infection.

In *Stat2*$^{-/-}$ mice, iNOS^{+} macrophages were elevated during super-infection. This contrasted with WT mice in which iNOS^{+} macrophages were only induced in the setting of bacterial challenge. In contrast, Arg1^{+} macrophages were decreased during both MRSA and super-infection in WT mice. However, in *Stat2*$^{-/-}$ mice the Arg1^{+} cells were increased in influenza-bacterial super-infection, but not MRSA infection, suggesting that the M2 induction during super-infection is driven by influenza infection. Chen et al. (15) correlated an increased number of M2 macrophages during influenza infection with suppression of the protective immune response to bacterial super-infection (50). In contrast, one study has shown that *S. aureus* priming increased M2 macrophages and anti-inflammatory responses to influenza challenge (51). In the current study, we found an increased number of Arg1^{+} macrophages associated with increased bacterial control in *Stat2*$^{-/-}$ mice during super-infection, and that WT mice suffered from impaired bacterial clearance despite the presence of Arg1^{+} at a level similar to that seen during MRSA infection alone. Also, we found that Arg1- and iNOS-expressing dual function macrophages were significantly higher in *Stat2*$^{-/-}$ mice. The presence of M1/M2 co-expressing macrophages has recently been described in response to *Toxoplasma gondii* infection (52), but to our knowledge is a novel finding in pulmonary host defense.

Promotion of bacterial killing by IFNγ is well established for intracellular pathogens such as *Mycobacterium tuberculosis* and *Toxoplasma gondii*. However, information regarding its role in bacterial killing of *S. aureus* is limited. Studies have shown that influenza-induced IFNγ inhibits pneumococcal control during super-infection (53). In this study, we found a trend in increased bacterial burden in IFNγ neutralization in WT mice during super-infection. However, no differences were observed in WT BMDMs in bacterial killing in response to IFNγ treatment. Observed discrepancies in the role of IFNγ during super-infection might be due to differences in the dose and the strain of bacteria. We found that improved bacterial control seen during influenza-bacterial super-infection in *Stat2*$^{-/-}$ mice was lost upon neutralization of IFNγ. This was associated with a loss of iNOS^{+} and iNOS^{+}Arg1^{+} dual-function cells, suggesting that increased IFNγ in *Stat2*$^{-/-}$ mice during super-infection mediates the protective phenotype by driving induction of M1-polarized macrophages. However, it is known that IFNγ increases neutrophil nitrite production and increases phagocytosis (54–56). It is also possible that other reactive oxygen species are involved in increased IFNγ-mediated bacterial clearance in *Stat2*$^{-/-}$ mice during super-infection. Similarly, we found that neutralization of Arg1 decreased bacterial clearance in *Stat2*$^{-/-}$ mice during super-infection in association with an attenuated number of iNOS^{+}Arg1^{+} dual function macrophages. Ultimately, bacterial control in *Stat2*$^{-/-}$ mice during super-infection was compromised when the number of iNOS^{+}Arg1^{+} dual function macrophages was diminished. Further, we confirmed this by decreased bacterial clearance upon both Arg1 and IFNγ neutralization in both WT and *Stat2*$^{-/-}$ mice.

Further, BMC studies showed increased bacterial control in both *Stat2*$^{-/-}$ BMC and *Stat2*$^{-/-}$ hematopoietic BMC mice during influenza-bacterial infection, confirming the role of macrophage STAT2 in suppressing bacterial control during influenza-bacterial super-infection. However, bacterial control in non-hematopoietic *Stat2*$^{-/-}$ mice is likely due to altered macrophage phenotype, as influenza-induced ISGs are reduced in these mice. Further, increased influenza viral burden in non-hematopoietic *Stat2*$^{-/-}$ mice indicated a role for stromal STAT2 signaling in inducing ISGs. Collectively, these data indicate a cell intrinsic role for STAT2 signaling in macrophages and an accessory role for stromal STAT2 signaling via regulation of ISG expression.

In addition to quantitative differences in critical macrophage phenotypes, we have demonstrated that macrophages from *Stat2*$^{-/-}$ mice showed important qualitative differences compared to WT cells. We demonstrated that BMDM from *Stat2*$^{-/-}$ mice had improved MRSA uptake at baseline. This increased efficiency was unaffected by any infection challenge. Increased uptake was associated with expression of multiple C-type lectin receptors (CLRs), which are primarily expressed in monocytes in the lung, and are involved in phagocytosis of a variety of pulmonary pathogens (57, 58). However, these receptors favor the entry of influenza by acting as a receptor for viral attachment (59, 60). In this study, we found increased expression of CLRs and increased phagocytic ability in BMDMs from *Stat2*$^{-/-}$ mice. These results suggest that, even though these receptors favor influenza infection, they may help control secondary bacterial infection in *Stat2*$^{-/-}$ mice by improving bacterial uptake. In addition to increased bacterial uptake, BMDM from *Stat2*$^{-/-}$ mice displayed increased bacterial killing compared to WT mice upon IFNγ treatment. Naive BMDMs from *Stat2*$^{-/-}$ mice also displayed increased *Nos2* and *Cxcl9* expression compared to cells from WT mice, suggesting that *Stat2*$^{-/-}$ macrophages have inherently increased expression of *Nos2* and enhanced bacterial killing ability compared to WT macrophages.

In summary, we have shown that STAT2 signaling decreases influenza viral burden and inflammatory immune responses during influenza infection, at the cost of inhibiting bacterial control during subsequent bacterial challenge by suppressing a distinct M1/M2 dual function macrophage population during influenza-bacterial super-infection. Together our data show a novel role of influenza induced type I IFN-mediated STAT2 signaling in inhibiting bacterial control through suppression of macrophage activity during influenza and influenza/MRSA super-infection. STAT2 and dual function M1/M2 macrophage activation may be a potential target for the treatment or prevention of influenza-bacterial super-infection.

ETHICS STATEMENT

All mouse experiments were approved by the University of Pittsburgh IACUC, Protocol #: 17071194; PHS Assurance Number: D16-00118.

AUTHOR CONTRIBUTIONS

RG, BL, JR-M, and JA designed the experiments. RG, BL, KM, HR, KR, SM, MC, PS, RE, MM, KMR, and JR-M performed the experiments. RG, BL, JR-M, and JA performed the analyses. RG, BL, HR, MM, KMR, JR-M, and JA drafted and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02151/full#supplementary-material

FIGURE S1 | No differences in Th17 response between WT and *Stat2*$^{-/-}$ mice following MRSA, and influenza-MRSA super-infection**.** WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza A/PR/8/H1N1 or PBS by oropharyngeal aspiration for 6 days then challenged with 5×10^{7} cfu of MRSA or PBS by oropharyngeal aspiration for one additional day. Frequency of **(A)** IL-17^{+} or **(B)** IL-22^{+} cells were determined by flow cytometry. The percentage of IL-17^{+} and IL-22^{+} cells were calculated from the percentage of CD3^{+}CD4^{+} T cells. WT or *Stat2*$^{-/-}$ mice were infected with influenza-MRSA as described above. Lung lobes were homogenized with PBS, and the levels of (**C**) IL-23, (**D**) IL-17 were measured by Luminex analysis, and **(E)** IL-22 levels measured by ELISA. Data are

represented as mean ± SEM. Data analyzed using two-tailed Student's *t*-test, ns, not significant.

FIGURE S2 | *Stat2*$^{-/-}$ mice have altered other STAT1 and STA6 signaling pathways during influenza-bacterial super-infection. WT or *Stat2*$^{-/-}$ mice were infected with either 100 PFU of influenza A PR/8/H1N1 or influenza for 6 days and then challenged with 5×10^7 cfu of MRSA by oropharyngeal aspiration for one additional day, **(A)** *Stat1*, **(B)** *Stat6,* **(C)** *Pparg* gene expression relative to *Hprt* was analyzed by RT-PCR. $N = 4$ per group. Data are represented as mean ± SEM. Data analyzed using two-tailed Student's *t*-test, $^*p \leq 0.05$, ns, not significant.

FIGURE S3 | M1 and M2 macrophages are altered in *Stat2*$^{-/-}$ mice in response to Arg1 and IFNγ neutralization during influenza-MRSA super-infection. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Mice were treated with 300 μg of anti-IFNγ antibodies or 300 μg of rat IgG isotype controls as described in methods. **(A)** Arg1$^+$F4/80+ producing cells, were determined by IHC, $N = 4$–13 per group. WT or *Stat2*$^{-/-}$ mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Mice were treated with Vehicle (DMSO) or N-hydroxy-nor-L-arginine (nor-NOHA) as described in methods. **(B)** iNOS+F4/80+ cells, $N = 6$–9 per group. Data are represented as mean ± SEM. Data analyzed using two-tailed Student's *t*-test, $^*p \leq 0.05$, $^{**}p \leq 0.01$, ns, not significant.

FIGURE S4 | *Stat2*$^{-/-}$ mice have deficient type I IFN responses with preserved type II IFN responses. **(A)** WT and *Stat2*$^{-/-}$ male 6–8 weeks mice were infected with 100 PFU of influenza A/PR/8/H1N1 by oropharyngeal aspiration for 6 days then challenged with 5×10^7 cfu of MRSA by oropharyngeal aspiration for one additional day. Overall gene expression was measured in lung by RNAseq analysis, Heat-map representing the fold induction of Interferon stimulated genes (ISGs) from WT and *Stat2*$^{-/-}$ mice following influenza-MRSA super-infection, $N = 4$ per group. WT or *Stat2*$^{-/-}$ mice were infected with either 100 PFU of influenza A PR/8/H1N1 or influenza for 6 days and then challenged with 5×10^7 cfu of MRSA by oropharyngeal aspiration for one additional day, **(B)** *Mx1*, **(C)** *Lhx2*, and **(D)** *Ccl12*, **(E)** *Cxcl9* gene expression relative to *Hprt* was analyzed by RT-PCR. $N = 3$–4 per group. Bone marrow-derived dendritic cells (1×10^6) were cultured *in vitro* and stimulated *in vitro* with IFNβ (6.5 units/mL) or IFNγ (20 ng/mL) for 24 h, **(F)** *Mx1*, **(G)** *Ccl12* gene expression in cell RNA was analyzed by qRT-PCR. The cells were treated in triplicates. Each data point from panel F and G were the number of replicates per treatment. Data are represented as mean ± SEM. Data analyzed using two-tailed Student's *t*-test, $^*p \leq 0.05$, $^{**}p \leq 0.01$, ns, not significant.

FIGURE S5 | Increased influenza burden in cells from non-hematopoietic compartments of *Stat2*$^{-/-}$ mice. WT BMC (Thy1.1 host, Thy 1.2 BM or Thy 1.2 host, Thy 1.1 BM), *Stat2*$^{-/-}$ BMC (*Stat2*$^{-/-}$ host, *Stat2*$^{-/-}$ BM), Hematopoietic *Stat2*$^{-/-}$ BMC mice (Thy 1.1 or Thy 1.2 host, *Stat2*$^{-/-}$ BM), non-hematopoietic *Stat2*$^{-/-}$ BMC (*Stat2*$^{-/-}$ host, Thy 1.1 or Thy 1.2 BM) were generated as described in methods. These mice were infected with 100 PFU of influenza for 6 days then challenged with 5×10^7 cfu of MRSA for one additional day. Mice were sacrificed and RNA expression of Influenza M protein was measured from the lungs by PCR, $N = 6$–9 per group. Data are represented as mean ± SEM. Data analyzed using One way ANOVA followed by Bonferroni test for multiple comparisons, $^{**}p \leq 0.01$, ns, not significant.

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Resolving Viral-Induced Secondary Bacterial Infection in COPD: A Concise Review

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Chronic obstructive pulmonary disease (COPD) is a leading cause of disability and death world-wide, where chronic inflammation accelerates lung function decline. Pathological inflammation is worsened by chronic bacterial lung infections and susceptibility to recurrent acute exacerbations of COPD (AECOPD), typically caused by viral and/or bacterial respiratory pathogens. Despite ongoing efforts to reduce AECOPD rates with inhaled corticosteroids, COPD patients remain at heightened risk of developing serious lung infections/AECOPD, frequently leading to hospitalization and infection-dependent delirium. Here, we review emerging mechanisms into why COPD patients are susceptible to chronic bacterial infections and highlight dysregulated inflammation and production of reactive oxygen species (ROS) as central causes. This underlying chronic infection leaves COPD patients particularly vulnerable to acute viral infections, which further destabilize host immunity to bacteria. The pathogeneses of bacterial and viral exacerbations are significant as clinical symptoms are more severe and there is a marked increase in neutrophilic inflammation and tissue damage. AECOPD triggered by a bacterial and viral co-infection increases circulating levels of the systemic inflammatory marker, serum amyloid A (SAA). SAA is a functional agonist for formyl peptide receptor 2 (FPR2/ALX), where it promotes chemotaxis and survival of neutrophils. Excessive levels of SAA can antagonize the protective actions of FPR2/ALX that involve engagement of specialized pro-resolving mediators, such as resolvin-D1. We propose that the anti-microbial and anti-inflammatory actions of specialized pro-resolving mediators, such as resolvin-D1 should be harnessed for the treatment of AECOPD that are complicated by the co-pathogenesis of viruses and bacteria.

Keywords: COPD-chronic obstructive pulmonary disease, exacerbation, influenza (flu), secondary infection, co-infection, resolvin D1 (RvD1), serum amyloid A (SAA), pneumococcus (*Streptococcus pneumoniae*)

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COPD PATIENTS ARE HIGHLY SUSCEPTIBLE TO RESPIRATORY INFECTIONS

Chronic obstructive pulmonary disease (COPD) is an umbrella term encompassing multiple lung pathologies (including emphysema, chronic bronchitis and bronchiolitis) that manifest into persistent and poorly reversible airflow limitation. It is a major chronic disease that is predicted to become the third leading cause of death world-wide by 2030 (1) and has a huge economic burden costing $50 billion annually in the USA alone (2). COPD is characterized as a chronic inflammatory condition due to the persistent accumulation of innate

and adaptive immune cells in the airways along with increased systemic inflammation (3). This inflammatory process is driven, in part by inhalation of highly noxious particles in cigarette smoke and biomass fuels that overwhelm protective detoxifying and anti-oxidant pathways. Inflammation is maintained through the continual recruitment and activation of leukocytes, which perpetuate a state of oxidative stress and local tissue damage. Neutrophilic inflammation is particularly prominent in COPD where neutrophils degranulate with increasing severity of COPD, resulting in uncontrolled release of proteolytic enzymes (neutrophil elastase and matrix metalloproteinase 9-MMP9) that further damage the lungs (4).

An important paradox in COPD is that despite the accumulation of leukocytes in the airways with increasing disease severity, there is still a major failure to adequately control and eradicate respiratory pathogens. As a consequence, the lower airways become persistently infected, where up to 50% of COPD patients are chronically colonized with potentially pathogenic bacteria including *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* (5, 6). Another major consequence of susceptibility to infection is that people with underlying severe disease frequently develop acute exacerbations of COPD (AECOPD). AECOPD are defined as "a sustained worsening of the patient's condition, from the stable state and beyond normal day-to-day variations, that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD" (7). AECOPD are predominately caused by acquisition of a new respiratory pathogen (8), which accelerate pathological remodeling leading to a more rapid decline in lung function (9). Severe exacerbations account for much of the health related expenditure for COPD (10) and the "frequent exacerbator" phenotype has been defined, where patients that fall into this category are at much greater risk of hospitalization and death (11).

Anti-inflammatory agents including inhaled corticosteroids in combination with bronchodilators are frequently used to treat COPD, however they have failed to reduce the rate of severe AECOPD requiring hospitalization (12). Inhaled corticosteroids, delivered alone or in combination with bronchodilators are also associated with a small but significant increased risk of serious adverse pneumonia events (13). *S. pneumoniae* is the major bacterial cause of community acquired pneumonia (CAP); accounting for up to 40–50% of bacteriologically examined cases. COPD is a common underlying chronic comorbid condition in CAP (14) and the severity of CAP is worse in COPD patients based on severity index and respiratory failure (15). Without radiological assessment, it is difficult to differentiate CAP from AECOPD, however CAP is generally associated with more severe illness and pulmonary consolidation. Polyvalent vaccination may also be less effective in COPD patients as the elevated relative risk for developing pneumococcal pneumonia in COPD (8-fold compared to controls) is only reduced by 50% with vaccination (16). With over 90 serotypes, the genetic diversity of *S. pneumoniae* means that strategies in addition to vaccination are needed to control this pathogen, particularly in people with chronic comorbid conditions, such as COPD.

DYSFUNCTIONAL OXIDANT PATHWAYS DISRUPT CLEARANCE OF RESPIRATORY PATHOGENS IN COPD

The persistence of bacterial infection in the lower airways of COPD patients is associated with impaired phagocytic function, where clearance of both *H. influenzae* and *S. pneumoniae* by alveolar macrophages was shown to be defective relative to control macrophages (17, 18). Oxidative stress is thought to drive impaired phagocytosis as activation of nuclear erythroid–related factor 2 (Nrf2) restores phagocytosis of bacteria by alveolar macrophages from patients with COPD (19). Nrf2 is a key transcription factor that regulates expression of a suite of cytoprotective and antioxidant enzymes. As COPD progresses in severity, inflammatory cells accumulate and become an important endogenous source of reactive oxygen species (ROS) independent of smoking status. Increased ROS production by activated leukocytes will also promote the peroxidation of polyunsaturated fatty acids and generation of reactive carbonyl species, such as 4-hydroxynonenol (4HNE) and malondialdehyde (MDA); both of which are increased in COPD (20). Levels of auto-antibodies against carbonyl-modified proteins correlate with severity of COPD (21). Importantly, reactive carbonyls can impair macrophage-mediated phagocytosis of bacteria by directly causing cytoskeletal instability and carbonyl modification of pseudopodia (22–24). Also, macrophages interact with carbonyl-modified extracellular matrix proteins and this interaction suppresses their phagocytic functions (25).

Immunity to common respiratory viruses is also disrupted in COPD patients. Around 30–50% of AECOPDs are associated with respiratory viruses including rhinovirus, respiratory syncytial virus (RSV) and influenza (26, 27). Experimental rhinovirus infection in subjects with COPD results in elevated systemic and airway inflammation (28). In addition, increased rhinovirus load was detected in COPD subjects compared to controls, which was associated with reduced interferon production (28). Using the same human experimental model, rhinovirus infection was also shown to markedly increase markers of oxidative and nitrosative stress (29). We have recently shown that single stranded RNA viruses (including influenza A virus, rhinovirus, RSV) and DNA viruses promote the production of ROS through activation of endosomal NADPH oxidase 2 (NOX2) (30). The internalization of viruses specifically triggered production of hydrogen peroxide within endosomes, where NOX2-dependent production of ROS suppressed antiviral signaling networks via modification of Toll-like receptor-7 (30). This process acts as a critical physiological brake to prevent an over exuberant anti-viral or humoral response that can contribute to autoimmune disease. Conversely, excessive ROS production in COPD has the potential to compromise essential anti-viral immune responses (such as type-1 interferon) during viral-induced exacerbations, although this has yet to be proven. We have however, targeted excessive ROS generation with the glutathione peroxidase (Gpx) mimetic and NOX2 oxidase inhibitor, ebselen and apocynin in an experimental AECOPD

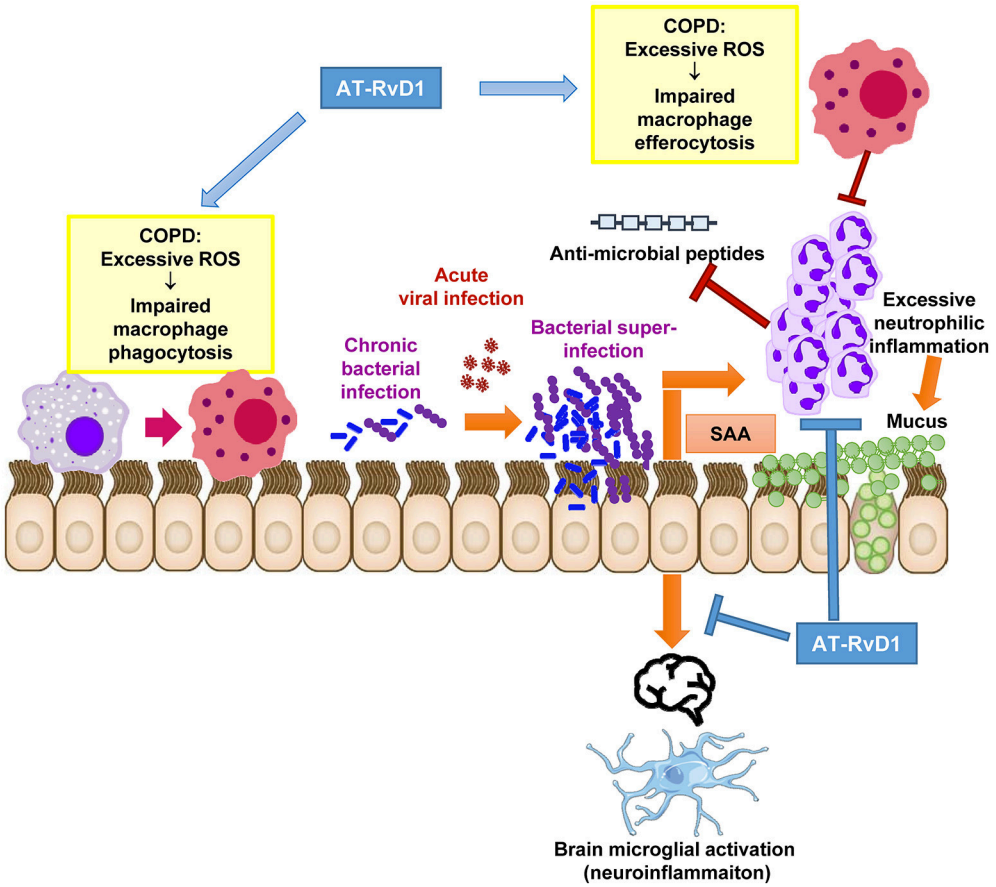

FIGURE 1 | Proposed therapeutic actions of AT-RvD1 during acute exacerbations in chronically infected COPD patients. Excessive inflammation and ROS are a characteristic feature of the COPD lung microenvironment. Excessive ROS directly impairs macrophage function preventing efficient phagocytosis of potentially pathogenic bacteria and efferocytosis (removal of dying neutrophils). This has deleterious effects on mucosal immunity and permits the establishment of chronic bacterial infection of the lower airways. Upon exposure to a newly acquired viral infection, which is a common trigger for AECOPD, the virus permits the further outgrowth of bacteria causing a bacterial super-infection in the lungs. SAA is significantly increased during co-infections and stimulates neutrophilic inflammation via FPR2/ALX-dependent mechanisms. Excessive neutrophilic inflammation can drive mucus hypersecretion and degrade anti-microbial peptides in the airways. We propose that the alternative FPR2/ALX agonist, AT-RvD1 can therapeutically intervene at critical pathological pathways that lead to bacterial super-infections. AT-RvD1 facilitates the resolution of inflammation during co-infections by improving the phagocytic clearance of bacteria and efferocytosis of apoptotic neutrophils in the lungs. It also potently suppresses neutrophil migration, thereby limiting tissue damage, mucus secretion and anti-microbial peptide degradation caused by ongoing inflammation. AT-RvD1 may also reduce neuroinflammation consequent to serious lung co-infections as brain microglia are activated by SAA in manner that is suppressed by AT-RvD1.

model (31). Here, we exposed mice to cigarette smoke prior to acute influenza A virus infection, which resulted in a greater viral lung burden (31). Furthermore, treatment with ebselen effectively reduced viral lung titres and lung inflammation, thus identifying the Gpx pathway as a novel therapeutic target for the treatment of viral-induced exacerbations (31).

VIRAL AND BACTERIAL CO-INFECTIONS INCREASE THE SEVERITY OF AECOPD

AECOPD associated with respiratory viruses including rhinoviruses have been shown to be frequently followed by secondary bacterial infections in COPD (32). A subsequent study revealed that acute rhinovirus infection during AECOPD promoted a significant rise in pathogenic bacteria, such as *H. Influenzae* from the pre-existing lung microbiota, which persisted for over 40 days (33). Hence, the classic secondary bacterial infection setting whereby an acute viral event leads to a secondary bacterial lung infection may not necessarily represent the natural course of respiratory co-infections in COPD. This is because many moderate/severe COPD patients are already chronically infected with multiple pathogenic bacteria before they encounter a viral pathogen. Acute flares or COPD exacerbations associated with the presence of bacterial and viral pathogens occur frequently, constituting around a quarter of infective exacerbations (8, 34, 35), and this rate may increase with the development of more sensitive assays to detect respiratory pathogens. Clinically, AECOPD associated with the presence of a bacterial and viral pathogen are significant as they result in

more severe events involving greater lung function impairment and longer hospitalization (8). Virus infection in the presence of chronic bacterial infection is also an important determinant of hospital readmission following the initial exacerbation (36). AECOPD associated with respiratory co-infections result in increased bacterial lung loads during the acute phase of the exacerbation (37). The viral-mediated outgrowth of bacteria is not restricted to rhinovirus and *H. Infuenzae* interactions as rhinovirus can also facilitate acquisition and transmission of *S. pneumoniae* (38). Influenza A readily triggers pneumococcal dispersion from nasopharyngeal biofilms deeper into the lower airways (39). In addition, rhinovirus has been shown to impair antibacterial responses to the TLR4 ligand, LPS and reduce uptake of *E. coli* bioparticles (40).

At a cellular level, severe exacerbations drive an airway inflammatory response involving increased neutrophilic inflammation (36). The amplified neutrophilic response during severe AECOPD is thought to directly compromise host immunity to bacterial respiratory pathogens. We have previously shown that the degranulation of neutrophil-derived proteinases, such as neutrophil elastase and MMP9 increase with the severity of COPD irrespective of elevated corticosteroid therapy (4). Increased levels of neutrophil elastase are also detected during AECOPD associated with the presence of a viral and bacterial pathogen (32). In this cohort, the levels of antimicrobial peptides were markedly reduced in exacerbations where viral and bacterial pathogens were detected, and they suggest that uncontrolled degranulation of neutrophil elastase actively degrades anti-microbial peptides in the lung, leading to outgrowth of bacteria (32). Neutrophils can also increase mucin production by activating epidermal growth factor receptor (EGFR) signaling (41), which involves protease-dependent release of membrane bound EGFR ligands, such as TGF-α (42, 43). In addition, neutrophil-derived myeloperoxidase can also catalyze the generation of hypochlorous acid that cross-links mucus to form hydrogels (44). Since mucus hypersecretion is significantly associated with an excess decline in lung function during AECOPD and an increased risk of subsequent hospitalization (45), we propose that an excessive neutrophilic response is directly driving mucus-mediated obstruction during AECOPD.

TARGETING BACTERIAL SUPER-INFECTIONS WITH SPECIALIZED PRO-RESOLVING MEDIATORS

Hence, a therapeutic strategy that dampens the exuberant neutrophilic response during AECOPD may not only be beneficial in reducing the risk of developing a secondary bacterial infection, but will also alleviate symptomatic lung function decline due to excessive mucus production and plugging. To support our claim, we have actively screened for inflammatory biomarkers that are altered during AECOPD and identified serum amyloid A (SAA) as an acute phase reactant that is markedly increased during severe episodes (34). Furthermore, we demonstrated that the circulating levels of SAA were significantly higher in AECOPD associated with the presence of a bacterial and viral co-infection (34). SAA serves multiple innate host defense roles during acute infection including opsonisation of gram-negative bacteria to facilitate more efficient phagocytosis (46). It can also promote the recruitment of leukocytes to the site of infection as it is a functional agonist for the Formyl peptide receptor 2 (FPR2/ALX), which stimulates chemotaxis (47). In COPD, we found a positive association between SAA expression and neutrophilic inflammation in lung tissue biopsies derived from COPD patients (48). We demonstrated that SAA-induced recruitment of neutrophils into the airways is dependent on Interleukin 17A (IL-17A) signaling (48, 49). SAA can also prolong neutrophil survival by suppressing the apoptotic machinery involving activation of the ERK and PI3K/Akt signaling pathways (50). Hence, production of SAA is normally protective and self-limiting with a sharp decline during the resolution phase of infection, however the persistent elevation during AECOPD triggered by co-infections may lead to the excessive recruitment of neutrophils.

The persistence of SAA expression during AECOPD will alter FPR2/ALX signaling in innate and mucosal cells that express this receptor. FPR2/ALX is a member of the G-protein coupled receptor (GPCR) superfamily characterized by seven putative trans-membrane domains that displays diverse ligand affinities, interacting with over 30 different ligands (51). By binding to distinct ligands that are temporally expressed, this receptor is a master regulator of acute inflammation and resolution of inflammation. Hence, there is a class switch from pro-inflammatory ligands (such as SAA and the leukotriene LTB_4) to pro-resolving mediators during the course of infection and acute inflammation (52). Lipoxin A_4 (LXA_4) is a specialized pro-resolving mediator that directly interacts with FPR2/ALX to initiate resolution pathways. This eicosanoid opposes leukocyte migration and activation by suppressing transendothelial (53) and transepithelial (54) migration and neutrophil degranulation (55). Furthermore, we have shown that LXA_4 can potently antagonize the recruitment of neutrophils into the lungs in response to SAA stimulation (56). LXA_4 also promotes tissue repair by stimulating basal cell proliferation required for wound healing following mucosal injury (57). In addition, LXA_4 stimulates more efficient macrophage-mediated efferocytosis of apoptotic neutrophils, which is an essential component to the resolution of inflammation (58, 59).

FPR2/ALX also interacts with an alternate specialized pro-resolving mediator called resolvin D1 (RvD1), which belong to the D series resolvins derived from the omega-3 fatty acid, docosahexaenoic acid (50). Like LXA_4, RvD1 and its more stable aspirin-triggered derivative (AT-RvD1) display potent anti-inflammatory actions in a number of disease models including cigarette smoke exposure and acute lung injury (60, 61). This eicosanoid is also a promising therapeutic target in bacterial pneumonia because it can stimulate macrophage phagocytosis of pathogenic bacteria (*E. coli*, *P. aeruginosa,* and *H. Influenzae)* and enhance neutrophil efferocytosis to prevent collateral lung injury (62, 63). More recently, we have evaluated the efficacy of AT-RvD1 in a co-infection model where mice were initially inoculated with *S. pneumoniae* and subsequently infected with influenza A virus. This model represents the AECOPD setting where the lower airways

are chronically infected with pathogenic bacteria prior to encountering a respiratory virus. Similar to classic bacterial super-infection models, acute viral infection resulted in a marked increase in pneumococcal lung load (64). We also observed a significant increase in neutrophil and monocyte infiltration into the lungs of co-infected mice and the lung pathology was consistent with severe pneumonia. Levels of SAA were strikingly increased in the serum and lungs of co-infected mice relative to mice infected with either pneumococcus or influenza A virus alone (64). Our approach to reducing excessive inflammation in co-infected mice was to therapeutically deliver exogenous AT-RvD1 during the acute phase of infection, where FPR2/ALX is the only characterized receptor for AT-RvD1 in mice. AT-RvD1 reduced pneumococcal lung load and potently reduced the degree of pneumonia or alveolitis, which was associated with a marked reduction in neutrophil and monocyte lung infiltration in co-infected mice (64). The reduction in neutrophilic inflammation was accompanied by a reduction in neutrophil elastase activity in the lungs. The AT-RvD1 mediated suppression of neutrophil elastase activity was concurrently associated with restoration of anti-microbial activity in the bronchoalveolar lavage (BAL) fluid of co-infected mice (64). AT-RvD1 is also known to stimulate the production of the anti-bacterial peptide lipocalin 2, thereby enhancing anti-microbial activity (62).

In addition to reducing the degree of localized pneumonia, specialized pro-resolving mediators, such as AT-RvD1 may also treat the central consequences of co-infection or bacterial super-infection. In the classic bacterial super-infection setting, *S. pneumoniae* can enter and proliferate in the brain, with the ensuing inflammatory response causing bacterial meningitis in severe cases (65). Whilst meningitis rarely occurs, severe respiratory infections will prolong sickness behavior including fever, malaise and fatigue. The onset of sickness behavior is mediated in part, by inflammatory cytokines, such as IL-1β produced at the primary site of infection, which then act centrally to stimulate neuroinflammation (66). Brain inflammation can also produce acute cognitive impairments, such as delirium, which is common in COPD patients (67, 68) and this is worsened during acute exacerbations (69). The circulating inflammatory mediators that impair cognitive function during AECOPD are not well-characterized. However, circulating SAA can readily cross the intact blood-brain barrier (70) and excessive production of SAA in a transgenic model resulted in greater deposition in the brain and an increase in brain inflammation (71). Consistent with this study, we found that in our co-infection model associated with a marked increase in circulating SAA, there was an increase in SAA immuno-reactivity, increased numbers of "activated" amoeboid-shaped microglia and inflammatory cytokine expression in the brain (72). Furthermore, under *in vitro* conditions SAA proved to be a potent stimulus for primary mouse microglia activation, and this response was markedly suppressed by the anti-inflammatory actions of AT-RvD1 (72).

CONCLUDING REMARKS

The likelihood of developing a co-infection or secondary bacterial infection is increased in COPD largely due to the ROS-dependent suppression of macrophage phagocytic function. Suppressed macrophage phagocytosis contributes to the establishment of chronic bacterial infection of the lower airways. Consequently, acute infection with a newly acquired respiratory virus, such as influenza A can promote uncontrolled bacterial outgrowth in the lung. This is observed clinically, as the detection of a respiratory virus and bacteria during AECOPD occurs frequently and is associated with an increase in exacerbation severity and neutrophilic inflammation. We have shown that the exuberant inflammatory response during co-infections are associated with elevated levels of SAA, which is an agonist for FPR2/ALX that promotes neutrophil migration and survival. Specialized pro-resolving mediators, such as lipoxins and D-series resolvins can counteract the pro-inflammatory actions of SAA. We provide compelling pre-clinical data to demonstrate that AT-RvD1 is a very effective therapeutic in the co-infection setting, where it potently suppressed leukocyte tissue accumulation in the lungs and concurrently improved pneumococcal clearance. Furthermore, co-infections markedly increase brain inflammation and AT-RvD1 can suppress the release of inflammatory cytokines from activated microglia. In summary, specialized pro-resolving mediators have great potential in the co-infection setting as they not only reduce local tissue inflammation and improve bacterial clearance at the site of infection, but they can also dampen central microglial inflammation that prolongs sickness behavior and cognitive dysfunction (**Figure 1**).

AUTHOR CONTRIBUTIONS

SB performed the literature review. HW, DA, and SB designed the figures. SB, SS, HW, DA, and RV organized, wrote and edited the manuscript.

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REVIEW
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Sepsis and Nosocomial Infection: Patient Characteristics, Mechanisms, and Modulation

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Sepsis is a leading cause of death worldwide. After initial trials modulating the hyperinflammatory phase of sepsis failed, generations of researchers have focused on evaluating hypo-inflammatory immune phenotypes. The main goal has been to develop prognostic biomarkers and therapies to reduce organ dysfunction, nosocomial infection, and death. The depressed host defense in sepsis has been characterized by broad cellular reprogramming including lymphocyte exhaustion, apoptosis, and depressed cytokine responses. Despite major advances in this field, our understanding of the dynamics of the septic host response and the balance of inflammatory and anti-inflammatory cellular programs remains limited. This review aims to summarize the epidemiology of nosocomial infections and characteristic immune responses associated with sepsis, as well as immunostimulatory therapies currently under clinical investigation.

Keywords: sepsis, compensatory anti-inflammatory response, priming, nosocomial infection, immunosuppression, SIRS, immunostimulation

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INTRODUCTION

In 1996, following several failed trials aimed at treating the systemic inflammatory response syndrome (SIRS) (1–3), Roger C. Bone first urged a paradigm shift toward understanding the "compensatory anti-inflammatory response (CARS)" in sepsis (4). Decades later, sepsis remains a leading cause of morbidity and mortality affecting over 30 million people worldwide each year (5), yet not a single immune modulating therapy is actively being used in the clinical setting today. Though mortality rates have declined over time with advances in supportive care, improvements in sepsis therapy are needed to combat persistently high mortality (6, 7). The most recent focus has been on delivery of precision medicine through immunomodulation of the altered host response in sepsis.

Early observations of immune dysfunction in the critically ill come from the trauma and surgical literature (8–10). Decreased cytokine responses to stimulation were later identified and associated with decreased survival in patients with septic shock (11). Though a correlation between sepsis and nosocomial infection has been clinically recognized for decades, it is mostly within the last 10–15 years that this clinical entity has been strongly associated with depressed host immune responses. Large observational studies examining the impact of secondary infection on morbidity and mortality in patients with sepsis are limited and at times data are conflicting. Here, we review the evidence for increased susceptibility to secondary infection in sepsis, mechanisms of depressed host immunity, and promising therapies to modulate the host response.

EPIDEMIOLOGY OF NOSOCOMIAL INFECTION AFTER SEPSIS

Nosocomial infections after sepsis are common. However, there are wide variations in the reported incidence and associated morbidity. Sepsis-related immunosuppression in the form of depressed cytokine responses and lymphocyte apoptosis has been hypothesized as the main factor contributing to this complication, though evidence supporting causation is found mainly in experimental models of sepsis. A number of small retrospective observational studies have demonstrated increased risk for nosocomial bacterial and fungal infections in patients with sepsis (12, 13). Likewise, reactivation of dormant viral infections has also been well-recognized and occurs concomitantly with other nosocomial infections (14). For the purposes of this review, we will focus on two large observational studies that have examined this topic in detail.

One large retrospective study estimated that 1 in 3 patients with sepsis will develop a nosocomial infection and half of these infections will occur in the lung (15). A larger prospective study found that 1 in 8 patients will develop nosocomial infection and one-quarter of these will be pulmonary infections (16). In both studies, nosocomial infection developed in the late phase of sepsis at a median of 9 days from admission. In the study by Zhao and colleagues, the most common site of secondary infection was pulmonary (52.5%) and there was no association between primary site of infection (e.g., pulmonary, abdominal, skin/soft tissue, urinary) and the development of secondary infection. In the study of van Vught and colleagues, the most common site of secondary infection was cardiovascular (35.3%). The distribution of primary and secondary infection sites in both studies were distinct, suggesting that secondary infection resulted from a new infectious insult rather than inadequately managed primary infection. Patient risk factors for development of nosocomial infection were similar and included older age, higher illness severity score, longer intensive care unit (ICU) length of stay (LOS), and respiratory insufficiency. ICU-specific exposures such as central venous catheterization and endotracheal intubation also increased risk. The most common causative pathogens were bacterial in both studies. As list of typical sites of nosocomial infection and pathogens are shown **Table 1**.

Nosocomial infection was associated with increased hospital LOS in both studies, but the effect on mortality varied between 15 and 21%. The adjusted absolute increase in mortality specifically attributable to nosocomial infection (population attributable mortality fraction) was only 2% (16). These data suggest that a significant portion of the difference in mortality after sepsis is actually due to competing factors such as higher admission illness severity rather than nosocomial infection. Other studies have made similar observations linking illness severity to outcome, rather than nosocomial infection (17). Furthermore, critically ill patients without sepsis had similarly high rates of nosocomial infection suggesting that ICU exposure, rather than sepsis itself, contributes largely to the development of nosocomial infections. However, infections in patients with sepsis were more commonly due to opportunistic pathogens (enterococci, *Pseudomonas aeruginosa* and viruses) implying there still may be a link to sepsis-related immunosuppression.

Both exposures and host susceptibility play a role in development of nosocomial infection. As such, differences among studies in nosocomial infection and mortality rates are likely due to differences in patient selection, ICU type, primary type of sepsis, infectious diagnostics/definitions, infection prevention practices, and geographical location. Regardless of their impact on mortality, nosocomial infections are common and remain a significant factor in morbidity during recovery from sepsis. In addition, they are a burden on the health care system and account for an additional $20,000–40,000 dollars per episode (18). Whether immunostimulatory therapies will reduce rates of secondary infection in patients with sepsis will be determined in ongoing clinical trials.

DOES THE BIPHASIC MODEL EXPLAIN THE HETEROGENEITY OF RESPONSE IN SEPSIS?

The Biphasic Model

The biphasic model of sepsis has been hypothesized for nearly two decades (19). This model depicts an initial hyperinflammatory response followed by prolonged immune paralysis resulting in morbidity and mortality. However, it is well recognized that the septic immune response does not fit a linear timeline of enhanced inflammation with progression to impaired immunity. Evidence to support this comes from multiple studies, including systematic reviews of gene expression microarray data from blood leukocytes over the course of sepsis (20). In this study, no clear immunosuppressive phase was identified. In fact, at any given timepoint pro- and anti-inflammatory genes were expressed simultaneously in the same patient. Similarly, others have demonstrated circulating anti-inflammatory mediators, such as IL-10, in conjunction with prototypical inflammatory cytokines (TNFα, IL-6, IL-8) at the onset of septic shock (21, 22). Sepsis is therefore a heterogeneous continuum of pro and anti-inflammatory immune programs occurring concurrently. There is also evidence supporting primed immune programs, discussed later in this section, that may occur during the course of sepsis or recovery. A revised model of the inflammation is therefore necessary to illustrate the simultaneous nature of these processes (**Figure 1**).

To complicate matters further, cytokines often do not behave in a dichotomized manner (23). A single cytokine may contribute to survival or death depending on the context in which it is examined (23). For example, IL-10 neutralization 24 h after cecal ligation and puncture (CLP) is protective against secondary *Pseudomonas aeruginosa* pneumonia (24). However, IL-10 neutralization performed prior to endotoxemia or CLP (25, 26) is lethal. A cytokine may also act pleiotropically depending on the environmental context. For instance, IL-10 has suppressive and priming effects depending on the adherent state of a monocyte (27). Non-adherent monocytes are actually primed by this "anti-inflammatory" cytokine to produce more TNFα, IL-6,

TABLE 1 | Primary and secondary sites of infection and etiology of secondary nosocomial infection in patients presenting with sepsis.

Infection site	Primary	Secondary
Pulmonary	48%	25.4%
Cardiovascular*	7.3%	35.3%
Abdominal	19%	15.9%
Neurological	2.2%	12.7%
Skin/Soft tissue	2.2%	3.9%
Urinary	4.3%	1.2%
Other¥	16.8%	19%
TYPICAL NOSOCOMIAL PATHOGENS		
Gram positive (45.2%)		
• *Staphylococcus epidermidis* (14.7%)		
• *Enterococcus faecalis* (12.0%)		
• *Enterococcus faecium* (6.3%)		
• *Staphylococcus aureus* (6.0%)		
• Others (6.2%)		
Gram negative (26.6%)		
• *Pseudomonas aeruginosa* (9.0%)		
• *Escherichia coli* (3.9%)		
• *Klebsiella pneumonia* (2.7%)		
• *Stenotrophomonas maltophilia* (2.7%)		
• Others (8.3%)		
Fungi (9.6%)		
• *Candida albicans* (2.7%)		
• *Candida glabrata* (1.2%)		
• Others (5.7%)		
Viruses (9.9%)		
• *Herpes simplex* (3.9%)		
• Cytomegalovirus (2.1%)		
• Others (3.9%)		

*Data from Van vught et al. (16) Table 2 and Supplementary Table 3. *Cardiovascular site of infection included bacteremia and catheter-related bloodstream infections ¥Other sites of infection included lung abscess, sinusitis, pharyngitis, tracheobronchitis, endocarditis, mediastinitis, myocarditis, postoperative wound infection, bone and joint infection, oral infection, eye infection, reproductive tract infection.*

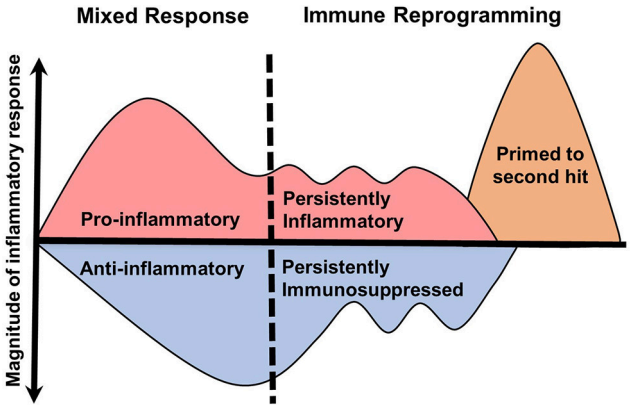


FIGURE 1 | Revised model of inflammation in sepsis. The traditional biphasic model of sepsis (19) plots the immune system on a timeline with an initial hyperinflammatory cytokine storm followed by hypoinflammatory immune paralysis. However, clinical evidence does not support well-demarcated immune phases. In this revised model, the initial immune response to sepsis is a continuous mix of pro- and anti-inflammatory processes that lead to specific immune reprogramming. These programs include persistently pro- or anti-inflammatory and primed responses. The duration and magnitude of each inflammatory program is likely result of many determining factors.

and IL-1ra upon endotoxin challenge *ex vivo*. The net effect of an inflammatory mediator is therefore highly contextual.

Moreover, the inflammatory response is tissue-specific (28). Differential expression of inflammatory mediators has been noted in mouse tissues in response to systemic endotoxin. IL-1α production is maximally increased in lung, spleen and liver, while IL-6 is increased in the heart, muscle, brain and kidney (29). In a rat model of CLP examining the molecular response to sepsis, though a subset of gene expression was shared, each organ had a distinct molecular fingerprint (30). Tissue-level responses to secondary stimulation may also be discrepant in the septic host. For example, hemorrhage prior to CLP in mice caused primed responses in alveolar macrophages and Kupffer cells, whereas splenic macrophages and peripheral blood mononuclear cells demonstrated decreased cytokine production consistent with endotoxin tolerance (31). The magnitude of response to an inflammatory stimulus is also variable, as *in-vivo* imaging of lipopolysaccharide (LPS)-induced NF-κB activation has shown heterogenous intracellular activation more prominently in the skin, lungs, spleen, and small intestine as compared to other organs (32). These observations illustrate two important conceptual points: (1) the (tissue) compartmental response to sepsis and other inflammatory stimuli is highly variable both in quality and magnitude and (2) primed and suppressed responses may be present simultaneously within the same organism following a single exposure. These experimental studies raise important questions that require further investigation in humans.

Priming

Primed immune responses in sepsis may contribute to the heterogenous inflammatory response and are not accounted for in the biphasic model. Priming requires an initial exposure to the host that results in an enhanced inflammatory response to secondary stimulation. For example, Kupffer cells are primed to produce more TNFα in response to endotoxin when femur fracture precedes CLP (33). Similarly, hemorrhage prior to CLP enhances production of plasma IL-6 and TNFα (31). Priming during sepsis may be a protective mechanism, as seen in a model of enteral *Enterococcus faecalis* infection (34). In this study, mice were pre-exposed to mild or severe sterile systemic inflammation using varying degrees of pancreatitis or thermal injury. Mice experiencing mild inflammation were protected from *E. faecalis* related mortality, this was associated with primed IL-12 production and enhanced phagocytic function in peritoneal macrophage.

Priming may also represent a mechanism of late organ injury in survivors of sepsis. It is well recognized that survivors of sepsis are at increased risk for long-term cognitive impairment (35), new cardiac events (myocardial infarction, cerebrovascular accident, sudden cardiac death) (36), and new renal failure (37). The cause of these complications remains unclear, though persistent inflammation and primed immune responses have been hypothesized to contribute. Murine CLP models have demonstrated enhanced TNFα production in splenic inflammatory monocytes and brain microglia for at least 2 weeks after sepsis (38, 39) suggesting a possible link between

primed cells and long-term organ dysfunction. Meanwhile, persistent inflammation may influence patient outcome, as observations of persistent elevation of IL-6 in patients with pneumonia have been associated with increased risk for long-term mortality due to cardiovascular disease or renal failure (40). Similarly, models of sepsis survival have demonstrated progressive atherosclerosis in the setting of low-grade circulating inflammation (41) and neurocognitive dysfunction associated with persistent neuroinflammation (38, 42) weeks to months after polymicrobial sepsis. In a mouse model of pneumococcal pneumonia, recruitment of inflammatory monocytes to the brain was associated with microglial activation and long-term cognitive impairment (43). When monocyte recruitment was abolished, neuroinflammation was reduced and cognitive impairment was improved. While these initial findings are exciting, the relationship between persistent inflammation, immune priming and long-term organ injury needs to be understood in more detail.

There is strong experimental evidence supporting the conclusion that the inflammatory response of sepsis is heterogeneous at a molecular, cellular, tissue compartment, and individual level. Though further studies are needed in human sepsis, the possibility of "within patient" compartment specific immune heterogeneity warrants consideration. A new conceptual model of the patient experiencing sepsis is required (**Figure 2**), as one may have reduced immune response on peripheral blood assays, yet in other compartments the net immune response may be mixed or primed. With the introduction of immune stimulating therapies, one must consider that disproportionately primed organs may be harmed by this therapy. Decisions on how to modulate the immune response may be informed by *ex vivo* stimulation assays, but consideration should be taken to survey for multiple cellular programs in multiple tissue compartments. In addition, these programs are not limited to the acute phase of sepsis, as immune reprogramming influences the entire clinical course including recovery (**Figure 3**). Pre-sepsis immune status is likely to be an important determinant of which predominant cellular program manifests during acute illness. Post-sepsis immune status may also be impacted by the preceding phases of illness. Likewise, chronic comorbidities may influence the magnitude and evolution of both responses. Despite the evidence supporting sepsis-related immunosuppression in this review, there are large pieces of the immune response to sepsis that remain a mystery and our understanding of sepsis heterogeneity is only in its infancy.

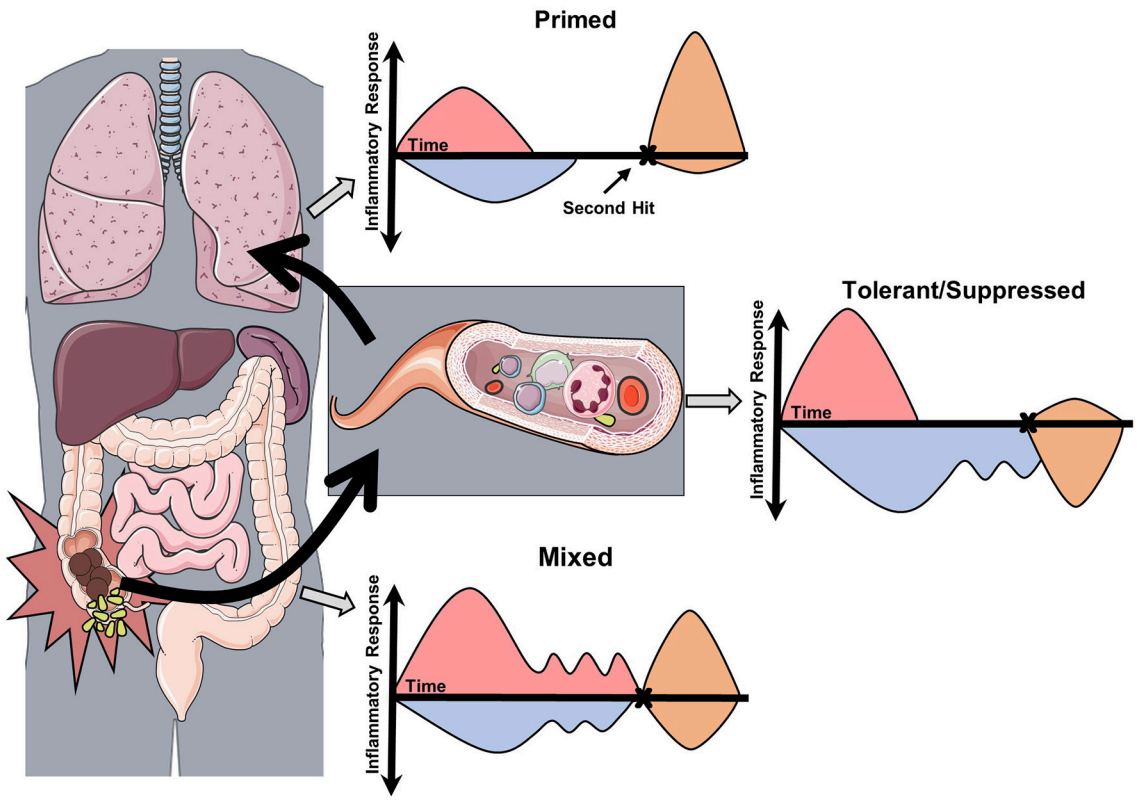


FIGURE 2 | Conceptual model of the compartmentalization and heterogeneity of sepsis. This conceptual model is derived from studies in experimental sepsis that have demonstrated tissue-specific inflammatory responses. In this model, acute sepsis in one compartment (abdomen) leads to specific and dynamic changes in proximal (blood) and distal (lungs) compartments. Assessment of the immune response by *ex vivo* stimulation assays (second hit) may then reveal the predominant cellular program. In this case, each compartment responds differently to secondary stimulation based on the severity and composition of the preceding inflammatory insult.

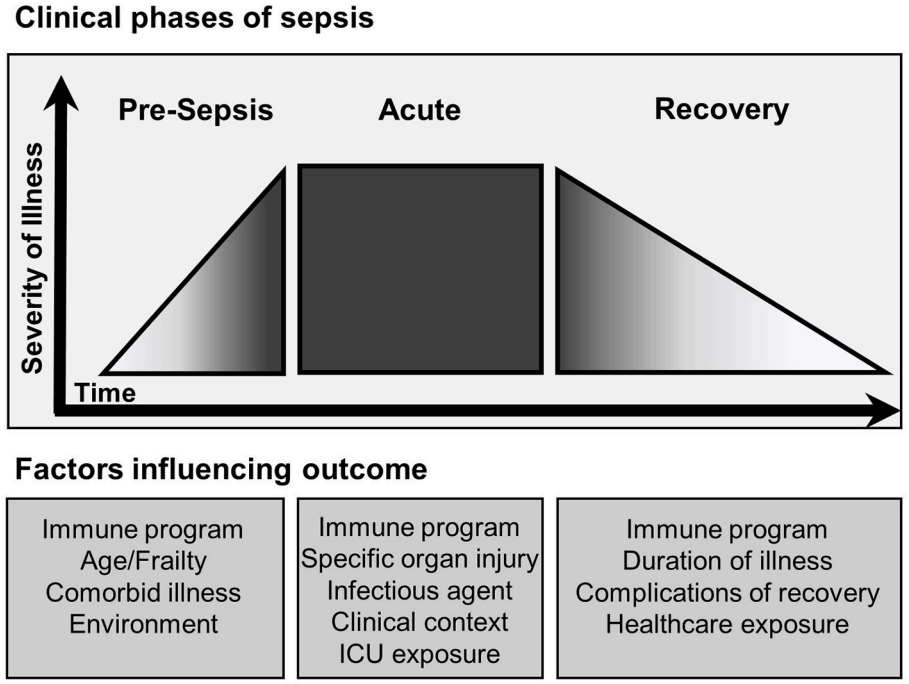


FIGURE 3 | Clinical phases of sepsis and factors influencing outcome. The clinical course of sepsis is characterized by accelerated progression in severity of illness leading to the development of clinical sepsis. The outcome of each clinical phase of sepsis is influenced by multiple factors. The pre-sepsis phase is influenced primarily by the baseline functional state of the patient. Pre-sepsis functionality directly affects the course of acute sepsis including onset, magnitude and duration. Furthermore, properties inherent to the type of sepsis and exposures occurring during management of acute sepsis continue to affect outcome. Recovery follows and is largely dependent on the severity of prior phases, though continued exposure to the healthcare system places patients at risk for nosocomial complications. Throughout each phase the specific immune program is heterogenous and influences outcome.

THE IMMUNOSUPPRESSIVE CELLULAR PROGRAM OF SEPSIS

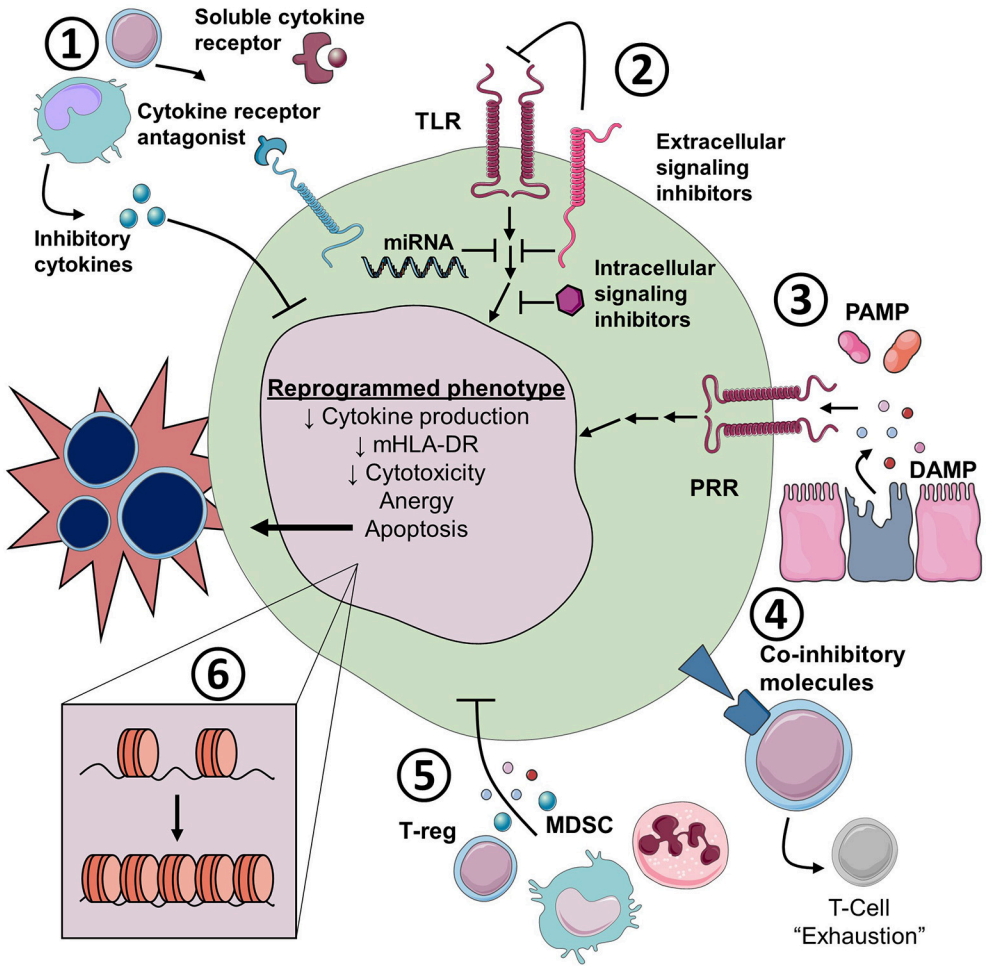
Critically ill patients with sepsis, trauma, and burns experience similar immunosuppressive phenotypes. Broadly, these include enhanced cellular apoptosis, suppressed cytokine production, decreased major histocompatibility complex (MHC) class II surface markers, reduced antigen presentation, anergy, and diminished cytotoxic effector cell function. Collectively, these septic leukocyte responses are known as the immunoparalysis or immune exhaustion. While these phenotypes may be associated with increased risk of nosocomial infections and death, they are not inclusive of broader immune changes in sepsis such as potentially primed cells. Moreover, though an exhausted cell may down-regulate cytokine responses, other cellular functions may be simultaneously upregulated (44, 45). As such, the term "cellular reprogramming" is more appropriate to describe general immunophenotypic changes occurring during sepsis. Several examples of immunosuppressive cellular program are described below and are summarized in **Figure 4**.

Cellular Apoptosis

Apoptosis of T and B lymphocytes has been demonstrated in models of sepsis (46), post-mortem analysis of septic patients (47–49), and in the circulation of patients with septic shock (50). Resultant lymphopenia is associated with increased mortality and risk of nosocomial infection (50, 51) and occurs commonly in patients with persistent critical illness (52). Enhanced apoptosis also occurs in myeloid and epithelial cells including blood monocytes (53, 54), dendritic cells (55), intestinal (48) and pulmonary epithelial cells (56), but not neutrophils (57). Apoptosis of monocytes may be a protective mechanism, as acute apoptosis of these cells has been associated with improved mortality (54). Broad reversal of lymphocyte apoptosis through caspase inhibition or over-expression of the antiapoptotic B-cell lymphoma 2 (Bcl-2) protein in experimental sepsis has subsequently been shown to improve mortality (58, 59). As such, improving sepsis-induced lymphocyte apoptosis and lymphopenia has been a primary target for immunomodulation.

Suppressed Cytokine Release

Immunodepression, immune tolerance, and immunoparalysis are all terms used to describe the decreased production of various cytokines, including TNFα, IL-1β, IL-6, and IFNγ, after *ex vivo* stimulation of leukocytes with endotoxin or other pattern recognition receptor (PRR) agonists. This phenotype in sepsis is similar to the *in vitro* phenomenon of endotoxin tolerance, whereby stimulating with high concentration endotoxin results in decreased responses upon secondary stimulation. In sepsis, decreased cytokine production in response to endotoxin stimulation has been demonstrated in whole blood (60),



Mechanism of reprogramming	
1.	Anti-inflammatory cytokine and soluble mediator production
2.	Negative regulators of TLR signaling
3.	Altered signaling through pattern recognition receptors
4.	Exhaustion via co-inhibitory molecule signaling
5.	Altered adaptive and innate populations
6.	Epigenetic reprogramming

FIGURE 4 | Cellular and molecular mechanisms of immune reprogramming in sepsis**.** TLR, toll-like receptor; miRNA, microRNA; PAMP, pathogen associated molecular pattern; DAMP, damage associated molecular pattern; PRR, pathogen recognition receptor; MDSC, myeloid derived suppressor cell; T-reg, regulator T-cell; mHLA-DR, monocyte Human Leukocyte Antigen-DR.

peripheral blood mononuclear cells (61), adherent monocytes (11), neutrophils (62), and NK cells (63). This phenotype occurs early in the course of sepsis and resolves in survivors. However, failure to resolve immune tolerance is associated with increased mortality (11), and expression of endotoxin-tolerant gene signature has been associated with sepsis induced organ failure (64). Though immune tolerance is associated with increased risk of nosocomial infection in trauma patients (65), the association in sepsis is less clear (66, 67).

Alterations in HLA-DR Expression and Other Surface Markers

Changes in cellular surface markers occurs during sepsis, with reduced expression of MHC class II molecules on monocytes being the most well studied. Low monocyte human leukocyte antigen (mHLA)-DR correlates with endotoxin tolerance and suppression of antigen-specific T cell responses (68). Early studies found increased rates of sepsis in trauma patients with low mHLA-DR expression (69). Subsequently, low mHLA-DR

on admission for sepsis or septic shock has been associated with increased mortality (70, 71) and nosocomial infections (72). Failure to restore mHLA-DR expression over the course of illness is also associated with worse outcome in patients with severe sepsis (66). While highly predictive of mortality and nosocomial infection, mHLA-DR expression dynamics are contextual and dependent on the infectious agent (73) with Gram-positive infections showing lower monocytic mHLA-DR expression than Gram-negative bloodstream infections. The reliability of mHLA-DR to predict outcome and its dynamics of expression are currently under investigation as part of two large observational cohort studies (74, 75). Neutrophil surface markers are also altered, including CD88 expression which is associated with reduced phagocytic function and increased risk for nosocomial infection (76). Importantly, the presence of multiple surface marker abnormalities (low mHLA-DR, low neutrophil CD88 and increased T regulatory cell markers) was most associated with nosocomial infection than any single marker alone (77).

Gene Expression Endotypes

Functional assays and cell surface marker assessments remain the standard method to assess immunosuppression in sepsis. However, whole-genome expression endotypes correlating with survival of sepsis have been discovered using advanced statistical techniques (78, 79). Davenport et al. found increased 14-day mortality and illness severity associated with a gene expression endotype that was characterized by functional changes in T cell activation, apoptosis, endotoxin tolerance, and down-regulation of HLA class II molecules. Variation in genomic DNA sequence was also associated with specific gene expression patterns, supporting a genetic mechanism for individual heterogeneity. In a follow-up study of patients with community-acquired pneumonia (CAP) and fecal peritonitis (FP), the genomic response to sepsis was largely similar between types of infection with only a modest number of genes differentially expressed between CAP and FP (80). Serial sampling over the course of sepsis demonstrated that patients may switch between the defined endotypes during the course of disease. These studies confirm what is known about clinical sepsis, that phenotype is both heterogenous and dynamic. Classification of patient immune responses quickly and dynamically remains a priority for precision medicine in sepsis. In addition, while mixed leukocytes in whole blood have been reliably used for gene expression analysis, cell-type specific gene expression in sepsis may reveal additional endotypes and help us understand therapies that may reliably be used to modify them.

Lung-Specific Changes in Immunity

The lung is uniquely and continuously exposed to the external environment and acts as a first line of defense against environmental pathogens, especially in critically ill patients with respiratory failure requiring mechanical ventilation. Consequently, the lung is a primary site for nosocomial infection in patients with sepsis. The alveolar macrophage (AM) represents the predominant immune effector cell in the alveolus. Similar to the dysfunction of blood monocytes, decreased TNFα production in AM of mice and humans with sepsis has been recognized for quite some time (81). In polymicrobial sepsis models, AM display both depressed cytokine responses to endotoxin challenge and decreased phagocytic capacity (82). Neutrophil recruitment to the alveolus is decreased (83) and recruited neutrophil ROS production is depressed (84). In an *Escherichia coli* pneumonia model, lung parenchymal dendritic cells demonstrated decreased antigen presenting capacity and reduced immunostimulatory responses during recovery from sepsis (85). Moreover, these depressed responses were specific to the pulmonary compartment and were mediated by local inflammatory factors released upon organ injury. Disruption of the epithelial barrier leads to alveolus permeability, leak, and decreased mucocilliary clearance all of which may predispose to development of nosocomial infection (86). General loss of pulmonary epithelial barrier function is noted with pulmonary epithelial cell apoptosis in polymicrobial sepsis models (87) and in patients with acute lung injury due to sepsis (56).

Anergy and Cytotoxicity

Anergy due to a failure of T cell proliferation or elaboration of cytokines in response to specific antigens has also been described. An increased risk of post-operative sepsis and death was initially described in patients with anergy to delayed-type hypersensitivity skin testing (88). Similarly, patients with lethal post-operative peritonitis had reduced T cell proliferation and secretion of both IL-2 and TNFα in response to CD3/CD28 cross-linking (89). In sepsis models, development of anergy is mediated via a population of TNF-related apoptosis-inducing ligand (TRAIL) expressing $CD8^+$ T cells (90). In humans, $CD4^+$, $CD25^+$, $CD127^{lo}$ regulatory T cells (Treg) have been associated with reduced mitogen responses and development of anergy (91). Depressed cytotoxic responses have been reported in various cell types. Impaired NK cell function with reduced IFNγ secretion and cytotoxicity has been reported in patients with sepsis (92), while others have found normal NK cytotoxic function in severe sepsis (93).

MECHANISMS OF ALTERED IMMUNITY

There are many potential mechanisms for altered immunity, both suppressed and primed, in patients with sepsis. Here we highlight several important mechanisms with a primary focus on mechanisms of immunosuppression (**Figure 4**).

Anti-inflammatory Cytokines and Soluble Receptors

Three decades of research examining the cytokine response in sepsis is too broad a topic to review here and extensive reviews have already been published on the subject (21). Several cytokines and anti-inflammatory mediators are associated with worse outcomes in septic patients. IL-10 suppresses the pro-inflammatory immune response through deactivating innate immune cells (94, 95). IL-10 is elevated early in the course of sepsis (96) and persistent elevations increase risk of death (97). As discussed previously, this cytokine has pleiotropic roles in experimental models of sepsis, though it may have a particular importance in sepsis-induced impairment of lung immunity (24).

IL-10 is actively secreted by multiple cell types that are expanded in septic patients including Treg and myeloid derived suppressor cells (MDSC), which are discussed in more detail below. Though IL-10 has direct immunosuppressive effects, its association with development of nosocomial infection is less straight forward (96). Enhanced IL-10 signaling has been associated with the development of nosocomial infection in at least one study (98).

Soluble receptors for cytokines are additional anti-inflammatory mediators that have been long recognized in sepsis. These molecules are shed cell-surface receptors that neutralize the activity of pro-inflammatory cytokines and are largely viewed as a protective mechanism. TNF soluble receptors I and II (sTNFR-I, sTNFR-II) levels are increased in septic patients and are associated with mortality (97, 99, 100). Though there is minimal data linking sTNFR to nosocomial infections in sepsis, they are chronically elevated in the elderly and therefore elevated levels may represent a predisposition to infection (101).

IL-1 receptor antagonist (IL-1ra) is a naturally occurring antagonist to IL-1. IL-1ra levels are significantly elevated in patients with septic shock (97, 102) and are associated with increased mortality (103). Recent retrospective analysis of the IL-1 pathway in a previously completed trial of anti-IL1 therapy in sepsis showed a mortality benefit of anti-IL1 antibody administration in patients with the highest levels of circulating IL-1ra (104). These data suggest that the levels of soluble receptors and receptor antagonists may be markers of mortality by indicating the magnitude of the pro-inflammatory response. In addition, recent data have suggested a link between an initial dysregulated hyperinflammation and subsequent development of nosocomial infection (98). Gene expression analysis of leukocytes from patients developing nosocomial infection has demonstrated overactivation of IL-1 signaling (16) supporting a potential relationship between elevation of IL-1ra and nosocomial infection in sepsis.

Pathogen Recognition Receptor Signaling Inhibitors

The pro-inflammatory host response to microbial mediators occurs through PRRs including the Toll-like receptor (TLR) family. Negative regulators of TLR signaling are induced during sepsis. These regulators selectively inhibit the downstream inflammatory response via interactions with one or multiple TLR pathways. Single immunoglobulin IL-1R-related protein (SIGIRR) interferes with binding of IL-1 and LPS extracellularly and interferes with complexing of IRAK-1 and TRAF-6 intracellularly, resulting in profound effects on NF-κB and MAPK-dependent signaling (105). MyD88 short (MyD88s) splice variant is upregulated in response to LPS and is defective in its ability to phosphorylate IRAK resulting in reduced NF-κB activation (106). Both SIGIRR and MyD88s expression were found to be elevated in septic monocytes and associated with depressed cytokine responses (107). Interleukin-1 receptor associated kinase-M (IRAK-M, also known as IRAK-3) negatively regulates TLR signaling through inhibiting the dissociation of IRAK-1 from the Toll-IL-1 signaling domain. In experimental sepsis, IRAK-M is upregulated in alveolar macrophages and mediates supressed cytokine responses and impaired clearance of *P. aeruginosa* (108). IRAK-M is also elevated in monocytes from septic patients (109). MicroRNAs (miRNA) are small non-coding RNA and have been found to exert negative regulatory effects on TLR signaling. Multiple miRNAs are dysregulated in sepsis. In particular, elevated circulating levels of miRNA 155 have been associated with poor outcome and expansion of regulatory T cells in patients with sepsis (110) indicating a possible link to sepsis immunosuppression and nosocomial infection.

Pathogen Associated Molecular Patterns

Pathogen associated molecular patterns (PAMPs) are exogenous microbial factors derived from infectious organisms that activate PRRs. In sepsis, PAMPs, such as cell wall and intracellular microbial components, are the primary factors initiating the inflammatory response. PAMPs are therefore critical to the reprogramming of immune cells in sepsis, this reprogramming is likely dependent on the specific antigen (PAMP) and receptor (PRR) combinations that are engaged on a particular cell. For example, *in vitro* stimulation of human monocytes with various PAMPs has demonstrated that the fungal cell wall component β-glucan induces primed (trained) responses through nucleotide-binding oligomerization domain-like receptors (NLRs) (111). In contrast, engagement of PAMP-TLR combinations, such as LPS with its receptor Toll-like receptor 4 (TLR4), induced predominantly tolerant programs with depressed cytokine production. Interestingly, the TLR ligands administered at low dose caused primed responses while inducing tolerance at higher doses, suggesting the presence of an inflammatory rheostat guiding secondary responses. In the context of sepsis, pathogen specific ligands such as LPS (Gram-negative organism) or lipotechoic acid (Gram-positive organism) and endogenous PRR ligands (discussed in the next section) form a complex network of PRR signaling that is likely to contribute to the inflammatory cellular program. In addition to exposure to infectious pathogens, sepsis and critical illness are associated with collapse of the host microbial community, a term known as dysbiosis. This occurs through a combination of ecological factors that are drastically altered in the critically ill (112). While there is no direct evidence linking the dysbiosis that occurs in septic patients to subsequent nosocomial infection, population level studies have demonstrated an increased risk of severe sepsis within 90 days following hospitalizations known to result in dysbiosis (113). A second study demonstrated an increased risk of severe sepsis and septic shock in the 90 days following a hospital-related antibiotic exposure (114). These studies suggest PAMP-PRR interactions via primary infection or continued dysbiosis may promote changes in the immune program that predispose critically ill patients to secondary infection and sepsis, although further investigation is required to establish causal relationships.

Damage-Associated Molecular Patterns

Damage-associated molecular patterns (DAMPs) are endogenous pattern recognition receptor agonists that initiate inflammatory responses but have distinct biological roles in non-inflammatory states. These proteins are released upon host injury, either passively from necrotic cells or actively secreted into the extracellular space (115). DAMPs are released during injured states, including sepsis, trauma, and burns. As such, DAMPs

are appealing candidates as mediators of altered immune programs observed in these patients. Several DAMPs have been shown to correlate with sepsis morbidity and mortality including S100A8/A9, high-mobility group box-1 (HMGB1), mitochondrial DNA, nuclear DNA, histones and heat shock proteins (HSP) (115). HMGB1 and S100A8/A9 are acutely elevated in patients presenting with sepsis and are associated with worse outcome (116, 117). Their functions are pleiotropic, including induction of immunosuppressive MDSC expansion and priming of immune cells in sepsis models (38, 39, 118). HMGB1 also stimulates the expansion of Treg in chronic inflammatory diseases (119). HSPs are intracellular molecular chaperone proteins that can have anti-inflammatory properties. Several HSPs (HSP27, 60, 70, 90) are increased in patients with sepsis and are associated with enhanced neutrophil oxidative activity and reduced apoptosis (120). HSP70 levels increase with the degree of hyperinflammatory response and are associated with increased risk of mortality in patients with sepsis (121, 122). HSP70 also promotes adaptive immune dysfunction through enhanced Treg suppressor activities and secretion of anti-inflammatory cytokines (123). In many of the studies referenced elevated levels of certain DAMPs persist in the circulation of survivors for many days, indicating a potential for ongoing modulation of the immune system after acute sepsis has resolved.

Expansion of Regulatory T Cells

Cellular subsets that have roles in homeostasis are expanded during sepsis and may contribute to nosocomial infection susceptibility. Treg are a T cell population that are able to negatively regulate the adaptive and innate immune response (124). There are several subsets of Treg which can be identified by cell surface markers. The $CD4^+$, $CD25^+$, $CD127^{lo}$ subset is one of several studied in sepsis. These cells are expanded in patients with sepsis (125) and contribute to lymphocyte anergy in septic shock patients (91). Higher Treg numbers are also associated with the development of nosocomial infections in critically ill patients with and without sepsis (77). In sepsis models, Treg expansion results in systemic immunosuppression potentiating tumor growth (126). Data is conflicting though as models of sepsis have demonstrated that antibody mediated depletion of Treg does not improve mortality (127) and adoptive transfer of $CD4^+$ $CD25^+$ Treg early in the course of sepsis actually improves bacterial clearance and mortality (128). Similarly, in one human study, increased Treg in patients with severe sepsis was associated with improved survival, though this finding may have been driven by higher total T cell counts in these patients (129). Further investigation such as depletion or inhibiting expansion of Treg in humans is required to establish a detrimental role for Treg in patients with sepsis.

TABLE 2 | Current clinical evidence for immunostimulation in patients with sepsis.

Therapy	Goal of therapy	Human Evidence	References
G-CSF/ GM-CSF	• Accelerate innate immune cell production • Restore mHLA-DR expression and cytokine production	• Enhanced resolution of infection[1] • Decreased length of ICU stay[1] • Minimal adverse events[1] • May be delivered directly to lung[2] • Pending results from phase III clinical trial[3]	1. Bo et al. (157) 2. Scott et al. (158) 3. NCT02361528
IFNγ	• Increase phagocytic capacity • Restore mHLA-DR expression and cytokine production	• Enhanced resolution of bacterial and fungal infection (case series)[1,2] • Pending results from phase IIIb trial[3]	1. Nalos et al. (159) 2. Delsing et al. (160) 3. NCT01649921
IL-7	• Accelerate lymphocyte production • Decrease lymphocyte apoptosis	• Well tolerated in phase IIb trial[1] • Increased $CD4^+$ and $CD8^+$ lymphocytes[1] • Increased T cell activation and trafficking[1]	1. Francois et al. (161)
Anti-PD-1/ PD-L1	• Reverse innate and adaptive immune exhaustion • Restore mHLA-DR expression and cytokine production	• Well tolerated in patients with sepsis and septic shock[1] • Trend toward sustained restoration of mHLA-DR[1] • Pending results from phase Ib trial[2]	1. Hotchkiss et al. (162) 2. NCT02960854
Tα1	• Restore mHLA-DR expression	• No adverse events reported in single RCT[1] • Trend toward improved 28-day mortality[1] • Ongoing phase III clinical trial[2]	1. Wu et al. (163) 2. NCT02883595
MSC	• Reduce inflammatory response • Decrease lymphocyte apoptosis • Increase phagocytic capacity	• No adverse events reported in a phase I clinical trial[1] • Ongoing phase II clinical trial[2]	1. McIntyre et al. (164) 2. NCT02883803

Tα1, Thymosin alpha 1; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage stimulating factor; IFNγ, interferon gamma; MSC, mesynchymal stem cell; NCT, clinicaltrials.gov identifier.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSC) are a heterogenous group of immature innate immune cells that exert predominantly suppressive effects. Their expansion is a component of the emergency myelopoietic response to injury, infection and malignancy (130). This population consists of a mix of immature granulocytes, monocytes, and dendritic cells with the ability to suppress T cell function through enhanced arginase, nitric oxide synthase, and reactive oxygen species activity (131). MDSCs can produce a number of pro- and anti-inflammatory cytokines upon secondary stimulation, including IL-10 (132). As such, they represent important effector cells in sepsis recovery. Their release is driven mainly by STAT-3 signaling through inflammatory mediators including IL-6 and colony stimulating factors. Activated STAT-3 also induces release of S100A8/9 which both prevents maturation of MDSCs and promotes additional expansion in a feed forward loop (118, 133). In mouse models of sepsis, MDSCs exert pleiotropic effects, with protective or detrimental properties depending on phase of sepsis in which they are examined (134, 135). MDSCs are expanded acutely in patients with sepsis (136, 137) and are associated with the development of nosocomial infection (138). Their persistence in severe sepsis and septic shock is also associated with increased risk for persistent critical illness and mortality (139).

Co-inhibitory Molecules

Co-inhibitory molecules of the B7-CD28 family function to maintain homeostasis in the host by negatively regulating the immune response (140). In sepsis, these molecules have been postulated to be responsible for immune exhaustion. Post-mortem studies of patients dying of sepsis has demonstrated elevations in co-inhibitory receptors PD-1 and CTLA-4 in splenic T cells, while the ligand PD-L1 was elevated in antigen presenting cells and tissue macrophages (47). T cells and dendritic cells isolated from the lung also expressed increased PD-1 and PD-L1, respectively. Circulating T cells in patients with severe sepsis demonstrated non-statistically significant elevations in PD-1, with a decrease in CTLA-4 (61). Others demonstrated marked elevation in T cell PD-1 and monocyte PD-L1 in septic shock. *In vitro* treatment with anti-PD1 antibody resulted in restoration of monocyte proinflammatory responses and decreased apoptosis of T cells (141). Furthermore, higher monocyte PD-L1 has been associated with mortality, while higher PD-1 and PD-L2 are associated with increased risk of nosocomial infection (142). Others have shown that monocyte PD-L1, and not T cell PD1, expression on day 3–4 of septic shock is an independent predictor of death (143). In mice, neutralization of PD-1 or PD-L1 24 h after sepsis reduced apoptosis of lymphocytes and improved survival (144, 145). In leukocytes isolated from septic patients, *in vitro* blockade of PD1 and PDL1 reverses T cell exhaustion and restores neutrophil and monocyte phagocytic function (146, 147).

Epigenetic Reprogramming

Epigenetic mechanisms of immune dysfunction have also been proposed. Histone modification at inflammatory loci alters the accessibility of DNA to transcription factors and therefore gene transcription. Alterations in chromatin structure are determined by gene activating and repressing histone marks, which are in turn regulated by chromatin modifying enzymes (CME) (148). Specific changes in chromatin are associated with exposure to inflammatory stimuli (149). Not surprisingly, chromatin remodeling occurs in the monocytes of septic patients (150). In models of sepsis survival, dendritic cell cytokine responses were suppressed for up to 6 weeks and were associated with alterations in histone H3 lysine-4 (H3K4) and histone H3 lysine-27 (H3K27) methylation at IL-12 promoter regions. These changes were also mediated by interactions with histone methyltransferases (151). We have demonstrated an IRAK-M mediated reduction in histone H4 acetylation and H3K4 methylation (H3K4me) in immune tolerant AM 24 h after induction of sepsis (152). These studies provide compelling evidence for epigenetic changes in sepsis leading to immunosuppressive phenotypes in both the acute and recovery phases of illness. As such dynamic changes in histones and their CMEs have high potential for use as prognostic markers and therapeutic targets. Epigenetic reprogramming of inflammatory cells may also result in primed phenotypes. This concept is also known as "trained immunity" whereby exposure to specific PAMPs (β-glucan, BCG) leads to specific modifications in chromatin structure and enhanced inflammatory responses to secondary stimulation (153, 154). These modifications include genome wide changes in H3K4me3, H3K4me1, and H3K27 acetylation. Our understanding of trained immunity and its potential implications in the pathophysiology of sepsis-related immune priming remains limited.

IMMUNOSTIMULATION

Though the immune response to sepsis is dynamic and contextual, there is a large body of evidence supporting an association between immunosuppressive cellular programs and poor outcomes in patients with sepsis. We know that supportive care is inadequate in addressing the complex immunology of sepsis. As such, immunostimulatory therapies have been evaluated to improve sepsis outcomes. Precision approaches in sepsis through immunomodulation require the development of immune monitoring strategies and suitable immunomodulating agents that can be deployed quickly and dynamically at the bedside. The first step is accurately predicting which patients are at risk for secondary infection and mortality related to immune exhaustion. Large observational studies aiming to predict poor outcomes through cellular phenotyping and cell surface marker expression are already underway (74, 75). Given the heterogenous and rapidly evolving immune programs occurring during sepsis, safety and tolerability of immune therapies with a focus on monitoring for hyperinflammatory consequences is essential. Moreover, assessment of long-term outcomes will be important. In addition, recent *in vitro* studies of PD-1/PD-L1 blockade in mononuclear cells from septic patients has demonstrated significant variability in response to immunostimulation (146, 147). Non-response of mononuclear cells to *in vitro* immunostimulation was associated with increased mortality (155). As such, response rates and mechanisms of response will also need to be examined in further detail, similar to the current practices of immune modulation in oncology (156).

There are a number of ongoing trials of immunostimulation in sepsis (**Table 2**).

Immunostimulatory Cytokines

Early clinical trials attempting to simulation the immune system used granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) to enhance phagocyte production, function and improve bacterial clearance. A meta-analysis of trials performed between 1998 and 2011 failed to find a mortality benefit of these therapies, though there was some improvement in clinical endpoints such as ICU LOS (157). IFNγ has similarly been used to stimulate the innate immune response in patients with bacteremia and chronic fungal infections (159, 160). Both are being used in patients with sepsis as part of ongoing phase III clinical trials.

IL-7 is potent inducer of the antiapoptotic protein Bcl-2 that has the ability to both stimulate lymphocyte production and reduce apoptosis. A recent multicenter, randomized and controlled phase IIb trial examined IL-7 administration in patients with septic shock and lymphopenia (161). Patients received low or high dosing regimens of IL-7 for a total of 4 weeks or until discharge. These investigators found an increase in $CD4^+$ and $CD8^+$ T cells, enhanced T cell activation and potentially T cell trafficking as compared to placebo. In this small trial, there was no difference in mortality or rate of nosocomial infection. The drug was well tolerated with minimal adverse events. Cytokine profiles were measured serially over the course of IL-7 administration and there were no signs of patients developing cytokine storm. Also, the effect of enhanced lymphocyte production persisted for weeks following treatment. These promising results will require confirmation in larger studies to determine if therapy is efficacious and without long-term consequences.

Checkpoint Inhibition

Checkpoint inhibitors such as anti-PD1 and anti-PD-L1, improve monocyte and lymphocyte function and reduce apoptosis through disruption of negative cell-cell interactions. These inhibitors have revolutionized management of malignancy and are now first line therapy for many types of cancer (165). However, immune related adverse events (iRAE) with checkpoint inhibition are not infrequent, with rates of grade $\geq$3 toxicity approaching 7% (166). Early human studies have demonstrated feasibility and reversal of sepsis induced immunosuppression with *in vitro* blockade (146, 147). A phase I clinical trial of the novel PD-L1 inhibitor, BMS-936559, has been completed (162). A phase I clinical trial of PD-1 inhibitor, Nivolumab, is currently ongoing (NCT02960854).

Endogenous Immunostimulatory Proteins

Thymosin alpha 1 (Tα1) is an endogenous thymic peptide that regulates the innate and adaptive immune system. Initial studies in sepsis have shown improvement in mHLA-DR (163). Continued study of this agent is ongoing in a phase III clinical trial (NCT02883595).

Cellular Therapies

Mesenchymal stem cells (MSCs) reduce mortality and organ dysfunction in models of sepsis through modulation of the inflammatory cascade, pathogen clearance and promotion of tissue repair (167). Following completion of a phase I clinical trial in septic shock, administration of MSCs appears to be safe (164). Further research is ongoing in a phase II clinical trial (NCT02883803).

CONCLUSIONS

The clinical phases of sepsis are associated with specific and dynamic changes in the immune programming of multiple cell types. Suppressed inflammatory responses to stimulation have been demonstrated extensively in patients with sepsis, however the dynamics, pathogen, and compartmental specificity of these findings requires additional investigation. Primed immune responses have also been demonstrated in animal models of sepsis survival. This cellular program has not been examined extensively in humans and its contributions to sepsis related morbidity and mortality remains unknown. Molecular mechanisms of immune reprogramming in sepsis still require further investigation. In particular the complexities of PAMP/DAMP-PRR interactions, the role of MDSC and T-reg, and alterations in the epigenome are prime targets for evaluation.

Patients at higher risk for nosocomial infection and mortality frequently experience an immunosuppressed state characterized by defects in immune tolerance, exhaustion, and apoptosis. While reversal of these immunosuppressive phenotypes has improved outcomes in animal models, a direct causal relationship between sepsis-related immunosuppression and nosocomial infection/death has not yet been established in humans. Furthermore, rates of nosocomial infection and its attributable mortality in sepsis may not be as high as previously estimated, suggesting that reasons why septic patients die despite best supportive care still need to be explored. Regardless, nosocomial infections are common and carry such significant morbidity that sepsis patients may benefit from immunostimulatory therapies. Early stage trials of immune therapy have shown reversal of leukocyte dysfunction and good safety profiles, both promising for the potential future of these therapies. However, as sepsis has long-term consequences that are still not well understood and immune therapies may have lasting effects, long-term outcomes of patients receiving immunostimulatory therapy require attention.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Coinfection With Influenza A Virus and *Klebsiella oxytoca*: An Underrecognized Impact on Host Resistance and Tolerance to Pulmonary Infections

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Pneumonia is a world health problem and a leading cause of death, particularly affecting children and the elderly (1, 2). Bacterial pneumonia following infection with influenza A virus (IAV) is associated with increased morbidity and mortality but the mechanisms behind this phenomenon are not yet well-defined (3). Host resistance and tolerance are two processes essential for host survival during infection. Resistance is the host's ability to clear a pathogen while tolerance is the host's ability to overcome the impact of the pathogen as well as the host response to infection (4–8). Some studies have shown that IAV infection suppresses the immune response, leading to overwhelming bacterial loads (9–13). Other studies have shown that some IAV/bacterial coinfections cause alterations in tolerance mechanisms such as tissue resilience (14–16). In a recent analysis of nasopharyngeal swabs from patients hospitalized during the 2013–2014 influenza season, we have found that a significant proportion of IAV-infected patients were also colonized with *Klebsiella oxytoca*, a gram-negative bacteria known to be an opportunistic pathogen in a variety of diseases (17). Mice that were infected with *K. oxytoca* following IAV infection demonstrated decreased survival and significant weight loss when compared to mice infected with either single pathogen. Using this model, we found that IAV/*K. oxytoca* coinfection of the lung is characterized by an exaggerated inflammatory immune response. We observed early inflammatory cytokine and chemokine production, which in turn resulted in massive infiltration of neutrophils and inflammatory monocytes. Despite this swift response, the pulmonary pathogen burden in coinfected mice was similar to singly-infected animals, albeit with a slight delay in bacterial clearance. In addition, during coinfection we observed a shift in pulmonary macrophages toward an inflammatory and away from a tissue reparative phenotype. Interestingly, there was only a small increase in tissue damage in coinfected lungs as compared to either single infection. Our results indicate that during pulmonary coinfection a combination of

seemingly modest defects in both host resistance and tolerance may act synergistically to cause worsened outcomes for the host. Given the prevalence of *K. oxytoca* detected in human IAV patients, these dysfunctional tolerance and resistance mechanisms may play an important role in the response of patients to IAV.

Keywords: coinfection, influenza A virus, *Klebsiella oxytoca*, disease tolerance, pulmonary infection

INTRODUCTION

During the influenza season an average of 20% of the human population is infected, with this percentage varying from year to year depending on the virulence of the strains circulating that season (18). Secondary bacterial pneumonia following influenza A virus (IAV) infection is a serious complication whose prevalence and severity correlates with the virulence of the influenza strain (3, 19). On average, 0.5% of previously healthy, young individuals and 2.5% of elderly or immunocompromised patients that contract IAV have bacterial coinfections; however, during times of influenza pandemic these numbers climb even higher and in the 1918 influenza virus pandemic up to 6.1% of all patients with IAV were thought to have secondary bacterial infections (20). In 1918, prior to the use of antibiotics, autopsies confirmed the presence of bacteria in up to 95% of fatalities (3, 21). In the 2009 pandemic between 18 and 34% of IAV patients in the ICU had a bacterial coinfection and up to 55% of fatalities were associated with bacterial coinfection (21, 22).

The bacteria that are most commonly implicated in coinfection with IAV are *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Legionella pneumophila*, *Pseudomonas* species, and *Klebsiella* species (18). The development and use of antibiotic treatment has increased the prevalence of antibiotic-resistant bacterial strains, such as methicillin-resistant *S. aureus* (MRSA), implicated in coinfection as well (18). However, due to the significant overlap in symptoms of pneumonia caused by influenza virus infection alone vs. coinfection, diagnoses of coinfection are difficult to make and often antibiotics are inappropriately administered (18). With the growing concern about antibiotic- and antiviral-resistant pathogens, it is clear that more emphasis needs to be placed on finding alternative therapies to treat coinfection. Currently the IAV vaccine, while it does impart some protection and can decrease the severity of symptoms, has variable effectiveness due to the antigen drift that occurs each season (3). Even with advances in treatments against pathogens such as vaccines, antivirals, and antibiotics, bacterial coinfection still represents a major threat to human health (23, 24).

Host resistance and host tolerance are two important factors that can determine the outcome of a patient following infection (4–8). The ability to successfully detect and eliminate pathogens is called host resistance while the ability to overcome the damaging effects caused by the pathogen and the immune response to that pathogen is known as host disease tolerance or resilience. If the host lacks either one of these properties, it becomes susceptible to infection (4–8). Bacterial coinfections can cause increased mortality due to alterations in either resistance or tolerance; for example, *S. pneumoniae* coinfections are characterized by an increased bacterial burden which overwhelms the host, whereas *L. pneumophila* coinfections cause mortality through a significant amount of tissue damage without an increase in pathogen burden (14, 25). IAV/*S. pneumoniae* coinfection may be an example of decreased resistance leading to alterations in tolerance as there is also increased tissue damage, but given the overwhelming bacterial burden it is challenging to separate out these two mechanisms (26). Because each type of IAV/bacterial coinfection can cause mortality through different mechanisms, it is important to study them individually to uncover the best way to treat them.

Up until now, the majority of studies on IAV/bacterial coinfection have focused on *S. pneumoniae* and *S. aureus*. While these are two of the most prevalent bacteria in coinfections with IAV, there are many other bacteria that have been vastly understudied (18). This includes *Klebsiella spp* which are gram-negative, opportunistic pathogens responsible for between 3 and 7% of all nosocomial infections including UTIs, septicemia, and pneumonia (17). Pneumonia caused by *Klebsiella spp* has up to a 50% fatality rate and the emergence of multi-drug-resistant strains has made it increasingly difficult to treat (17, 27). Among this genus is *Klebsiella oxytoca* which is a pathobiont in the human microbiome and an underrecognized contributor to hospital-acquired pneumonia in immunocompromised patients (28). The involvement of *K. oxytoca* in bacterial coinfections with IAV has of yet been unclear. A recent study from Gao et al. identified the presence of *K. oxytoca* in one H7N9 patient from a cohort in China in 2013 (29). Data presented here indicates that its prevalence is potentially underestimated and therefore should be a target for further study. Our lab has detected an increased presence of *K. oxytoca* in nasopharyngeal swabs from patients who tested positive for IAV in Rhode Island during the 2013–2014 influenza season and this finding prompted us to investigate the immunological responses that occur during coinfection with IAV and *K. oxytoca*.

To study the pathogenesis of IAV/*K. oxytoca* coinfection we developed a mouse model in which we observed increased mortality in coinfected animals compared to singly-infected controls. Within our model system, we saw a heightened inflammatory response following coinfection but despite an increase in immune cell infiltrate, there was a delay in bacterial clearance. In addition, we observed an increase in tissue damage as a result of coinfection, perhaps caused by a shift in macrophage polarization away from a tissue reparative phenotype. As such, this model is an excellent vehicle to study host resistance and tolerance since both are impacted as a result of coinfection. Our work studying coinfections with IAV and the previously

underrecognized *K. oxytoca* highlight the complex relationship between host resistance and tolerance and suggest the need for further study of these systems.

RESULTS

Detection of *Klebsiella oxytoca* in Nasopharyngeal Swabs From IAV Patients

While pneumonia caused by *K. oxytoca* has recently been reported in one IAV-infected patient (29), the overall prevalence of *K. oxytoca* among IAV patients is as yet unknown. In order to investigate this, we looked for the presence of *K. oxytoca* in nasopharyngeal swabs from a cohort of patients admitted to the Memorial Hospital in Rhode Island during the influenza season of 2013–2014. Our findings show that among patients that tested positive for IAV there was a significantly higher proportion that also tested positive for *K. oxytoca* (14.00%) compared to those patients that tested negative for IAV (3.88%), implying that infection with IAV increases susceptibility to *K. oxytoca* colonization (**Table 1**). While these data show a clear association of IAV patients with *K. oxytoca*, it is unknown whether these patients had an active secondary infection with *K. oxytoca* or whether IAV infection enhances susceptibility to *K. oxytoca* colonization without causing infection. We also looked for the presence of *S. pneumoniae* in this cohort and found a similar trend to *K. oxytoca* in which a higher percentage of IAV-positive patients also tested positive for *S. pneumoniae* (20.00%) compared to IAV-negative patients (8.53%) (**Table 1**). *S. pneumoniae* is commonly implicated in secondary bacterial infections with IAV and is known to cause increased morbidity and mortality in these cases. Our findings showed similar patterns in the association of IAV patients with *K. oxytoca* as *S. pneumoniae,* which led us to question whether *K. oxytoca* is likewise able to alter host responses during coinfection with IAV to cause worsened outcomes.

TABLE 1 | Influenza patients are more susceptible to bacterial colonization by *Streptococcus pneumoniae* and *Klebsiella oxytoca*.

	Influenza–	Influenza+
S. pneumoniae –	91.47%	80.00%
S. pneumoniae +	8.53%	20.00%
Total number of patients	129	50
K. oxytoca –	96.12%	86.00%
K. oxytoca +	3.88%	14.00%
Total number of patients	129	50

Nasopharyngeal swabs from a total of 179 patients were tested for the presence of influenza as well as S. pneumoniae and K. oxytoca. Of the 50 patients that tested positive for influenza, 10 (20.00%) also tested positive for S. pneumoniae and 7 (14.00%) tested positive for K. oxytoca. In contrast, of the 129 patients that tested negative for influenza, only 11 (8.53%) also tested positive for S. pneumoniae and 5 (3.88%) tested positive for K. oxytoca. These results indicate that infection with influenza leads to an increased association with several bacterial species. Statistics were calculated by Fisher's exact test with P = 0.0403 for S. pneumoniae and P = 0.0391 for K. oxytoca.

Coinfected Mice Exhibit Increased Inflammation and Cellular Infiltrate Early After Bacterial Infection

In order to investigate the host response to infection during IAV and *K. oxytoca* coinfection, we developed a mouse model in which a sublethal dose of IAV was administered followed by a sublethal dose of *K. oxytoca* 5 days after IAV. First, we assessed whether coinfection induced changes to the inflammatory response early following bacterial infection as has been observed in other coinfection models (9, 25, 30–32). We measured the concentrations of a panel of inflammatory cytokines and chemokines in the bronchoalveolar lavage fluid (BALF) including IL-6, TNFα, IFNγ, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL5, and CXCL10 (**Figure 1**). These cytokines and chemokines are essential in the innate immune response to both bacteria and viruses. On day 1 post-coinfection, there was an early increase in the production of TNFα during coinfection that was not observed in either singly-infected group whereas levels of IL-6 were equal between IAV-infected and coinfected groups (**Figure 1A**). We also saw a significant amplification in the production of all chemokines measured during coinfection compared to any other group (**Figure 1B**). The only reduction in cytokine levels that we observed in the coinfection was in IFNγ; however, IFNγ during coinfection was still significantly increased over the group infected with *K. oxytoca* alone (**Figure 1A**). By day 3, IFNγ levels in the coinfected lungs overtook those seen in the singly-infected groups and reached the level seen in IAV-infected lungs on day 1, indicating a delay in the kinetics of IFNγ during coinfection (**Figure 1C**). At day 3 post-coinfection, most chemokines remained elevated in the coinfected lungs, although these levels were decreased overall from day 1 with the exception of CCL2 (**Figure 1D**). Only CCL5 and CXCL5 concentrations were higher in the group with *K. oxytoca* alone than coinfection (**Figure 1D**). These results indicate that the coinfected lung was able to sense the presence of both pathogens and increase production of multiple inflammatory signals in response. This also shows that there is an early, robust response on day 1 that tapers but remains elevated by day 3 post-coinfection.

After observing that coinfection with IAV/*K. oxytoca* was characterized by a significant amplification of inflammatory cytokines and chemokines, we next investigated how this inflammatory milieu might affect which innate immune cells traffic to the lung and the magnitude of their recruitment as compared to either single viral or bacterial infections. Using a flow cytometric panel of markers to identify different innate immune cell subsets, we identified $Ly6G^{-}F480^{+}CD11c^{+}$ macrophages which include both alveolar macrophages and macrophages that upregulate CD11c as they infiltrate the lungs, $Ly6G^{-}F480^{+}CD11c^{-}Ly6C^{+}$MHC II^{-} inflammatory monocytes, $Ly6G^{-}F480^{+}CD11c^{-}Ly6C^{+}$MHC II^{+} infiltrating inflammatory macrophages, and $Ly6G^{+}F480^{-}$ neutrophils (**Figures 2A–C**). For identification of lung macrophages, we compared expression of CD11c and Siglec-F (known alveolar macrophage markers) and found that on day 1 post-coinfection, all macrophages that expressed CD11c also expressed Siglec-F and were therefore all alveolar macrophages, but on day 3

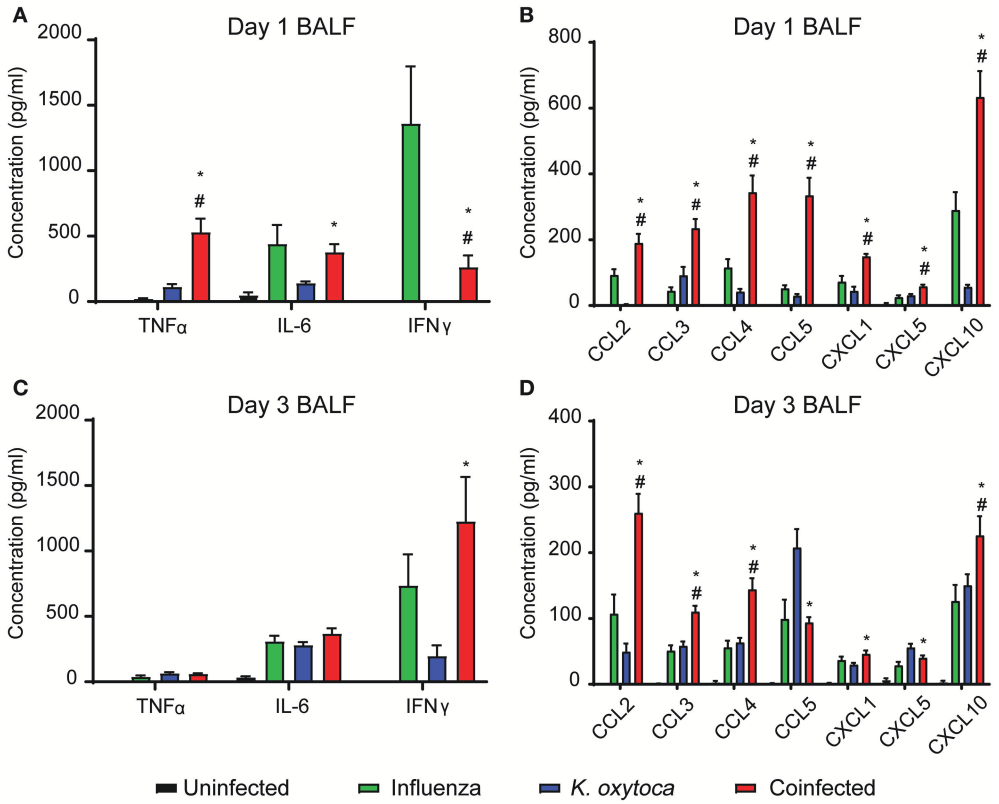

FIGURE 1 | Coinfected mice exhibit increased inflammation early after bacterial infection. Protein concentrations of a panel of cytokines and chemokines were measured in the bronchoalveolar lavage fluid (BALF) of mice on days 1 and 3 following *K. oxytoca* infection (days 8 and 10 following IAV). Day 1 cytokine levels **(A)** and chemokine levels **(B)** are depicted for all 4 groups. Day 3 cytokine **(C)** and chemokine **(D)** levels are depicted for all 4 groups. # denotes $P \leq 0.05$ between coinfected and IAV groups. * denotes $P \leq 0.05$ between coinfected and *K. oxytoca* groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests. Error bars represent SEM. Data are combined from at least four independent experiments with at least four mice per group.

there was a percentage of $CD11c^+$ cells in the coinfected group that did not express Siglec-F, potentially representing a population of infiltrating macrophages that upregulate CD11c as they repopulate the lungs following infection (**Supplemental Figure 2**) (33). For complete description of gating strategies see **Supplemental Figures 1**, **2**.

We first examined cell subsets in the BALF (**Figure 2**). The $Ly6G^-F480^+CD11c^+$ macrophage population did not show any notable changes in number throughout the course of any single or dual infection. However, neutrophil numbers increased dramatically during coinfection. One day post-coinfection, coinfected lungs showed similar neutrophil numbers to *K. oxytoca*-infected lungs; however, by 3 days post-coinfection, neutrophil numbers from coinfected lungs were increased greater than three-fold over *K. oxytoca*-infected animals and greater than seven-fold over IAV-infected animals (**Figures 2A,D**). This delayed but significant increase in the recruitment of neutrophils was likely in part caused by the early induction of many chemokines that recruit neutrophils, such as CXCL1 and CXCL5, at day 1 post-coinfection (**Figure 1B**). In addition, both the inflammatory monocyte and infiltrating macrophage populations expanded significantly during coinfection at both days 1 and 3 post-coinfection in the BALF when compared to other groups (**Figures 2B,C,E,F**).

We next determined changes in innate immune cells that infiltrated into the lung parenchyma during infection (**Figure 3**). In the lung tissue on day 1 post-coinfection, we observed a significantly greater number of neutrophils (**Figures 3A,D**), inflammatory monocytes (**Figures 3B,E**), and infiltrating macrophages (**Figures 3C,F**); however, by day 3 there were no significant differences in these populations between the infected groups which may be indicative of these cells trafficking through the lungs on day 1 to reach the alveolar space by day 3 (**Figures 3C,D–F**).

Amplified Innate Immune Responses Do Not Enhance Resistance in Coinfected Mice

Following our observations that coinfected lungs had a significantly heightened initial immune response compared to singly-infected lungs, we reasoned that this response was an attempt by the host to eliminate the dual pathogen burden. Therefore, we measured viral and bacterial loads in the lung

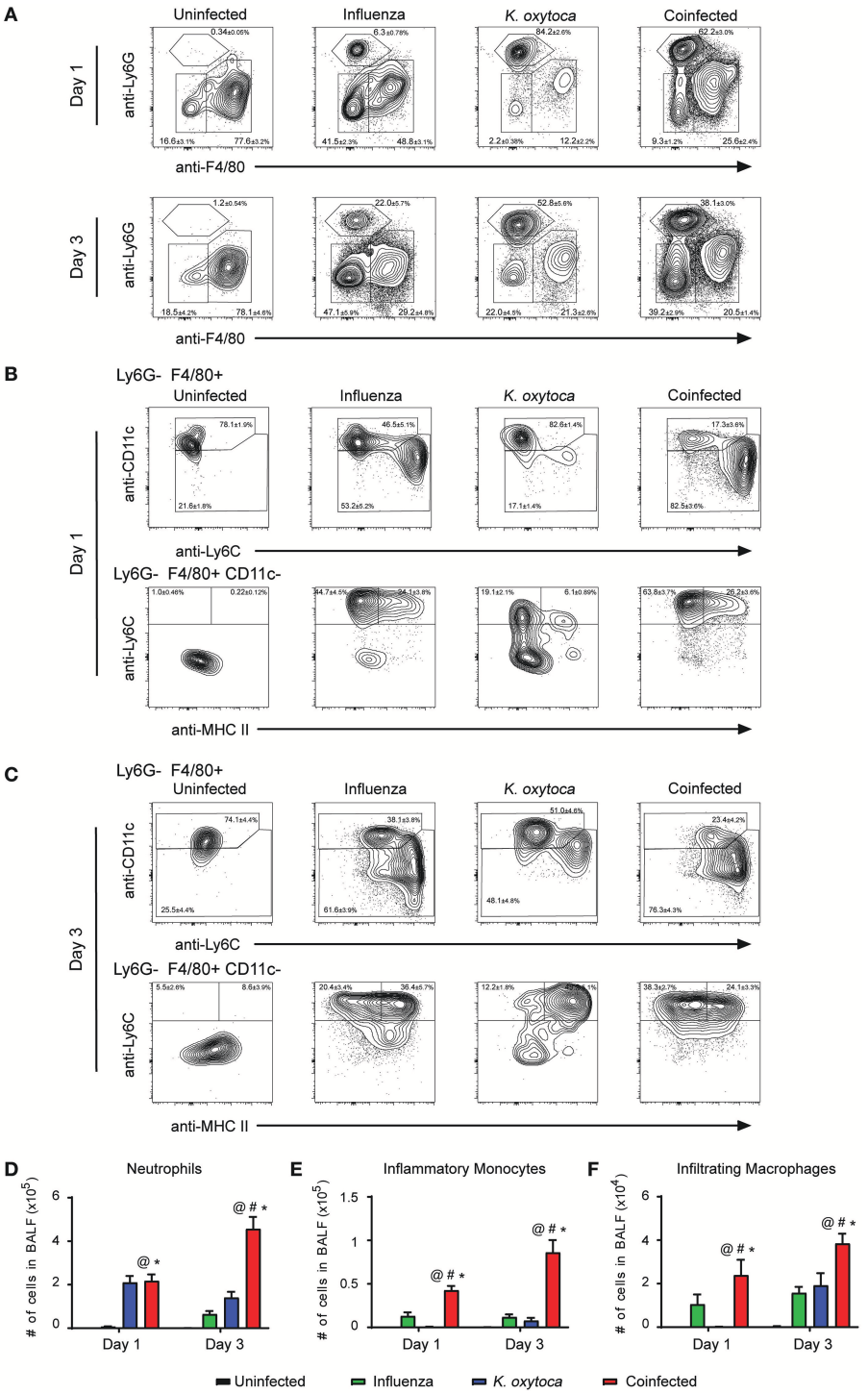

FIGURE 2 | Immune cell infiltrate is increased in the BALF of coinfected mice. Innate immune cell populations were quantified in the BALF of mice on days 1 and 3 post-*K. oxytoca* infection. We identified neutrophils as Ly6G$^+$F480$^-$ cells and separated the F480$^+$ population according to expression of CD11c with alveolar and repopulating macrophages identified as Ly6G$^-$F480$^+$CD11c$^+$ cells. The CD11c$^-$ population was further separated by expression of Ly6C and MHC II with inflammatory monocytes identified as Ly6G$^-$F480$^+$CD11c$^-$Ly6C$^+$MHC II$^-$ cells and infiltrating macrophages identified as Ly6G$^-$F480$^+$CD11c$^-$Ly6C$^+$MHC II$^+$ cells **(A–C)**. The total number of neutrophils **(D)**, inflammatory monocytes **(E)**, and infiltrating macrophages **(F)** from days 1 and 3 were calculated according to their percentages of the total population and the total number of cells collected in each BALF. @ denotes $P \leq 0.05$ between coinfected and uninfected groups. # denotes $P \leq 0.05$ between coinfected and influenza groups. * denotes $P \leq 0.05$ between coinfected and *K. oxytoca* groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests. Error bars represent SEM. Data are combined from at least four independent experiments with at least four mice per group.

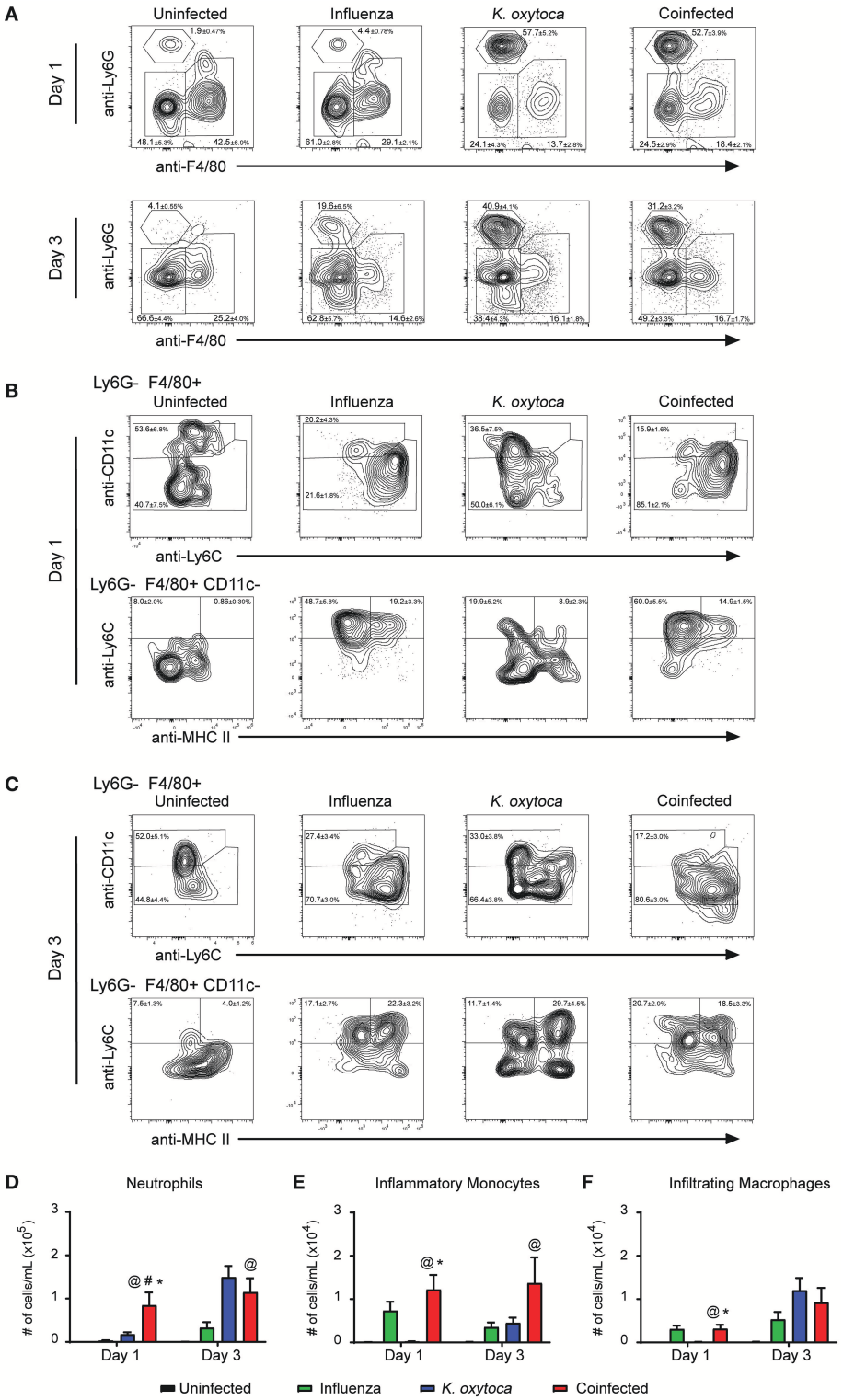

FIGURE 3 | Immune cell infiltrate is increased in the lungs of coinfected mice. Innate immune cell populations were quantified in the lungs of mice on days 1 and 3 post-*K. oxytoca* infection. We identified neutrophils as Ly6G$^+$F480$^-$ cells and separated the F480$^+$ population according to expression of CD11c with alveolar macrophages and repopulating macrophages identified as Ly6G$^-$F480$^+$CD11c$^+$ cells. The CD11c$^-$ population was further separated by expression of Ly6C and MHC II with inflammatory monocytes identified as Ly6G$^-$F480$^+$CD11c$^-$Ly6C$^+$MHC II$^-$ cells and infiltrating macrophages identified as

(Continued)

FIGURE 3 | Ly6G$^-$F480$^+$CD11c$^-$Ly6C$^+$MHC II$^+$ cells **(A–C)**. The total number of neutrophils **(D)**, inflammatory monocytes **(E)**, and infiltrating macrophages **(F)** from days 1 and 3 were calculated according to their percentages of the total population and the total number of cells collected in each lung. @ denotes $P \leq 0.05$ between coinfected and uninfected groups. # denotes $P \leq 0.05$ between coinfected and influenza groups. * denotes $P \leq 0.05$ between coinfected and *K. oxytoca* groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests. Error bars represent SEM. Data are combined from at least four independent experiments with at least four mice per group.

throughout coinfection to determine if the immune response was able to effectively clear or control the pathogens. On day 1 post-coinfection, both viral and bacterial burdens were comparable between the coinfected and respective singly-infected groups (**Figures 4A,B**). However, by day 3 post-coinfection, although viral load remained equal between the coinfected and virally-infected groups, the coinfected mice displayed delayed bacterial clearance compared to the *K. oxytoca*-infected animals (**Figures 4A–C**). While most of the *K. oxytoca*-infected mice had cleared the bacteria by day 3, only 30% of coinfected mice had no detectable bacteria in their lungs (**Figures 4B,C**). Interestingly, despite delayed clearance during coinfection, all of the mice with detectable bacteria had similar bacterial burdens, regardless of whether or not they had a prior IAV infection. Since neither viral load nor bacterial colonies increased between days 1 and 3 in the coinfected lungs (**Figures 4A,B**), it appears that the immune response mounted was able to prevent both pathogens from overwhelming the host but had a defect in the early clearance of bacteria.

Increased Innate Immune Responses Impact Host Tolerance During Coinfection

To determine the effects of delayed clearance of *K. oxytoca* on the overall health of coinfected animals, we monitored the survival of coinfected mice as compared to singly-infected mice and observed that while all singly-infected groups were able to overcome infection, there was a significant decrease in survival of the coinfected animals starting just 3 days post-coinfection (**Figure 4D**). We also measured weights of these animals throughout an extended period of time following coinfection and found that all singly-infected animals lost weight but were able to recover back to their starting weights within 15 days of infection (**Figure 4E**). Coinfected mice exhibited a decrease in body weight comparable to the IAV-infected group but at an accelerated rate starting 1 day post-coinfection until several of the mice succumbed to disease (**Figure 4E**). With only a mild defect in host resistance as seen through delayed clearance of *K. oxytoca* in coinfected lungs, we questioned whether this phenomenon was responsible for the increased morbidity and mortality of these mice. Another possibility was that the worsened outcomes observed during coinfection were not as a result of uncontrolled pathogen replication but rather an inability of the host to tolerate the damage done by the massive immune response to the pathogens. To test this hypothesis, we measured the concentration of albumin in the BALF of infected mice as an indicator of vasculature leakage and therefore tissue damage in the lung (34). We found that as early as day 1 post-coinfection there was a significantly greater concentration of albumin in the lungs of coinfected mice as compared to singly-infected animals, and this observation was even more profound at day 3 post-coinfection (**Figure 4F**). Additionally, we looked at lactate dehydrogenase (LDH) release into the BALF as a measure of cell death and found that there was also increased LDH in coinfected BALF although this trend was not statistically significant (**Supplemental Figure 2**).

Macrophage Populations in Coinfected Mice Are More Pro-Inflammatory Compared to Singly-Infected Mice

After observing mild defects in both host resistance and tolerance during coinfection, we aimed to determine how the innate immune cells might be contributing to this progression of disease. Alveolar macrophages are the prominent cell type patrolling the lungs and are therefore often the first cells to encounter an invading pathogen (35). Alveolar macrophages will recognize and phagocytose pathogens, which triggers the release of a plethora of inflammatory cytokines and chemokines to attract other immune cells to the lung to help fight the infection (36). When they are not responding to pathogens, alveolar macrophages play an important role in maintaining homeostasis at steady state as well as mediating the return to homeostasis at the resolution of infection (35, 37, 38). They do this through the release of anti-inflammatory agents and factors that promote tissue repair, as well as by aiding in the catabolism of surfactant (35, 37–39). We hypothesized that during coinfection alveolar macrophages and macrophages that repopulate the lung following infection might play a role in shifting the balance toward pro-inflammatory and away from tissue repair processes. To test this, we explored changes in MHC II and CD206 expression on Ly6G$^-$F480$^+$CD11c$^+$ macrophages as these are two markers of antigen presenting and tissue reparative phenotypes, respectively (35). We found that in a naïve lung, Ly6G$^-$F480$^+$CD11c$^+$ macrophages were almost exclusively CD206$^+$MHC II$^-$ and at 1 day post-coinfection, Ly6G$^-$F480$^+$CD11c$^+$ macrophages from the BALF and lungs of both singly-infected groups remained predominantly CD206$^+$ with a small percentage expressing MHC II as well (**Figures 5A,C,D**). By day 3 post-coinfection the Ly6G$^-$F480$^+$CD11c$^+$ macrophages from the IAV-infected BALF exhibited higher MHC II and lower CD206 expression (**Figures 5B,E**). Ly6G$^-$F480$^+$CD11c$^+$ macrophages in the *K. oxytoca*-infected BALF at day 3 were still predominantly CD206$^+$ (**Figures 5B,E**). In the lung at day 3, all infected groups showed an expansion in the MHC II$^+$ population (**Figures 5B,F**). In coinfected BALF, there was a significant reduction in the number of CD206$^+$

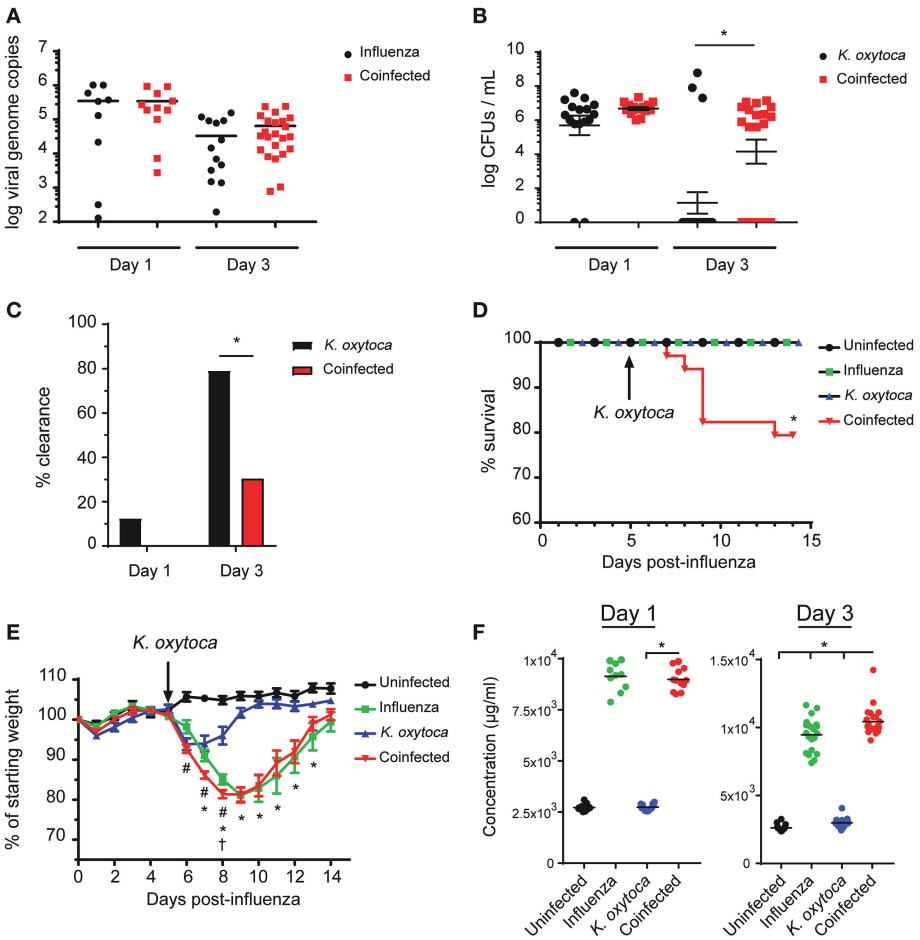

FIGURE 4 | Coinfected mice exhibit increased morbidity and mortality as well as decreased resistance and tolerance**.** Viral burden as measured by viral genome copies in lung tissue on days 1 and 3 **(A)**. Bacterial burden as measured by colony-forming units (CFUs) in 1 mL of lung homogenate on days 1 and 3 **(B)**. Percentage of mice with no detectable bacteria in their lungs at day 1 and 3 **(C)**. Survival of naïve, singly-infected and coinfected mice was measured over the course of 2 weeks **(D)**. Weights were monitored during this time and are expressed as percentages of the starting weight of each mouse prior to infection **(E)**. Concentration of albumin in the BALF on days 1 and 3 post-*K. oxytoca* **(F)**. In **(E)** # denotes $P \leq 0.05$ between coinfected and IAV groups while * denotes $P \leq 0.05$ between coinfected and *K. oxytoca* groups. † denotes day at which coinfected mice begin to exhibit decreased survival. In **(C,D,F)** * denotes $P \leq 0.05$ between indicated groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests **(A,E,F)**, Mann-Whitney *U*-test on non-transformed data **(B)**, Fisher's exact test **(C)**, and log rank tests **(D)** where appropriate. Error bars represent SEM. Data are combined from at least three independent experiments with at least four mice per group.

macrophages on day 1, while by day 3 there was also a significantly higher number of MHC II$^+$ macrophages in the BALF (**Figures 5C,E**). Ly6G$^-$F480$^+$CD11c$^+$ macrophages in the lung followed this trend but these results were not statistically significant. Additionally, we explored the capacity of the pulmonary macrophage populations to contribute to the inflammatory environment in the lung, particularly on day 1 post-coinfection (**Figure 1**). To do this, we looked for changes in TNFα production by Ly6G$^-$F480$^+$CD11c$^+$ and Ly6G$^-$F480$^+$CD11c$^-$ Ly6C$^+$MHC II$^+$ macrophages on day 1 post-coinfection (**Figures 6A–D**). On day 1 post-coinfection, the Ly6G$^-$F480$^+$CD11c$^+$ macrophage population is made up entirely of alveolar macrophages, as indicated by their Siglec-F expression (**Supplemental Figure 2**) while the Ly6G$^-$F480$^+$CD11c$^-$ Ly6C$^+$MHC II$^+$ macrophages are those that are infiltrating into the lung. The Ly6G$^-$F480$^+$CD11c$^+$ alveolar macrophages exhibit a significant increase in their production of TNFα as compared to any singly-infected group (**Figures 6A,C**). On day 1, the Ly6G$^-$F480$^+$CD11c$^-$ Ly6C$^+$MHC II$^+$ infiltrating macrophage population is absent in uninfected as well as *K. oxytoca*-infected BALF; however, comparing this population between IAV-infected and coinfected BALF, there is a significant increase in the production of TNFα during coinfection (**Figures 6B,D**). Also, it is notable that the Ly6G$^-$F480$^+$CD11c$^-$Ly6C$^+$MHC II$^+$ infiltrating macrophages never express CD206 and are therefore not likely to be exhibiting a reparative phenotype (**Figures 6B,D**). These data indicate that both the Ly6G$^-$F480$^+$CD11c$^+$ alveolar macrophages and the Ly6G$^-$F480$^+$CD11c$^-$Ly6C$^+$MHC II$^+$ infiltrating macrophages are likely important contributors to

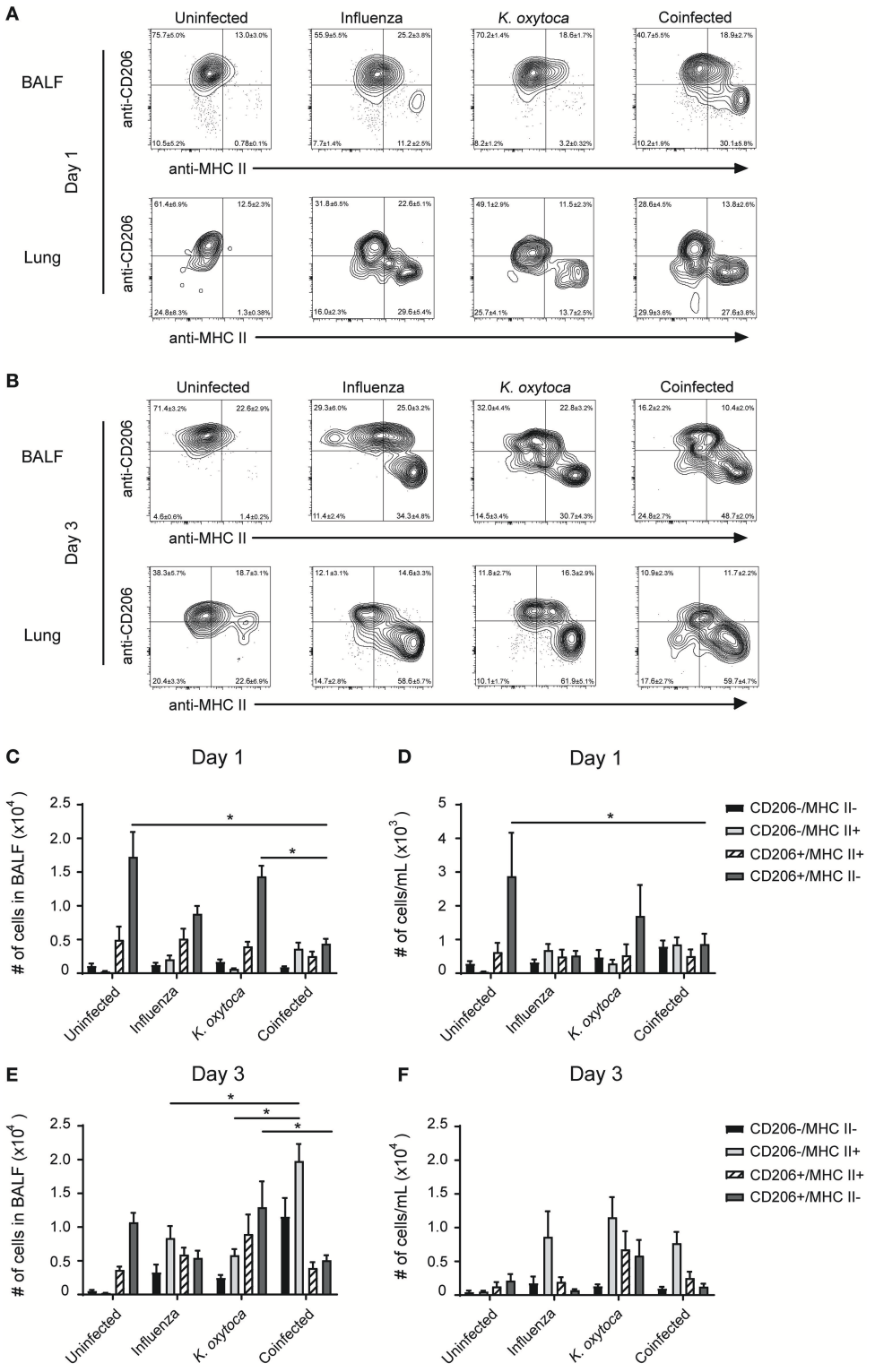

FIGURE 5 | Macrophage populations in coinfected mice have increased MHC II and decreased CD206 compared to singly-infected mice. Ly6G^{-}F480^{+}CD11c^{+} macrophages in the BALF and lung tissue were analyzed for the expression of the MHC II and CD206 on days 1 and 3 post-*K. oxytoca* infection **(A,B)**. Macrophage subsets were classified as CD206^{-}MHC II^{+}, CD206^{+}MHC II^{+}, CD206^{+}MHC II^{-}, and CD206^{-}MHC II^{-} and quantified in the BALF **(C,E)** and lung tissue **(D,F)** on days 1 and 3 post-*K. oxytoca*. * denotes $P \leq 0.05$ between indicated groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests. Error bars represent SEM. Data are combined from at least four independent experiments with at least four mice per group.

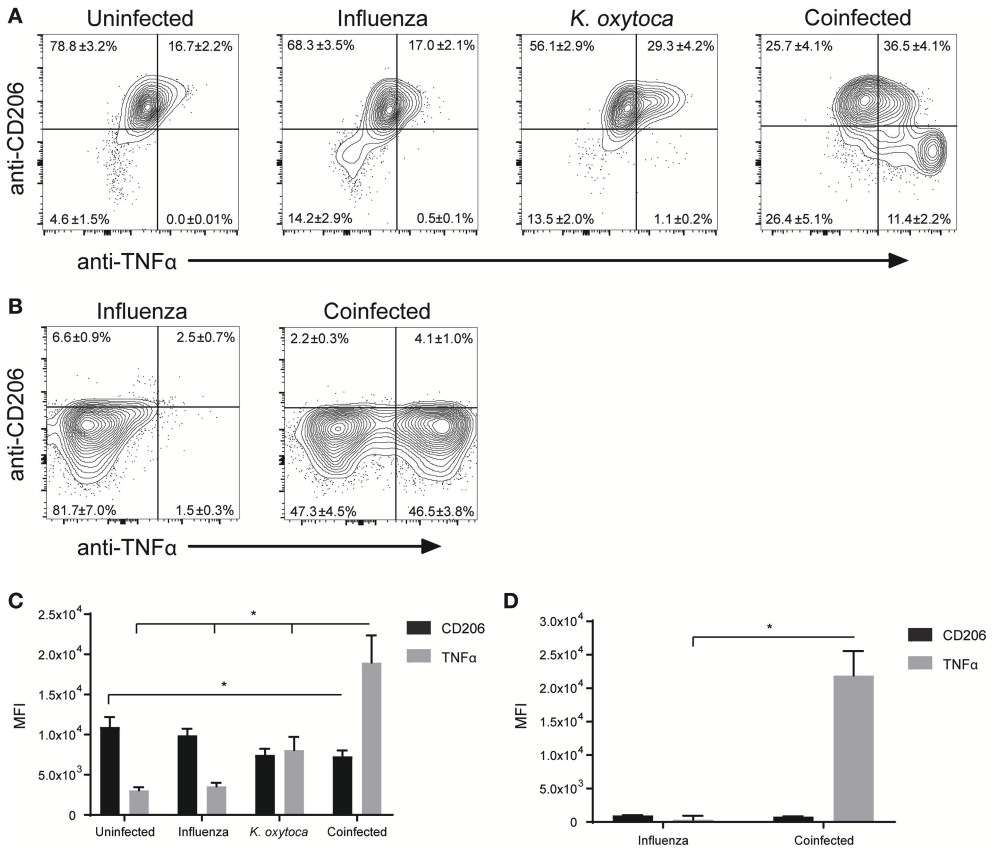

FIGURE 6 | Macrophage populations in coinfected lungs produce more TNFα than during single infections. $Ly6G^{-}F480^{+}CD11c^{+}$ **(A,C)** and $Ly6G^{-}F480^{+}CD11c^{-}Ly6C^{+}MHC\ II^{+}$ **(B,D)** macrophages in the BALF were analyzed on day 1 post-coinfection for expression of CD206 and production of TNFα as measured by mean fluorescent intensity (MFI). *denotes $P \leq 0.05$ between indicated groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests. Error bars represent SEM. Data are combined from at least four independent experiments with at least four mice per group.

the early, heightened inflammatory response during coinfection. Overall, these data showed that pulmonary macrophages from coinfected mice exhibit a more accelerated shift toward a pro-inflammatory phenotype when compared to singly-infected macrophages (**Figures 5**, **6**). These results indicate that pulmonary macrophages play an important role in propagating the prolonged inflammatory response and delaying the reparative processes necessary to return to homeostasis following coinfection.

DISCUSSION

Thus far the vast majority of research on IAV/bacterial coinfections has focused on the bacteria *S. pneumoniae* or *S. aureus*; however, our data demonstrate that patients with IAV infection have a higher risk of association with the previously underrecognized bacteria *K. oxytoca*. Although it is unknown whether this increased association is due to increased susceptibility to infection with *K. oxytoca* or colonization without active infection, we decided to investigate the potential effects of coinfection with *K. oxytoca* since we saw similar trends of association as with IAV and *S. pneumoniae* which is known to result in severe coinfections with IAV. In addition, *K. oxytoca* was recently implicated in a study of IAV infection (29). Therefore, in order to explore the effects of coinfection with IAV and *K. oxytoca*, we developed a mouse model of IAV/*K. oxytoca* coinfection in which we observed that coinfected mice had increased morbidity and mortality when compared to singly-infected mice, as measured by survival and weight loss. As early as 1 day after coinfection, mice that had been previously infected with IAV had increased levels of several inflammatory cytokines and chemokines in the lung. The early induction of the inflammatory response that ultimately recruits innate immune cells is likely orchestrated by early pattern recognition receptor (PRR) signaling from pulmonary epithelial and endothelial cells (40–42). It is known that prior influenza infection can lead to a cytokine storm during secondary bacterial infection which begins with early pathogen-sensing by the epithelial cells and leads to massive infiltration of immune cells (40, 41). In conjunction with these findings, we observed an early increase in the levels of inflammatory cytokines day 1 post-coinfection in mice that were coinfected compared to mice with only one infection. In addition, following this increase in cytokine and chemokine

levels in our model there was an increase in cellular infiltration into the lungs in coinfected mice by day 3 post-coinfection. Interestingly, this increased inflammatory infiltrate did not result in improved resistance to infection, as coinfected mice did not have a significant reduction in either viral or bacterial burden. Rather, 3 days after coinfection there was a decrease in the rate of clearance of *K. oxytoca* in mice that had an ongoing viral infection compared to those that did not. These results suggest a potential defect in the ability of the infiltrating immune cells to clear the bacteria. Although the increased inflammation in the lung during coinfection might have been expected to be accompanied by resultant lung damage, there was only a modest, albeit significant, increase in albumin in the BALF from coinfected mice, indicating a minor increase in vascular permeability compared to singly-infected lungs. It is unknown if this slight increase in vasculature leakage is enough to tip the balance toward increased morbidity and mortality or if there is another cause of decreased tolerance.

There are many examples of IAV/bacterial coinfection in which an overwhelming pathogen burden leads to a damaging inflammatory response (9–13). Some models of coinfection with IAV are characterized by an early, acute inflammatory response with increased production of TNFα among other cytokines. These models demonstrate that prior infection with IAV leads to bacterial overgrowth, tissue damage due to the heightened immune response, and ultimately decreased survival (9, 25, 30–32). Our model of IAV/*K. oxytoca* coinfection echoed the same types of inflammatory immune responses as have been seen in similar coinfection models. In contrast though, IAV/*K. oxytoca* coinfection did not result in increased bacterial or viral burden at early time points and bacterial burden was controlled despite a delay in clearance at later time points. These results indicate that decreased host resistance may not be solely responsible for decreased survival during coinfection with IAV/*K. oxytoca* and that perhaps there are other mechanisms which play a role in determining the outcome of the host during this particular coinfection.

Most studies of IAV/*S. pneumoniae* or IAV/*S. aureus* coinfections demonstrate that IAV infection suppresses the initial innate immune response to bacteria. IAV has been shown to impair neutrophil phagocytic activity and reactive oxygen species production which leads to increased susceptibility to bacterial infection (9, 43, 44). In addition, type I IFN production during IAV/bacterial coinfection has been shown to suppress CCL2, CXCL1, and CXCL2 levels and subsequently inhibit the recruitment of monocytes and neutrophils (45, 46). Type I IFN has also been demonstrated to suppress type 17 immunity and therefore increase susceptibility to secondary bacterial infection (47, 48). It has been reported that high levels of IFNγ suppress the expression of the scavenger receptor MARCO leading to decreased phagocytosis of bacteria by alveolar macrophages (49, 50). The only indication of immunosuppression in our model was a small drop in IFNγ levels 1 day after *K. oxytoca* infection in coinfected lungs compared to IAV infection alone; however, the levels in coinfected animals were higher than those seen in animals infected with bacteria alone. Also by day 3 post-coinfection, IFNγ levels in the coinfected mice increased and were higher than the other groups. Elevated IFNγ production at day 3 also corresponded to the time at which we observed a delay in bacterial clearance, which may indicate that IFNγ suppressed bacterial clearance at this later timepoint in the coinfected group. In contrast, *K. oxytoca*-infected mice produce less IFNγ and have no defect in bacterial clearance, supporting the notion that IFNγ may hinder clearance during coinfection.

Neutrophils are often essential in the response to bacterial infection, playing important roles in rapid clearance of the bacteria; however, their role during viral/bacterial coinfection has been less clear with some studies arguing their importance for tissue protection while others demonstrate more pathogenic roles (9, 44, 51). Our results showed that during IAV/*K. oxytoca* coinfection there was massive infiltration of neutrophils without a reduction in bacterial or viral load. One possible explanation for this outcome is that IAV infection impairs neutrophil phagocytic or bactericidal functions so that the neutrophils that are recruited to the lung following coinfection are less able to clear bacteria than naïve neutrophils. It has been shown that neutrophils from IAV-infected lungs have impaired phagocytosis as well as production of reactive oxygen species (ROS) and that increasing production of ROS by neutrophils and macrophages can reduce susceptibility to secondary bacterial infections (43, 44, 52). IAV infection has also been shown to diminish the production of granulocyte-colony stimulating factor (G-CSF), which is known to activate neutrophils, and administering this cytokine following IAV infection restores neutrophil bactericidal function (9). It has also been shown that during coinfection with IAV and *S. pneumoniae*, neutrophils produce excessive neutrophil extracellular traps (NETs) which cause extensive lung damage without any reduction in pathogen burden (53). In addition, neutrophils during IAV/bacterial coinfection have been shown to have accelerated apoptosis, which can cause tissue damage if not effectively cleared (54). This knowledge, coupled with our results of macrophages downregulating the efferocytic mannose receptor CD206, may point to a source of damage in our coinfection model (55). With this in mind, it is reasonable to suspect that neutrophils in IAV/*K. oxytoca* coinfected lungs are also dysfunctional, which might explain the delay in bacterial clearance and increase in tissue damage in these animals.

Lastly, many studies show that excess damage and the inability for the lung to return to homeostasis can cause decreased survival (14–16). Our data demonstrate that IAV/*K. oxytoca* coinfected lungs have increased vasculature leakage and tissue damage as indicated by a small but significant increase in albumin in the BALF as compared to IAV-infected lungs, whereas lungs infected with bacteria alone have virtually no lung damage. Although this increase in tissue damage is modest, it is possible that there is a threshold for the amount of damage that can be done to the lung and still allow for function and this small difference is a tipping point in morbidity and mortality during coinfection. One potential factor involved in the inadequate repair response following IAV/*K. oxytoca* coinfection is the shift in phenotype of pulmonary macrophages away from their native, homeostatic state. Macrophages are phenotypically flexible cells that can

perform a variety of different roles depending on a number of environmental cues. On one side of the spectrum of macrophage phenotypes are M1 macrophages which have been defined by their role in recognizing certain bacterial and viral pathogens and generating inflammatory signals in response (55, 56). On the other side of the spectrum are M2 macrophages, which have been defined by their role in maintaining homeostasis through anti-inflammatory and tissue protective actions. However, these classifications are very broad and often do not accurately describe the nuanced states that macrophages can shift between (56). Alveolar macrophages normally play important roles during the resolution of infection to clear inflammatory agents and apoptotic cells and to remodel tissue (36–38, 55–57). However, pulmonary macrophage populations are also altered during inflammatory states, including infection and damage (58–60). The changes that occur to macrophage populations during IAV/bacterial coinfection have not been well studied. During coinfection with IAV/*K. oxytoca*, alveolar macrophages increase their production of TNFα and take on a more inflammatory phenotype. In addition, there is also a significant influx of macrophages and monocytes to the alveolar space during coinfection that tend to have a more inflammatory phenotype and likely also contribute to a shift in the macrophage population away from a reparative phenotype. Regardless of the origin of the pulmonary macrophages, whether they are alveolar macrophages or macrophages that have infiltrated and are repopulating the lung, it is evident that the population as a whole takes on a new role during coinfection that is directed more toward driving inflammation and is likely less conducive to repair. Several studies have demonstrated the important functions that macrophages play during lung infection. It has been demonstrated that the return to homeostasis mediated by macrophages is essential to survival of influenza infection and that mice suffer worsened outcomes in the absence of macrophages that promote tissue repair (61, 62). In addition, CD206 expression on macrophages is known to play important roles in removing potentially harmful extracellular enzymes generated during infection such as myeloperoxidases which are produced by neutrophils and cause tissue damage (63). CD206 is also an important mediator of phagocytosis of pathogens including influenza virus and *Klebsiella pneumoniae*, a bacterial species related to *K. oxytoca* (64–66). We observed downregulation of the mannose receptor CD206 in macrophages from coinfected lungs which could be indicative of a larger functional defect in important repair processes as well as recognition and clearance of pathogens. The overall shift in pulmonary macrophages toward a pro-inflammatory phenotype could be part of an ongoing effort to clear the bacteria that persists during coinfection. It is also likely that the influx of inflammatory cells is not only contributing to the damage done in the lung but also preventing the return to homeostasis by propagating and prolonging the inflammatory response. It is also possible that there are other factors that lead to decreased survival in conjunction with the increased lung damage so that lung function may be compromised independently of vascular leak. Another possibility is that additional organs are impacted by the coinfection. Although there is no evidence of systemic spread of either pathogen (data not shown), there could be a systemic effect that compromises organ function. In addition, although we see no evidence of overwhelming pathogen burden at early timepoints post-coinfection, it is also possible that there is a loss of control of either the virus or the bacteria at later timepoints, in some mice, which may contribute to the death of these coinfected animals. This seems unlikely though considering that the majority of mice that succumb to infection do so within the first 3 days post-coinfection where we observed no evidence of overwhelming pathogen burdens.

While other models of coinfection point to major perturbations to either host resistance or tolerance as the cause of increased mortality, IAV/*K. oxytoca* coinfection is instead characterized by more subtle alterations in both of these host responses that work synergistically to decrease survival. These data, in conjunction with previously published studies, demonstrate that the impact of IAV infection on host resistance and tolerance responses is dependent on the bacteria that comprises the secondary infection. Although we observed similar trends as have been previously reported in other coinfections, our data demonstrate that even slight dysfunction of these host responses can lead to poor disease outcomes and it is likely that there are other mechanisms of both host resistance and tolerance that factor into determining the outcome of coinfection. Understanding how IAV impacts the response to a secondary bacterial infection is crucial in producing more effective treatments for these complex pulmonary infections.

MATERIALS AND METHODS

Nasopharyngeal Swab Sampling

The survey study was approved by the IRB (institutional review board) at Memorial Hospital of RI before any samples were obtained. The study samples were residual, spent, clinical samples of nasopharyngeal washings obtained in 0.9% normal saline from patients with influenza-like illnesses. These patient samples were obtained through the emergency room and acute care outpatient clinics during the influenza season of 2013–2014. The clinical laboratory performed rapid diagnostic antigen detection methods and by standard PCR methodologies for influenza virus. The research samples were obtained from spent samples prior to their final disposal. A waiver of informed consent by IRB approval was granted as the samples were patient de-identified by the clinical laboratory staff before providing the samples for the research study. The samples were maintained at −80C until further study.

Mice

All animal studies were approved by the Brown University Institutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Animals of the National Institutes of Health. The University is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Brown University's PHS

Assurance Number: D16-00183 (A3284-01), expiration date July 31, 2022. The USDA Registration Number is 15-R-0003. Brown University IACUC was approved on September 28, 2016, and the animal protocol number is 1308000011. C57BL/6J mice were purchased from The Jackson Laboratory. Mice used were female and 7–9 weeks old.

Pulmonary Infection

Mice under anesthesia and analgesia by ketamine (70–100 mg/kg) and xylazine (20–40 mg/kg) injection were administered IAV intranasally in a volume of 30 μL using a sterile saline vehicle. Mice were infected with 300 PFU influenza A virus (A/WSN/33 (H1N1)) strain. Influenza A virus was obtained from Akiko Iwasaki at Yale University. It was propagated using MDCK cells using standard procedures. *Klebsiella oxytoca* was cultured from glycerol bead stocks in 50 mL Todd-Hewitt broth overnight. The next day 1 mL of the overnight culture was diluted in 50 mL Todd-Hewitt broth and allowed to grow to log phase before being washed and resuspended in a sterile saline vehicle. Mice were infected intranasally with 10^6 CFU in a volume of 30 μL. Coinfected mice were administered 300 PFU IAV followed by 10^6 CFU *K. oxytoca* 5 days later. Mice were monitored daily for a minimum of 3 days, and every other day for the remainder of the experiment, except for survival and weight monitoring which was conducted daily.

Confirming Klebsiella Oxytoca Identity

Bacterial DNA was isolated from a culture grown as described above using the QIAamp UCP Pathogen Mini Kit (Qiagen). Genomic DNA was sonicated to a median size of 300 bp using a Covaris S220 instrument. Fragmented DNA was subsequently prepared into sequencing libraries using the Ovation Ultralow Library System V2 from Nugen according to the manufacturer's instructions. The library was then sequenced on an Illumina HiSeqX machine in the 2x150 bp configuration, yielding a total of 3,234,751 paired end reads. Raw reads were deposited in the NCBI Short Read Archive (SRA) under accession number SRP148653. Reads were trimmed of Illumina adapters and low quality bases using Trimmomatic (67) and then assembled using SPAdes (version 3.11.0) (68). Preliminary Sanger sequencing of the 16S rRNA gene and Blastn analysis of individual contigs suggested that this strain was related to *Klebsiella oxytoca*. Therefore, we calculated average nucleotide identity of our scaffolds (ANI) based on MUMmer (69) using the web-based tool JSpeciesWS (70) using four completely sequenced genomes of *K. oxytoc*a as a reference. This analysis found that our strain, which we named JK01, was in fact *K. oxytoca* and shared >99% average nucleotide identity with strains CAV1335 and CAV1099. However, this strain was less similar to CAV1374 and KONIH1 (∼92.6% ANI). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QMBO00000000. The version described in this paper is version QMBO01000000.

BALF Collection

Bronchoalveolar lavage fluid (BALF) was collected via exposing the trachea, inserting a BD Venflon IV catheter into the trachea, removing the needle and inserting a 1 mL syringe with PBS. 1mL of PBS was flushed into the lung and collected. BALF was then centrifuged to isolate cells which were counted on a Moxi Z Automated Cell Counter (Orflo) and used for flow cytometry analyses and cell-free supernatants were collected for cytokine analyses and albumin content quantification.

Lung Tissue Cell Collection

For isolation of cells from lungs, the right superior and middle lobes were perfused with 15 ml of PBS. The lung tissue was cut into small pieces and incubated for 60 min at 37°C in 2 ml of digestion media containing type 4 collagenase (Worthington Biochemical Corporation) and DNAse I (Sigma-Aldrich). Digested lung tissue was then sieved passed through a 70 μM cell strainer and washed with PBS. After washing the cell pellet was resuspended in 1.5 ml 44% Percoll/0.15M NaCl and layered over 1 ml of 56% Percoll/0.15M NaCl. Percoll layers were centrifuged at room temperature for 20 min at 600 g with minimal acceleration and deceleration to form a gradient with a band of cells at the interphase which were then collected and washed with 10 mL PBS. Isolated lung cells were counted on a Moxi Z Automated Cell Counter (Orflo) and used for flow cytometry analyses.

BALF Albumin Content Quantification

Cell-free supernatants taken from BALF were tested for the concentration of albumin using the BCG Albumin Assay Kit (Sigma Aldrich) according to the manufacturer's instructions, using a dilution series of albumin standard to determine a standard curve which was used to calculate measurements of albumin in each sample. Absorbances were measured for each sample at 620 nm on a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices) using SoftMax Pro 6.4 software.

Viral Quantification

Viral genome copies were measured using RNA isolated from the unperfused right inferior lobe using the ReliaPrep™ RNA Tissue Miniprep System according to the manufacturer's instructions (Promega). RNA then underwent PCR with random hexamers (Invitrogen). Viral genome copies from the cDNA were then measured via qPCR using the forward primer (5′-CATGGAATGGCTAAAGACAAGACC-3′), the reverse primer (5′-CCATTAAGGGCATTTTGGACA-3′), and the probe (5′-[6-FAM]TTTGTGCCCA[BHQ1a-Q]-3′) specific for the M gene of influenza A viruses. qPCR was run on a Roche LightCycler® 96 Real-Time PCR System and analyzed with the LightCycler® 96 software.

Bacterial Quantification

Unperfused superior lobes of mouse lungs were harvested and homogenized by a gentleMACS™ Dissociator (miltenyi Biotec) in 1mL of PBS. The homogenate was immediately serial diluted by 10-fold up to six times. 5 μL of each dilution were then plated on a sheep's blood agar plate per sample. The plates were then incubated under 37°C overnight and the colonies were counted as a measurement for the bacterial load in the infected lungs.

Flow Cytometry Analysis of Cell Subsets

The following antibodies were used to identify cell subsets: Ly6C eFluor© 450 (clone HK1.4, eBioscience), F4/80 eFluor© 660 (clone BM8, eBioscience), CD11c Brilliant Violet™ 711 (clone N418, BioLegend), MHC II PerCP-eFluor© 710 (clone M5/114.15.2, eBioscience), CD206 PE-Dazzle™ 594 (clone C068C2, BioLegend), TNFα Alexa Fluor® 488 (clone MP6-XT22, eBioscience), Siglec-F PE (clone E50-2440, BD Biosciences) and Ly6G PE/Cy7 (clone 1A8, BioLegend). Dead cells were excluded from analyses using Fixable Viability Dye eFluor© 506 (eBioscience). For surface staining, cells were first washed with 1x PBS then incubated with Fixable Viability Dye diluted in 1x PBS for 20 min at room temperature. Cells were washed again with 1x PBS and then treated with anti-CD16/CD32 Fc receptor blocking antibody (clone 2.4G2) in 1x PBS (1% FBS) for 10 min on ice. Surface staining antibodies were then added and incubated for 30 min on ice. Cells were washed, then fixed and permeabilized with BD Cytofix/Cytoperm™ for 20 min on ice (BD Biosciences). Then cells were washed and stained with intracellular antibodies in the permeabilization buffer from the BD Cytofix/Cytoperm™ kit on ice for 30 min before undergoing a final wash and resuspension with 1x PBS (1% FBS). Samples were acquired on an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher) or a FACSAria Flow Cytometer (BD Biosciences) and downstream analyses were performed using FlowJo v10 software (Tree Star, Inc.). Isotype, fluorescence minus one, and unstained samples were used as controls to determine positive and negative gating of experimental samples. Viable cells were determined by first gating out doublets and debris using forward and side scatter properties and then selecting for cells with low staining with Fixable Viability Dye eFluor© 506 (**Supplemental Figure 1A**). Total cell numbers of each cell subset were determined by multiplying initial cell counts obtained on the Moxi Z Automated Cell Counter (Orflo) by the percentage of total viable cells.

Cytokine Analysis

Cytokine concentrations were determined in BALF using a custom LEGENDplex bead-based immunoassay (BioLegend) according to manufacturer instructions. The samples were acquired on an Attune NxT Acoustic Focusing Cytometer (Thermo Fisher) and the data files were analyzed by LEGENDPlex Data Analysis Software (Vigenetech). IL-6 concentrations in BALF were determined via the BD OptEIA™ ELISA set according to manufacturer instructions (BD Biosciences).

LDH Cytotoxicity Assay

The LDH cytotoxicity assay was conducted using the Pierce™ LDH Cytotoxicity Assay Kit according to the manufacturer's instructions on BALF samples from days 1 and 3 post-coinfection. Absorbances were measured for each sample at 490 nm and 680 nm on a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices) using SoftMax Pro 6.4 software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Fisher's exact test, log rank, ANOVA followed by Tukey's multiple comparison tests, and *t*-tests were performed where appropriate.

AUTHOR CONTRIBUTIONS

KL and AJ contributed to the conception and design of the study; KL performed the animal experiments, analyzed the data, performed statistical analysis, and made the figures; JM-L, DC, and PB identified and sequenced the *K. oxytoca* strain; SO obtained and provided the nasopharyngeal patient samples; KL and AJ wrote the manuscript; All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02377/full#supplementary-material

Supplemental Figure 1 | Flow cytometry gating strategy. First, viable cells were determined by gating out doublet cells based on FSC-A/FSC-H and SSC-A/SSC-H and then cellular debris/dead cells were gated out on FSC-A/SSC-A. From there, viable cells were gated on their low staining of viability dye **(A)**. Viable cells were further separated into cell subsets **(B)**. First, viable cells were gated based on Ly6G and F4/80. $Ly6G^{+}F4/80^{-}$ cells were called neutrophils. $Ly6G^{-}F4/80^{+}$ cells were further gated on CD11c. $Ly6G^{-}F4/80^{+}CD11c^{+}$ cells were called alveolar and repopulating macrophages. $Ly6G^{-}F4/80^{+}CD11c^{-}$ were further separated based on expression of Ly6C and MHC II. $Ly6G^{-}F4/80^{+}CD11c^{-}Ly6C^{+}$MHC II^{-} cells were called inflammatory monocytes and $Ly6G^{-}F4/80^{+}CD11c^{-}Ly6C^{+}$MHC II^{+} were called infiltrating macrophages. $Ly6G^{-}F4/80^{+}CD11c^{+}$ macrophages and $Ly6G^{-}F4/80^{-}CD11c^{-}Ly6C^{+}$MHC II^{+} macrophages were further separated by their expression of CD206 and MHC II or TNFα in order to determine polarization. To establish the identity of $Ly6G^{-}F4/80^{+}CD11c^{+}$ cells, we compared their expression of Siglec-F, a known alveolar macrophage marker, and CD11c on days 1 **(C)** and 3 **(D)** post-coinfection in the BALF.

Supplemental Figure 2 | An LDH assay was conducted on BALF from days 1 and 3 post-coinfection as a measure of cytotoxicity in the lungs. * denotes $P \leq 0.05$ between indicated groups. Data were analyzed with ANOVA followed by Tukey's multiple comparison tests. Error bars represent SEM. Data are combined from at least four independent experiments with at least four mice per group.

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IFNAR2 Is Required for Anti-influenza Immunity and Alters Susceptibility to Post-influenza Bacterial Superinfections

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Influenza virus infections particularly when followed by bacterial superinfections (BSI) result in significant morbidities and mortalities especially during influenza pandemics. Type I interferons (IFNs) regulate both anti-influenza immunity and host susceptibility to subsequent BSIs. These type I IFNs consisting of, among others, 14 IFN-α's and a single IFN-β, are recognized by and signal through the heterodimeric type I IFN receptor (IFNAR) comprised of IFNAR1 and IFNAR2. However, the individual receptor subunits can bind IFN-β or IFN-α's independently of each other and induce distinct signaling. The role of type I IFN signaling in regulating host susceptibility to both viral infections and BSI has been only examined with respect to IFNAR1 deficiency. Here, we demonstrate that despite some redundancies, IFNAR1 and IFNAR2 have distinct roles in regulating both anti-influenza A virus (IAV) immunity and in shaping host susceptibility to subsequent BSI caused by *S. aureus*. We found IFNAR2 to be critical for anti-viral immunity. In contrast to *Ifnar1*$^{-/-}$ mice, IAV-infected *Ifnar2*$^{-/-}$ mice displayed both increased and accelerated morbidity and mortality compared to WT mice. Furthermore, unlike IFNAR1, IFNAR2 was sufficient to generate protection from lethal IAV infection when stimulated with IFN-β. With regards to BSI, unlike what we found previously in *Ifnar1*$^{-/-}$ mice, *Ifnar2*$^{-/-}$ mice were not susceptible to BSI induced on day 3 post-IAV, even though absence of IFNAR2 resulted in increased viral burden and an increased inflammatory environment. The *Ifnar2*$^{-/-}$ mice similar to what we previously found in *Ifnar1*$^{-/-}$ mice were less susceptible than WT mice to BSI induced on day 7 post-IAV, indicating that signaling through a complete receptor increases BSI susceptibility late during clinical IAV infection. Thus, our results support a role for IFNAR2 in induction of anti-IAV immune responses that are involved in altering host susceptibility to BSI and are essential for decreasing the morbidity and mortality associated with IAV infection. These results begin to elucidate some of the mechanisms involved in how the individual IFNAR subunits shape the anti-viral immune response. Moreover, our results highlight the importance of examining the contributions of entire receptors, as individual subunits can induce distinct outcomes as shown here.

Keywords: IFNAR2, IFNAR1, Influenza, *Staphylococcus aureus*, superinfection

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INTRODUCTION

Influenza A virus (IAV) causes one of the most common respiratory infections worldwide. While infection from IAV can be detrimental on its own, more typically, anti-viral immune responses induced during IAV infection alter host susceptibility to bacterial superinfections (BSI). Over the past decades, *Staphylococcus aureus* has become one of the predominant bacteria involved in BSIs leading to increased morbidity and mortality, especially during IAV pandemics (1–3). A better understanding of the anti-viral immune factors that are involved in altering the host susceptibility to BSI is essential for decreasing the morbidity and mortality associated with post-IAV BSI.

It is well-known that type I IFNs, including a single IFN-β and 14 IFN-α's, have an established role in the anti-viral immunity to influenza and are known to regulate host susceptibility to the subsequent BSIs. Because type I IFNs are thought to require recognition by the heterodimeric IFNAR1/IFNAR2 receptor, depletion of the IFNAR1 subunit (*Ifnar1*$^{-/-}$) is assumed to render mice unresponsive to type I IFN signaling. However, previous studies on the role of IFNAR1 in influenza infection and post-influenza BSI models showed conflicting results. Absence of IFNAR1 either in knockout mice or by antibody blockage resulted in a range of responses. These responses varied from increased mouse susceptibility to infection (4–7), no significant antiviral effect (8), no effect on survival from influenza disease (9), or reduced susceptibility by the means of better controlled inflammation (10). Recent reports have also demonstrated that IFN-β can ligate to either IFNAR1 or IFNAR2 individually, while IFN-α appeared to only ligate to IFNAR2, and that the two different IFN-β/IFNAR subunit complexes can transduce expression of distinct sets of genes (11). Additional evidence for the existence of differential signaling by IFNAR1 and IFNAR2 is supported by an absence of sepsis morbidity in *Ifnar1*$^{-/-}$ mice compared to high morbidity in *Ifnar2*$^{-/-}$ and WT mice. These conflicting results prompted us to investigate whether the individual IFNAR subunits are sufficient for interferon signaling, and whether they have non-redundant roles in mediating host immunity to IAV infections and subsequent BSIs. Here, we report that despite some redundancies, IFNAR1 and IFNAR2 have distinct roles in regulating both anti-IAV immunity and in shaping host susceptibility to a subsequent *S. aureus* BSI. Importantly, these results highlight the need to understand the contribution of individual IFNAR subunits to the infections mediated by either virus or bacteria alone, or together in the context of BSI.

MATERIALS AND METHODS

Mice

Male and female wild type (WT) C57BL/6 (CD45.2), *Ifnar1*$^{-/-}$, and *Ifnar2*$^{-/-}$ (Ifnar2tm1(KOMP)Vlcg)mice (originally purchased from Jackson Laboratories or UC Davis KOMP Repository) were bred and maintained at Montana State University (Bozeman, MT) Animal Resources Center under pathogen-free conditions. All mice used in this study were between 6 and 8 weeks of age at the initiation of experiment. Mice were weighed and monitored for signs of morbidity and mortality. All care and procedures were in accordance with the recommendations of the NIH, the USDA, and the *Guide for the Care and Use of Laboratory Animals* (8th ed.) (12). Animal protocols were reviewed and approved by the MSU Institutional Animal Care and Use Committee (IACUC). MSU is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC; number 713).

Inoculations and Challenge

Nonsurgical intratracheal (i.t.) inoculations were performed as described previously for all inoculations and challenges (13). Virus inoculation: mice were inoculated with 100 μL of PBS or 0.1LD_{50} (1,500 plaque forming units [PFU]) of IAV strain A/PR8/8/34 (PR8; H1N1). Bacterial challenge: mice were inoculated with 100 μL of PBS or 10^8 CFU of LAC strain of *S. aureus* (MRSA pulsed-field type USA300; was a kind gift from Jovanka Voyich at MSU). Our previously described procedure for determining CFUs (13) and PFUs (14) was followed on lung homogenate samples. Phosphorylation of STAT3 (P-STAT3) inhibition: mice were inoculated 6 h after IAV infection with 100 μL containing 100 μg of FLLL32 [Cayman Chemical, (15)] in 10% DMSO. Control mice were inoculated with equal volume of PBS with 10% DMSO. Mouse recombinant IFN inoculation (mrIFN): mice were inoculated with either 10^4 IU mrIFN-β (PBLassay bioscience) or with 2.5×10^5 IU mrIFN-αA in 100 μL PBS (BioLegend) at 0, 3, and 6 h post-IAV infection.

Preparation of BALF Samples and Cytokine Analyses

Mice were sacrificed by intraperitoneal (i.p.) administration of 90 mg/kg of body weight sodium pentobarbital. Bronchoalveolar lavage (BAL) was performed by washing the lungs with 3 mM EDTA in PBS (16) and cellular composition was determined by hemocytometer cell counts and differential counts of cytospins after staining with Quick-Diff solution (Siemens; Medical Solutions Diagnostics, Tarrytown, NY). Cell-free BAL fluid (BALF) was used to determine levels of IL-13 (4–500 pg/ml) and IFN-α (31.3–20.00 pg/ml) using ELISA kits (Ready-SET-Go; eBioscience, San Diego, CA), and levels of IL-1α (Minimal Detectable Concentration (MDC); 1.3 pg/mL), IFNγ (MDC; 0.8 pg/mL), TNFα (MDC; 1.9 pg/mL), IL-1β (MDC; 2.8 pg/mL), IL-10 (MDC; 2.1 pg/mL), IL-6 (MDC; 0.9 pg/mL), IFN-β (MDC; 4 pg/mL) using the LEGENDplex mouse inflammation panel (BioLegend, San Diego, CA). LEGENDplex panel was acquired on LSRII running FACS-Diva software (both obtained from BD Bioscience) and analyzed using BioLegend data analysis software. Results are from ≥4 mice per group (biological replicates) and 2 technical replicates per mouse.

qRT-PCR

Mice were inoculated with IAV or PBS as described above and euthanized 24 h after inoculation. Lungs were homogenized and RNA was extracted immediately using Trizol reagent and chloroform method per manufacturer's protocol. RNA was reverse transcribed with QuantiTect reverse transcription kit (Qiagen, USA). Primers for all murine genes of interest were

designed, unless denoted, with PrimerQuest (IDT) and all were manufactured by IDT, USA. Sequences are:

STAT3 (17) Fwd: GGATCGCTGAGGTACAACCC
STAT3 Rev: GTCAGGGGTCTCGACTGTCT
STAT6 (18) Fwd: TCTCCACGAGCTTCACATTG
STAT6 Rev: GACCACCAAGGGCAGAGAC
STAT1 Fwd: GACCCTAAGCGAACTGGATAC
STAT1 Rev: TGTCGCCAGAGAGAAATTCGTGT
STAT2 Fwd: CGGCCAACAGGTGAAATTAAG
STAT2 Rev: GGGACTTACAAAGGAGCAGAA
IRF3 Fwd: CCCACAGTGCTACTGATACC
IRF3 Rev: GTCACACCAGACTTAGGAATGT
IRF7 Fwd: TATGCAAGGCATACCTGGAG
IRF7 Rev: CGATGTCTTCGTAGAGACTGTT

rpl13a fwd: CTCTGGAGGAGAAACGGAAGGAAA, rev: GGTCTTGAGGACCTCTGTGAACTT. All reactions were performed on Roche LightCycler 96 real-time PCR detection system with iTaq universal SYBR green supermix (Bio-Rad, Hercules, CA). The $\Delta\Delta C_t$ method was used to assess changes in mRNA abundance, using rpl13a as the housekeeping gene. Results presented are the mean and standard deviation from three biological and three experimental replicates.

Survival, Morbidity, LDH, and Albumin

Mice were weighed on a daily basis and assessed for signs of morbidity and mortality. Morbidity measures were as follow: 0, normal; 1, hunched back or ruffled fur; 2, both hunched back and ruffled fur; 3, not moving over a 5 min period. Lactate dehydrogenase (LDH) in the cell-free BALF was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) and albumin in the cell-free BALF was measured using QuantiChrom BCG Albumin Assay Kit (BioAssay Systems) following the manufacturers protocols.

Primary Alveolar Epithelial Cell Harvest

After mice were euthanized, their lungs were perfused with PBS containing gentamycin (10 μg/mL) injected into the heart. Lungs were then lavaged with PBS containing 3 mM EDTA, filled with 1 mL PBS containing Dispase (50 units/mL) and Elastase (5 units/mL) to degrade extracellular matrix proteins and tied off. Lungs were incubated at 37°C while shaking for 1 h and following incubation elastase activity was terminated by addition of fetal bovine serum (FBS) and Dnase (4 units/mL) was also added to the lung homogenate samples to ensure degradation of extracellular DNA. The lungs were then dissected and aspirated through syringes to create a single cell suspension. The cell suspension was filtered (100 and 50 μm) and separated by a discontinuous Percoll gradient consisting of a heavy layer (5.2 mL Percoll, 50 μL FBS, 3.8 mL water, 1 mL 10× PBS/100 mM HEPES/55 mM Glucose/pH 7.4) and a light layer (3.6 mL Percoll, 50 μL FBS, 6 mL HBSS, 0.4 10 × PBS/100 mM HEPES/55 mM Glucose/pH 7.4) by centrifuging at 2,000 rpm for 20 min. Cells were collected, washed and non-epithelial cells were removed by incubating collected cells in a plate pre-coated with IgG (5 ug/mL) for 30 min. Epithelial cells were collected, washed and transferred to plates pre-coated with fibronectin (Calbiochem, 50 μg/mL) in DMEM/F-12 media (Hyclone) containing 1.5 g/L $NAHCO_3$, 5 mL insulin/transferrin/sodium selenite (Gibco), 1 μg/mL hydrocortisone (Sigma), 1 mM L-glutamine, 10% FBS, and Pen-Strep. Upon confluency, cells were passaged and expanded for use.

Western Blot

For whole lung protein fractions, mice were euthanized 24 h after inoculation as described above, lungs were homogenized, and protein was extracted using RIPA buffer (50 mM Tris-HCl pH 7.5, 2 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 50 mM NaF). Primary alveolar epithelial cells were grown to ∼80–90% confluence on T-75 flasks and were treated with IAV (MOI 1) or PBS. At 24 h after treatment cells were washed with ice-cold PBS and cytoplasmic protein fraction was extracted with Buffer A (10 mM HEPES pH 8.0, 0.5% NP-40, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 200 mM sucrose). The tubes were incubated for 30 min on ice, and then centrifuged at 14,000 rpm at 4°C and the supernatant was collected. The pellets were resuspended in 30 μl of RIPA buffer and incubated on ice for 30 min. Then they were centrifuged at 14,000 rpm at 4°C and the supernatant was collected as the nuclear protein fraction. Proteins were quantified using BCA (Pierce). Proteins were run on a 10% SDS-Page gel, transferred to nitrocellulose, and stained for either STAT3 (Cell Signaling; D1B2), P-STAT3 (Cell Signaling; Tyr705, D3A7), or beta-actin (BioLegend; Poly6221). Protein levels were normalized to beta-actin and fold change was calculated over WT PBS.

Statistical Analyses

Reported results are means ± SD of ≥4 mice/group from a single experiment. Each experiment for which results are presented in the manuscript was independently performed at least twice with similar results. The differences between treatment groups were analyzed by analysis of variance (ANOVA) or Student's *t*-test (two-tailed) using GraphPad Prism software V.7.0d. For the differences in survival Kaplan-Meier curves were plotted and analyzed using GraphPad Prism software (Version 4.0; La Jolla, CA) using Gehan-Breslow-Wilcoxon (Logrank) test. Statistical differences with $P < 0.05$ were considered significant.

RESULTS

Absence of IFNAR2 Causes Increased Morbidity and Mortality in Response to IAV Infection

It is well-known that type I IFNs are involved in anti-viral immunity to influenza and involved in BSI outcome. As type I IFNs are recognized by and signal through the heterodimeric IFNAR1/IFNAR2 receptor, depletion of the IFNAR1 subunit (*Ifnar1*$^{-/-}$) has been assumed to eliminate type I IFN signaling in mice. However, IFN-β has recently been shown to ligate the IFNAR subunits independently resulting in distinct gene induction (11), suggesting that the individual receptors may have distinct functions following IFN recognition. Since the *Ifnar2*$^{-/-}$ mice have not been characterized in terms of susceptibility to IAV or BSI, we first sought to determine the role of IFNAR2 throughout the course of IAV infection. We found that *Ifnar2*$^{-/-}$

mice were more susceptible to IAV infection than WT mice indicated by earlier weight loss (**Figure 1A**) and significantly reduced survival (**Figure 1B**). Unlike *Ifnar1*$^{-/-}$ mice, that in our hands had similar viral load to WT mice throughout influenza disease (9), *Ifnar2*$^{-/-}$ mice had increased viral burden on both days 3 and 7 of IAV infection compared to WT mice (**Figure 1C**). These and our previously published results imply that the presence of IFNAR2 is sufficient for controlling viral infection in *Ifnar1*$^{-/-}$ mice (9), but the presence of IFNAR1 in *Ifnar2*$^{-/-}$ mice is not. Both weight loss and morbidity can be due to increased cellular recruitment into the lung, but also inflammation resulting from cellular and/or tissue damage. At day 3 post-IAV, when the virus titer is typically at its peak (19), neither the pattern of cellular recruitment into the lung (**Figure 1D**) nor the extent of lung damage as measured by LDH (**Figure 1E**) and albumin (**Figure 1F**) were different between *Ifnar2*$^{-/-}$ and WT mice. This suggests that IFNAR2 deficiency does not affect cell recruitment or lung integrity early during IAV infection. At day 7 post-IAV, when mice experience the peak of body weight loss, *Ifnar2*$^{-/-}$ mice had increased overall cell numbers with significantly more neutrophils recruited to the lung compared to WT mice (**Figure 1D**). The increased number of neutrophils in *Ifnar2*$^{-/-}$ mice corresponded to an increase in LDH (**Figure 1E**), but not in albumin (**Figure 1F**) when compared to WT mice.

Absence of IFNAR2 Alters the Inflammatory Environment During IAV

Due to the impaired anti-viral immune response in *Ifnar2*$^{-/-}$ mice and enhanced neutrophil recruitment later during IAV infection, we sought to determine whether these mice exhibit reduced anti-viral cytokines in the lung throughout IAV infection. At day 3 post-IAV we found a decrease in IFNγ and IL-6 levels in the *Ifnar2*$^{-/-}$ mice compared to WT mice (**Figure 2A**). The *Ifnar2*$^{-/-}$ mice at day 3 post-IAV also had about 1 log less of each IFN-β and IFN-α in their lungs compared to WT mice. At day 7 post-IAV there was an increase in the inflammatory cytokines IL-1α, IFNγ, and IL-6 and a decrease in the anti-inflammatory cytokine IL-10 compared to WT mice (**Figure 2B**). The increase in inflammatory cytokines at day 7 post-IAV in *Ifnar2*$^{-/-}$ mice compared to WT mice correlated with the increased neutrophil recruitment and cellular damage (LDH) found in these mice at that time (**Figures 1D,E**). These results imply that induction of a proinflammatory response early after IAV infection is reduced in the absence of IFNAR2, whereas later after IAV, lack of IFNAR2 resulted in exacerbated lung inflammation.

Absence of IFNAR2 Signaling Limits IAV Burden, but Does Not Alter Susceptibility to BSI at Day 3 Post-IAV

Previously we found that while IFNAR1 was not required for anti-IAV immunity (9), it was important for controlling host susceptibility to *S. aureus* BSI. Specifically, we demonstrated that *Ifnar1*$^{-/-}$ mice were more susceptible to BSI than WT mice on day 3 post-IAV, but less susceptible on day 7 post-IAV (9). Because our results thus far indicate that IFNAR2 is important for anti-viral immunity to IAV, we next sought to determine whether the increased IAV-susceptibility of *Ifnar2*$^{-/-}$ mice affected their BSI susceptibility, and whether *Ifnar2*$^{-/-}$ mice exhibit a similar pattern of BSI susceptibility as *Ifnar1*$^{-/-}$ mice. To this end we found that *Ifnar2*$^{-/-}$ mice superinfected with *S. aureus* on day 3 post-IAV had comparable lung bacterial burden to *Ifnar2*$^{-/-}$ mice infected with *S. aureus* alone (**Figure 3A**). Bacterial burden of either the BSI-*Ifnar2*$^{-/-}$ mice or the *Ifnar2*$^{-/-}$ mice infected with *S. aureus* alone did not significantly differ from that of *S. aureus*-only infected WT control mice. This suggested that IFNAR2 deficiency has no effect on host susceptibility to respiratory infection with *S. aureus,* whether introduced alone or as BSI on day 3 post-IAV. Because we previously found that IAV-infected *Ifnar1*$^{-/-}$ mice were more susceptible to day 3 BSI than either their mock-inoculated littermates or IAV-infected WT mice (9), collectively our results indicate that presence of IFNAR1 is sufficient to protect mice from altered BSI susceptibility at day 3 post-IAV. Previously we reported that IFNAR1 deficiency had no effect on either lung viral burden or influenza disease progression both prior to and following BSI (9). In contrast to these findings in *Ifnar1*$^{-/-}$ mice, here we found that lung virus burden of *Ifnar2*$^{-/-}$ mice was significantly higher than that of WT mice both prior and 24 h after BSI induced on day 3 post-IAV (**Figures 1C**, **3B**). This suggested that despite increased IAV susceptibility of mice in the absence of IFNAR2, BSI early after IAV infection did not exacerbate IAV disease in *Ifnar2*$^{-/-}$ mice. Along these lines, BSI at day 3 post-IAV did not further exacerbate body weight loss and morbidity of IAV-infected *Ifnar2*$^{-/-}$ mice (**Figures 3C,F**), further indicating that IFNAR2 may be more important for viral immunity than bacterial immunity. When compared to BSI-WT mice the BSI-*Ifnar2*$^{-/-}$ mice had similar levels (**Figure 3D**) and types (**Figure 3E**) of cells recruited to the lung during BSI, indicating that the *Ifnar2*$^{-/-}$ mice do not have a defect in cellular responses during a BSI. The superinfected *Ifnar2*$^{-/-}$ mice also did not have significantly altered LDH (**Figure 3G**) or albumin (**Figure 3H**) levels in the BALF compared to WT mice. This suggests that at day 3 BSI with *S. aureus* the absence of IFNAR2 did not have a substantial effect on damage, similar to what we found early during IAV infection alone (**Figure 1**). Importantly, although IFNAR2 is not required for protection from day 3 BSI post-IAV, our results thus far demonstrate that when examined individually, the IFNAR subunits can lead to distinct outcomes.

Ifnar2$^{-/-}$ Mice Have Reduced Susceptibility to Day 7 BSI Post-IAV

Unlike BSI at day 3 post-IAV, mice and humans are known to be more susceptible to BSI around day 7 post-IAV infection. Our laboratory and others have shown this susceptibility to depend on IFNAR1 with *Ifnar1*$^{-/-}$ mice being less susceptible to BSI at day 7 post-IAV compared to WT mice (7, 9, 20, 21). Thus, we next sought to determine whether IFNAR2 plays a similar role as IFNAR1 in the susceptibility to day 7 post-IAV BSI. *Ifnar2*$^{-/-}$ mice, similar to what we found for *Ifnar1*$^{-/-}$ mice (9), were protected from BSI at day 7 post-IAV as compared

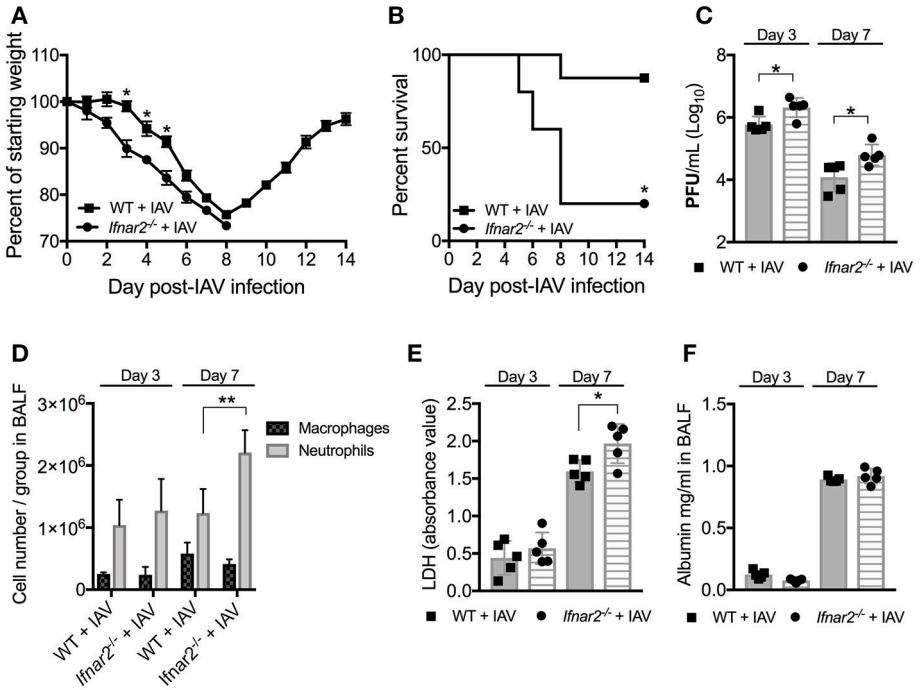

FIGURE 1 | *Ifnar2*$^{-/-}$ mice are more susceptible to IAV. WT and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0. **(A)** Weights were monitored daily and are represented as percent of starting body weight depicted as average/group (Significance represents WT+IAV compared to *Ifnar2*$^{-/-}$+IAV). **(B)** Survival is represented as percent survival/group. PFUs **(C)** were determined on day 3 or day 7 post-IAV. **(D)** Differential counts were determined from cell-free BALF on day 3 or day 7 post-IAV. LDH **(E)** and Albumin **(F)** were analyzed from cell-free BALF on day 3 or day 7 post-IAV. Data shown are mean ± SD results of ≥4 animals per group from one representative experiment. $^{**}P > 0.01$; $^{*}P > 0.05$.

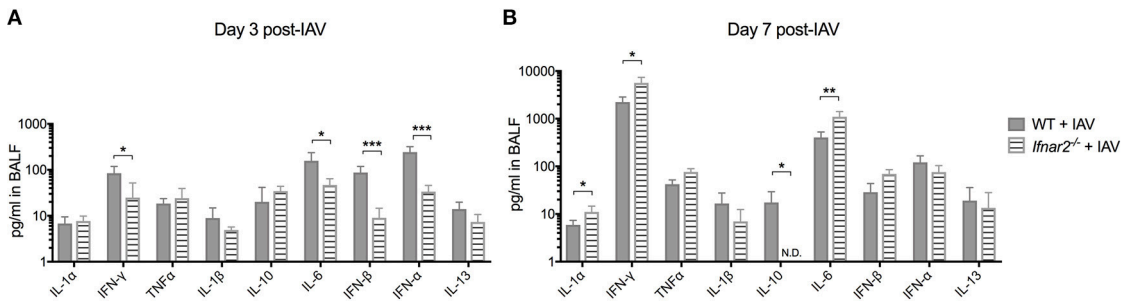

FIGURE 2 | Absence of IFNAR2 initially reduces the inflammatory environment during IAV. WT and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0. Indicated cytokines were measured in cell-free BALF collected on **(A)** day 3 or **(B)** day 7 post-IAV. Data shown are mean ± SD results of ≥4 animals per group from one representative experiment. N.D. is not detectable. $^{**}P > 0.01$; $^{*}P > 0.05$.

to *S. aureus-Ifnar2*$^{-/-}$ mice (**Figure 4A**). Like mice deficient in IFNAR1 signaling (9), when compared to WT mice *Ifnar2*$^{-/-}$ mice had similar levels of virus following BSI at day 7 post-IAV (**Figure 4B**). While *Ifnar2*$^{-/-}$ and WT mice had similar virus burden at 24 h post-BSI (**Figure 4B**), singly infected *Ifnar2*$^{-/-}$ mice at day 7 post-IAV infection showed 1 log increase in the lung viral burden when compared to IAV-only infected WT mice (**Figure 1C**). This suggests that in the absence of IFNAR2, superinfection on day 7 prevents further increases in viral burden. Although the IAV infected *Ifnar2*$^{-/-}$ mice had increased early weight loss compared to WT mice prior to BSI on day 7 (**Figure 4C**), these mice had a similar percentage of weight loss in response to *S. aureus* challenge (**Figure 4C**). However, IAV infected *Ifnar2*$^{-/-}$ mice had increased morbidity compared to IAV infected WT mice both before and after challenge with *S. aureus* (**Figure 4F**), further suggesting that IFNAR2 signaling may be involved in regulating anti-viral immunity. At 24 h after day 7 BSI, WT, and *Ifnar2*$^{-/-}$ mice had similar numbers (**Figure 4D**) and types (**Figure 4E**) of cells in the lung. This again was interesting because at day 7 post-IAV infection,

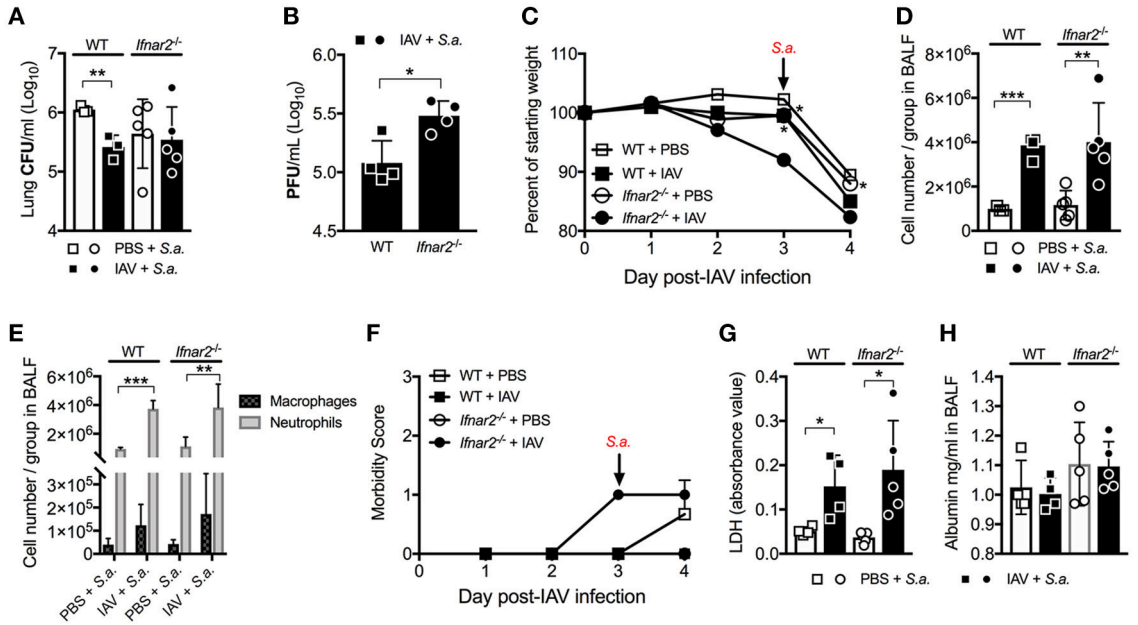

FIGURE 3 | Absence of IFNAR2 does not alter susceptibility to day 3 BSI post-IAV. WT and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0 and challenged with *S. aureus* on day 3. Lung CFUs **(A)** and PFUs **(B)** were determined 24 h post-*S.a.*-challenge. **(C)** Weights were monitored daily and are represented as percent of initial body weight depicted as average/group (Significance represents WT+IAV or *Ifnar2*$^{-/-}$ compared to *Ifnar2*$^{-/-}$+IAV). Cell number **(D)** and differential counts **(E)** were determined from cell-free BALF 24 h post- *S.a.*-challenge. **(F)** Mice were monitored and scored daily for signs of morbidity as described in methods section. Average daily score for each group is depicted. LDH **(G)** and Albumin **(H)** were analyzed in the cell-free BALF collected at 24 h post- *S.a.*-challenge. Data shown are mean ± SD results of ≥4 animals per group from one representative experiment. ***P >0.001; **P >0.01; *P > 0.05.

Ifnar2$^{-/-}$ mice showed an increase in number of neutrophils when compared to IAV-infected WT mice at that time (prior to *S. aureus* challenge; **Figure 1D**). This suggests that the increased number of neutrophils recruited during IAV and prior to BSI, may contribute to the decreased susceptibility of the *Ifnar2*$^{-/-}$ mice to BSI at day 7, as neutrophils are known to be involved in anti-*S. aureus* immunity (22, 23). Similar to BSI at day 3 post-IAV, BSI at day 7 post-IAV did not significantly increase levels of LDH (**Figure 4G**) or albumin (**Figure 4H**) in the lung. These results indicate that like IFNAR1, presence of IFNAR2 is detrimental to BSI at day 7 post-IAV. These results also suggest that response to bacterial challenge may be beneficial for the late anti-viral response in the *Ifnar2*$^{-/-}$ mice.

Ifnar2$^{-/-}$ Mice Have Decreased Inflammatory Cytokines at Day 3 and Increased IFNγ at Day 7 Post-IAV BSI

Since IFNAR2 deficiency had no effect on numbers and types of recruited cells in response to BSI either at day 3 or day 7 post-IAV, we next sought to determine whether cytokines produced by WT and *Ifnar2*$^{-/-}$ mice after BSI contribute to differences in BSI severity. In response to day 3 BSI, *Ifnar2*$^{-/-}$ mice had less IFNγ, TNFα, and IL-6 compared to BSI-WT mice, indicating that the *Ifnar2*$^{-/-}$ mice develop a less inflammatory lung environment (**Figure 5A**). The reduction in inflammatory cytokines in response to BSI at day 3 in *Ifnar2*$^{-/-}$ mice did not correspond to less cells or less damage (**Figures 3D,G**), suggesting there is another mechanism involved. Moreover, the reduction in inflammatory cytokines we found in day 3 BSI-*Ifnar2*$^{-/-}$ mice (**Figure 5A**) did not occur in *Ifnar2*$^{-/-}$ mice in response to single infection with *S. aureus* alone (**Supplemental Figure 1**). As it relates to interferon responses, the *Ifnar2*$^{-/-}$ mice also had increased IFN-α on day 3 BSI compared to WT mice. In response to day 7 BSI, *Ifnar2*$^{-/-}$ mice only had an increase in IFNγ compared to BSI-WT mice (**Figure 5B**). These results suggest that the decreased susceptibility of *Ifnar2*$^{-/-}$ mice to day 7 BSI is not due to an altered lung environment as there were no major changes in cytokine levels associated with anti-viral or anti-bacterial immunity. Together, these results suggest that the inflammatory environment on day 3 and day 7 may be more related to IAV outcome than the outcome of BSI or single *S. aureus* infection.

STAT3 Contributes to the Increased Susceptibility of *Ifnar1*$^{-/-}$ Mice, but Not *Ifnar2*$^{-/-}$ Mice to Day 3 BSI Post- IAV

IFN-β and the IFN-α's are known to have different affinities for IFNAR1 and IFNAR2 and to induce different gene expression profiles depending on their concentration and timing (11, 24). Thus, we next wanted to determine whether engagement of either IFNAR subunit by IAV resulted in induction of distinct anti-viral pathways. We found that both IFNAR subunits were required for the early expression of STAT1/2 and IRF3/7 to the levels found in WT mice (IFNAR$^{+/+}$) following viral recognition

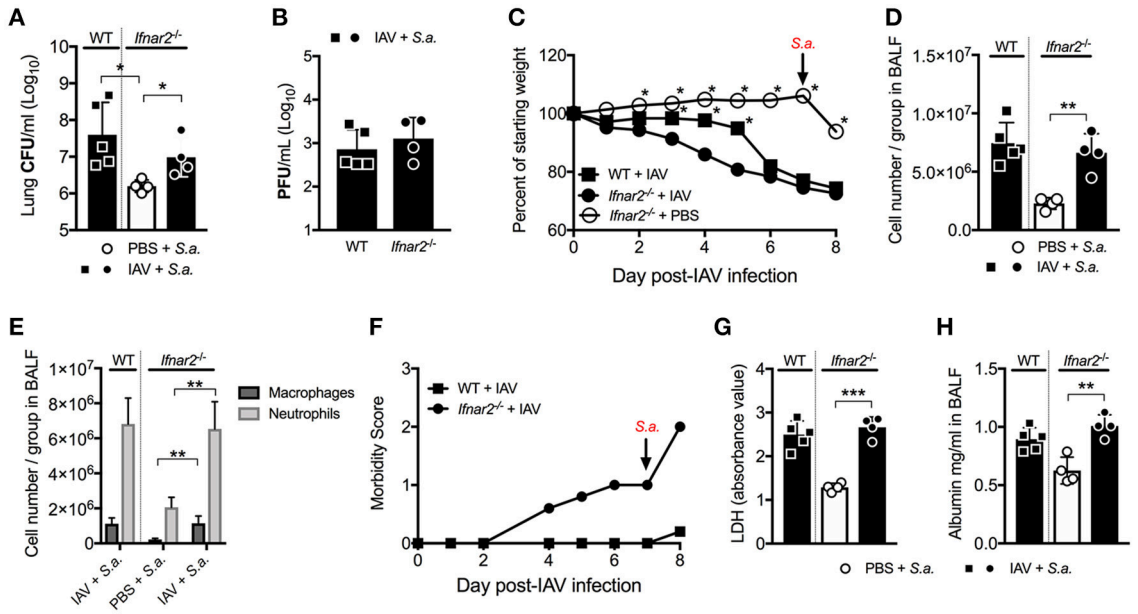

FIGURE 4 | *Ifnar2*$^{-/-}$ mice have reduced susceptibility to day 7 BSI post-IAV. WT and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0 and challenged with *S. aureus* on day 7. Lung CFUs **(A)** and PFUs **(B)** were determined 24 h post-*S.a.*-challenge. **(C)** Weights were monitored daily and are represented as percent of starting body weight depicted as average/group (Significance represents WT+IAV or *Ifnar2*$^{-/-}$ compared to *Ifnar2*$^{-/-}$+IAV). Cell number **(D)** and differential counts **(E)** were determined from cell-free BALF 24 h post- *S.a.*-challenge. **(F)** Morbidity was assessed and monitored daily. Average daily score for each group is depicted. LDH **(G)** and Albumin **(H)** were analyzed in the cell-free BALF collected at 24 h post- *S.a.*-challenge. Data shown are mean ± SD results of ≥4 animals per group from one representative experiment. ***P >0.001; **P >0.01; *P > 0.05.

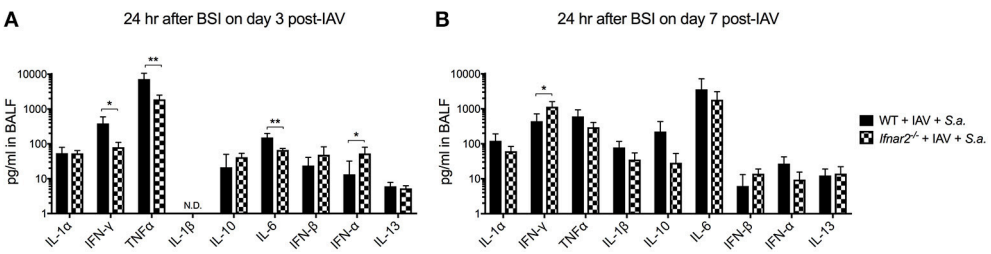

FIGURE 5 | *Ifnar2*$^{-/-}$ mice have decreased inflammatory cytokines at day 3 and increased IFNγ at day 7 post-IAV BSI. WT and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0 and challenged with *S. aureus* on day 3 **(A)** or day 7 **(B)**. Indicated cytokines were measured in cell-free BALF 24 h after *S.a.* challenge on day 3 post-IAV. N.D. is not detectable. **P >0.01; *P > 0.05.

(**Figure 6A**). Interestingly, early IAV infection in *Ifnar1*$^{-/-}$ mice resulted in a significant increase in STAT3 expression in the lung compared to both WT and *Ifnar2*$^{-/-}$ mice. This increase in expression of STAT3 in *Ifnar1*$^{-/-}$ mice corresponded to the high level of nuclear STAT3 protein found in *Ifnar1*$^{-/-}$ primary pulmonary epithelial cells regardless of IAV infection (**Figure 6B**). This indicated that STAT3 activation in *Ifnar1*$^{-/-}$ mice may have a role in early anti-viral immunity and subsequent BSI severity at day 3 in these mice. To address this possibility, we treated the IFNAR subunit-knockout mice with a STAT3 phosphorylation inhibitor (15) 6 h after IAV infection and determined their susceptibility to BSI at day 3 post-IAV. We found that P-STAT3 inhibition in both the WT and *Ifnar1*$^{-/-}$ mice resulted in reduced susceptibility to BSI, 0.5 log and 1 log (**Figure 6C**), respectively, compared to untreated littermate mice. Additionally, we found that P-STAT3 inhibition did not alter susceptibility of *Ifnar2*$^{-/-}$ mice to BSI on day 3, indicating that IFNAR1 and IFNAR2 induce disparate mechanisms in response to viral infection. Upon analyzing the cell abundances in the BALF of the WT, *Ifnar1*$^{-/-}$ and *Ifnar2*$^{-/-}$ mice we only found the P-STAT3-inhibited *Ifnar1*$^{-/-}$ mice to have decreased levels of neutrophils (**Figure 6D**). To determine whether P-STAT3 inhibition altered the anti-viral state of mice at 24 h after day 3 BSI, we analyzed their cytokine profiles. When compared to untreated littermates, the P-STAT3 inhibition did not alter cytokines produced by WT mice (**Figure 6E**), but resulted in decreased IFNγ and IL-6 in *Ifnar2*$^{-/-}$ mice (**Figure 6E**). The decreased IFNγ and IL-6 in P-STAT3-inhibited *Ifnar2*$^{-/-}$ mice

did not appear to significantly affect BSI outcome in these mice (**Figure 6C**). In *Ifnar1*$^{-/-}$ mice superinfected on day 3, P-STAT3 inhibition resulted in decreased TNFα and a 10-fold increase in IL-13 levels compared to untreated superinfected *Ifnar1*$^{-/-}$ mice. These results are consistent with our previous findings that treatment with mrIL-13 reversed the increased day 3 BSI susceptibility and resulted in decreased neutrophil accumulation in *Ifnar1*$^{-/-}$ mice (9). Therefore, our results expand our previous findings by demonstrating that STAT3 contributes to increased susceptibility of *Ifnar1*$^{-/-}$ mice to BSI at day 3 post-IAV at least in part by inhibition of IL-13 production.

IFN-β Signaling Through *Ifnar2* Rescued Mice From Morbidity and Mortality Upon Lethal IAV Infection

Our results thus far indicate that engagement of either IFNAR subunit individually differentially shapes both anti-IAV immunity and BSI susceptibility. These two IFNAR subunits are known to bind IFN-α's and IFN-β with different affinity inducing distinct gene profiles (11, 25). We found that IFN-αA, but not IFN-β inhibited production of IL-13 resulting in increased host susceptibility to BSI and that IFN-αA was involved in mediating increased susceptibility to *S. aureus* (9). Therefore, here we sought to determine whether IFN-αA and IFN-β have different effects on protection from IAV infection while signaling via distinct IFNAR subunits. IFNαA treatment of mice deficient in either IFNAR subunit (**Figure 7A** orange traces) had no effect on IAV-induced body weight loss when compared to either their untreated littermates or WT mice (open symbols). Treatment of mice that have functional IFNAR2 (*Ifnar1*$^{-/-}$ mice) with mrIFN-β (black triangles) protected these mice from morbidity and increased their survival compared to IAV-infected littermates (open triangle) or IAV-infected WT mice (open squares) (**Figures 7A,B**). Treatment of mice without functional IFNAR2 (*Ifnar2*$^{-/-}$ mice) with mrIFN-β (black circles) did not protect these mice from morbidity or mortality when compared to mrIFN-treated *Ifnar1*$^{-/-}$ mice. The mrIFN-β treatment of *Ifnar2*$^{-/-}$ mice however, did slightly but not significantly accelerate and worsen body weight loss when compared to untreated *Ifnar2*$^{-/-}$ mice (open circles). These results indicate that presence of IFNAR2 and signaling by IFN-β are sufficient to induce a protective anti-IAV state. Our results also suggest that IFN-β produced in the *Ifnar1*$^{-/-}$ and WT mice following IAV infection (**Figures 2A**, **6A**) is not sufficient to provide protection and that the protective effects only occur when IFN-β is present at the very beginning of viral infection (9).

DISCUSSION

Type I IFNs play a role in determining influenza and BSI severity. While cell surface receptor for type I IFNs consists of two distinct subunits (IFNAR1 and IFNAR2), almost all research on determining the role of type I IFNs in susceptibility to influenza and subsequent BSIs has focused solely on the involvement of IFNAR1 (using *Ifnar1*$^{-/-}$ mice). To this end our lab and others have shown that IFNAR1 is important in regulating time-dependent susceptibility to BSI during IAV infection (4, 7, 9). In the last decade, a number of reports demonstrated the ability of IFNAR1 and IFNAR2 to bind individual IFNs independent of one another, with different affinities, and subsequently causing induction of different gene (11, 24, 25). These reports alluded that the receptor subunits have distinct functions in relation to disease outcomes (25). Here, we begin to unravel how the absence of IFNAR2 affects IAV and BSI severity.

Our study revealed that IFNAR2 is required for effective anti-IAV immune responses, particularly as it relates to protection from influenza-mediated morbidity and mortality. While our results combined with work by others (5) suggest that a complete IFNAR receptor is important for protection from influenza, the accelerated morbidity and mortality, as well as increased viral burden of the *Ifnar2*$^{-/-}$ mice indicate that IFNAR2 plays a larger role than IFNAR1 in regulating the anti-viral response. That IFNAR2 deficiency resulted in increased neutrophil recruitment and an increased level of LDH production by day 7 of IAV infection further suggests that IFNAR2 may also control damage response during viral infections. Further studies are necessary to elucidate the exact mechanism by which IFNAR2 regulates anti-IAV immunity.

As it relates to host susceptibility to post-IAV BSIs, we demonstrate that IFNAR2 has both similar and distinct roles in BSI susceptibility compared to IFNAR1. Specifically, we found that IFNAR1 signaling in the absence of IFNAR2 (*Ifnar2*$^{-/-}$ mice) was sufficient to prevent the increased BSI susceptibility that we previously found to occur in *Ifnar1*$^{-/-}$ mice at day 3 post-IAV (9), suggesting that IFNAR1 alone is able to control bacterial burden more than IFNAR2 alone at day 3 post-IAV. In regards to BSI induced at day 7 post-IAV, *Ifnar2*$^{-/-}$ mice shared the same decrease in susceptibility as has been previously found for *Ifnar1*$^{-/-}$ mice (4, 9). As WT mice are more susceptible to day 7 BSI than either IFNAR subunit knockout mice, our combined results from *Ifnar2*$^{-/-}$ mice and previous results from *Ifnar1*$^{-/-}$ mice (4, 7, 9) suggest that a complete IFNAR is required for the increased susceptibility phenotype and that absence of either receptor subunit is sufficient to provide protection.

Conventional type I IFN signaling utilizes STAT1/2 heterodimer (26). A side by side comparison of intracellular signaling molecules known to be engaged in type I IFN cascade revealed preferential engagement of STAT3 upon IAV infection of *Ifnar1*$^{-/-}$, but not *Ifnar2*$^{-/-}$ or WT mice. We found that this engagement of STAT3 contributed to the increased BSI susceptibility of *Ifnar1*$^{-/-}$ mice at day 3 post-IAV. Our previous work demonstrated that the *Ifnar1*$^{-/-}$ day 3 BSI phenotype was at least in part due to the absence of IL-13 as increased susceptibility to BSI at that time post-IAV could be reversed by treating the *Ifnar1*$^{-/-}$ mice with mrIL-13, leading to a reduction in neutrophils and bacterial burden (9). Here, we found that P-STAT3 inhibition in *Ifnar1*$^{-/-}$ mice prior to day 3 BSI similarly reduced the level of neutrophil recruitment and caused a 10-fold increase in IL-13 in response to day 3 BSI. STAT3 has been previously reported to be involved in regulating inflammatory mediators and subsequent neutrophil trafficking

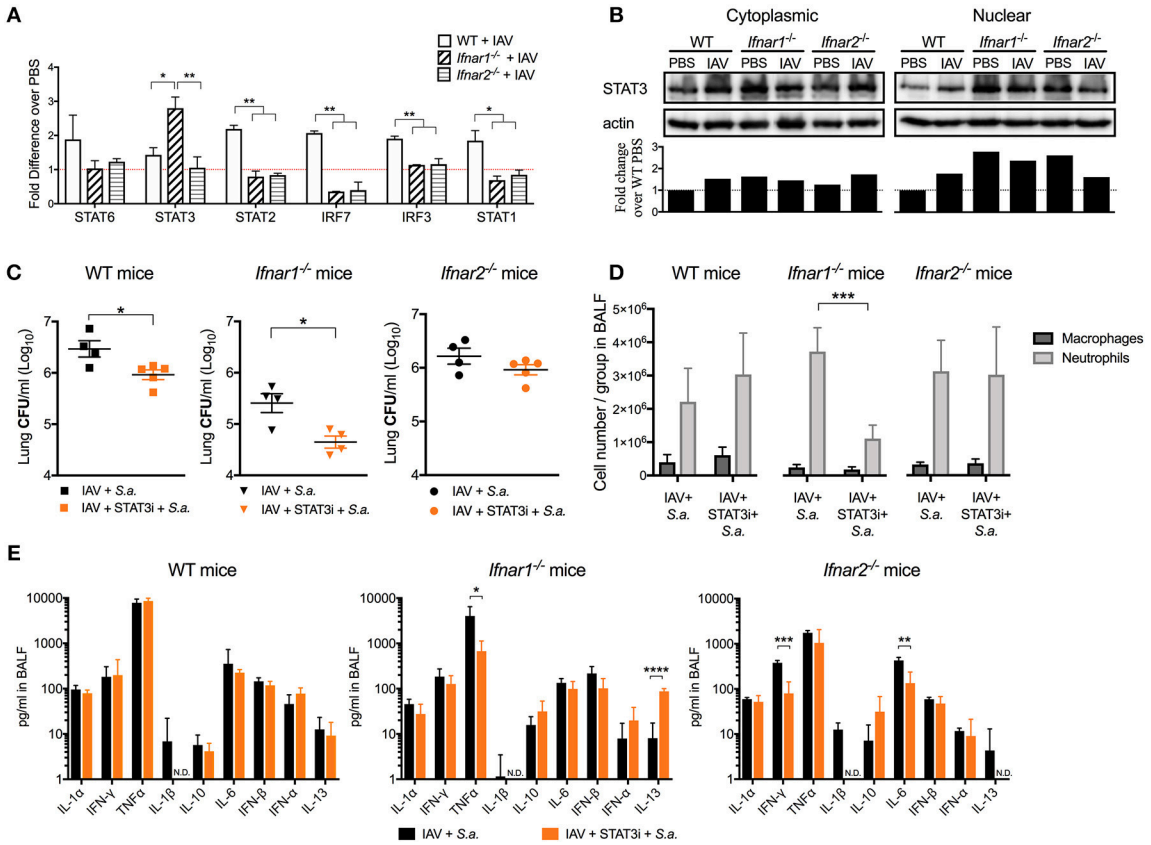

FIGURE 6 | STAT3 contributes to the increased susceptibility of day 3 BSI-*Ifnar1*$^{-/-}$ mice post-IAV. **(A)** WT, *Ifnar1*$^{-/-}$, and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0 and gene expression from RNA isolated from the whole lung was analyzed. **(B)** STAT3 protein was analyzed from cytoplasmic and nuclear fractions isolated from WT, *Ifnar1*$^{-/-}$, and *Ifnar2*$^{-/-}$ primary alveolar epithelial cells infected with IAV or inoculated PBS for 24 h. STAT3 was normalized to b-actin protein level and fold change over WT-PBS was calculated (Whole western blots: **Supplemental Figures 2–5**). **(C–E)** WT, *Ifnar1*$^{-/-}$, and *Ifnar2*$^{-/-}$ mice were infected with IAV on day 0, inoculated with STAT3 inhibitor or PBS 6 h post-IAV, and challenged with *S. aureus* on day 3. **(C)** Lung CFUs, **(D)** differential counts from the cell-free BALF, and **(E)** indicated cytokines from cell-free BALF were analyzed 24 h post- *S.a.*-challenge. Data shown are mean ± SD results of ≥4 animals per group from one representative experiment. N.D. is not detectable. ***P >0.001; **P >0.01; *P > 0.05.

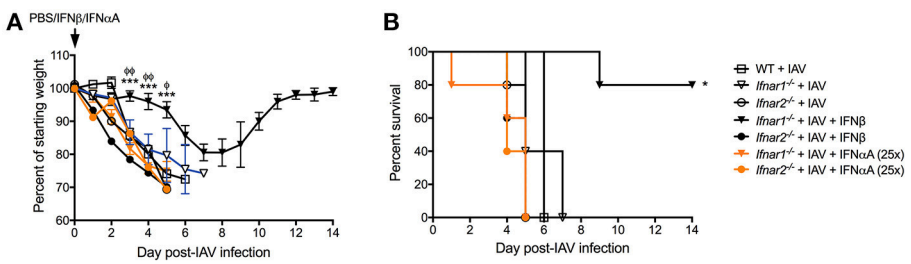

FIGURE 7 | IFN-β can rescue influenza-mediated morbidity and mortality in *Ifnar1*$^{-/-}$ mice. WT, *Ifnar1*$^{-/-}$, and *Ifnar2*$^{-/-}$ mice were infected with IAV or PBS on day 0 and treated with PBS, rIFN-β, or rIFN-αA at 0, 3, and 6 h post-IAV. **(A)** Weights were monitored daily and are represented as percent of starting body weight depicted as average/group (Significance represents *Ifnar1*$^{-/-}$+IAV+β compared to *WT or $^{\varphi}$*Ifnar1*$^{-/-}$+IAV). **(B)** Survival is represented as percent survival/group Data shown are mean ± SD results of ≥4 animals per group from one representative experiment. ***P > 0.001; $^{\varphi\varphi}P$ > 0.01, $^{\varphi}P$ > 0.05.

during infection, where inhibition of STAT3 reduced neutrophil chemokines and recruitment (27, 28). During viral infection, type I IFN signaling through IFNAR induces STAT1/STAT2 activity, but also leads to the induction of STAT3, which is thought to provide negative feedback keeping the IFN response under control (29). STAT3 deficiency was found to enhance anti-viral activity and gene expression in response to type I IFNs (30). Thus, it is tempting to speculate that in the presence

of IFNAR2, the STAT3 activation in IAV-infected *Ifnar1*$^{-/-}$ mice does not allow for induction of an anti-IAV immune response. Our data imply that STAT3 induction in *Ifnar1*$^{-/-}$ mice may be involved in preventing the increase in IL-13 that is required for controlling neutrophil recruitment and bacterial killing. How IFNAR1- or IFNAR2-induced STAT3, whether in conjunction with STAT1 or other STATs, is involved in viral and BSI susceptibility remains unknown.

Differential signaling through a single cytokine receptor is not a new concept in immunology. Results of interactions between cytokines and their cognate receptors can vary depending on the specific tissue and cellular environment, availability of the substrate and/or receptor, and affinity and avidity of the interactions (31). There are multiple examples where differential cellular processes induced by different cytokines occurs through a shared common receptor (31–33). As for type I IFNs, IFN-β, and the IFN-α's have been shown to induce distinct signaling pathways depending on their abundance, with all IFNs inducing "robust" genes (anti-viral) at low concentrations, and only IFN-β inducing "tunable" (anti-proliferative) at higher, still physiologic, concentrations (34–36). Importantly, de Weerd and colleagues established that type I IFNs are able to bind the individual IFNAR subunits independently (11). Specifically, they found that IFN-β ligates IFNAR1 independently of IFNAR2 and also the opposite, that IFN-β can ligate IFNAR2 independently of IFNAR1. However, human IFNα2, which shares high homology with mouse IFN-αA, was only able to form a stable complex with the extracellular domain of IFNAR2, which is the high-affinity portion of the IFNAR receptor. Here, we demonstrate that the morbidity and mortality associated with IAV infection of mice lacking IFNAR1 can be rescued by the administration of mrIFN-β at the time of IAV infection, but IFN-β treatment did not rescue mice lacking IFNAR2. These results suggest that the presence of IFNAR2 is required to generate a protective anti-viral response to IAV infection when stimulated with IFN-β, but that presence of IFNAR1 is not. Which response, whether it be the robust or tunable, and which of those genes are important for providing protection to IAV infection by IFNAR2 in the *Ifnar1*$^{-/-}$ mice will provide insight into how these receptor subunits are regulating the immune response to IAV. A better understanding of how the IFN-β-IFNAR2 complex in the absence of IFNAR1 can lead to protection will improve our knowledge of viral immunity.

Collectively our results demonstrate that either IFNAR subunit is sufficient for interferon signaling *in vivo*. As we began to elucidate differences in how the individual subunits shape the anti-viral immune response we found that IFNAR2 plays a non-redundant role in induction to anti-viral immunity. As such, we found IFNAR2 to be essential for both decreasing the morbidity and mortality associated with IAV infections and in altering subsequent host susceptibility to BSI. While further studies will determine the intracellular signaling mechanisms utilized by individual IFNAR subunits and whether these subunits have distinct outcomes in other viral and bacterial infections, results presented here set a stage for these mechanistic studies by emphasizing the importance of understanding the contributions of the entire receptor to disease outcomes.

ETHICS STATEMENT

All animal experiments were approved by the Montana State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KMS designed and performed experiments, analyzed data, and contributed to writing of the paper. KL and LJ designed and performed experiments and analyzed data. KS, HC, JW, and HH performed experiments. AR-A designed experiments, analyzed data, and contributed to writing of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02589/full#supplementary-material

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REVIEW
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Contribution of Dendritic Cell Responses to Sepsis-Induced Immunosuppression and to Susceptibility to Secondary Pneumonia

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Dendritic cells (DCs) are bone marrow derived cells which continuously seed in peripheral tissue. During infection, DCs play an essential interface between innate and adaptive immunity. Pneumonia is a lung inflammation triggered by pathogens and is characterized by excessive release of inflammatory cytokines that activate innate and acquired immunity. Pneumonia induces a rapid and protracted state of susceptibility to secondary infection, a state so-called sepsis-induced immunosuppression. In this review, we focus on the role of DCs in the development of this state of immunosuppression. Early during inflammation, activated DCs are characterized by decreased capacity of antigen (cross)- presentation of newly encountered antigens and decreased production of immunogenic cytokines, and sepsis-induced immunosuppression is mainly explained by a depletion of immature DCs which had all become mature. At a later stage, newly formed respiratory immature DCs are locally programmed by an immunological scare left-over by inflammation to induce tolerance. Tolerogenic Blimp1+ DCs produce suppressive cytokines such as tumor growth factor-B and participate to the maintenance of a local tolerogenic environment notably characterized by accumulation of Treg cells. In mice, the restoration of the immunogenic functions of DCs restores the mucosal immune response to pathogens. In humans, the modulation of inflammation by glucocorticoid during sepsis or trauma preserves DC immunogenic functions and is associated with resistance to secondary pneumonia. Finally, we propose that the alterations of DCs during and after inflammation can be used as biomarkers of susceptibility to secondary pneumonia and are promising therapeutic targets to enhance outcomes of patients with secondary pneumonia.

Keywords: dendritic cells, pneumonia, inflammation, immunity, innate, mucosal immunity, steroids, intensive care units

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Lung infection is a one of the main cause of mortality and morbidity worldwide (1). The overall death rate for patients with such infections was 2.6 million deaths worldwide in 2015, which is the leading infectious cause of death (2). However, the consequences of these infections cannot be reduced to the direct mortality from primary infection. Indeed, in critically ill patients recovering from a first severe sepsis (e.g., pneumonia or peritonitis), the risk for developing pneumonia reaches 30 to 50% (3)

and in critically ill patients cured from primary pneumonia the early relapse with the same pathogen is up to 20%. (4). One of the main hypothesis to explain this susceptibility to infections is that patients with severe sepsis acquire a state of immunosuppression as evidenced by different host response during community-acquired and hospital-acquired pneumonia (5).

SEPSIS INDUCED IMMUNOSUPPRESSION

Development of severe immune defects in immune-competent septic patients, a phenomena so-called "sepsis-induced immunosuppression" (6) has been associated with the risk of secondary pneumonia. During sepsis, the production and the release of pro-inflammatory cytokines is a necessary physiological phenomenon that activates the defense against bacterial infections and ensures injured tissue healing. To limit the risk of immunopathology observed during an overwhelming systemic inflammatory response syndrome (SIRS), whose main complication is a multi-organ failure syndrome (7), innate immunity cells rapidly develop a systemic compensatory anti-inflammatory response (CARS). This CARS aims to restore the state of immune homeostasis but either its prolongation or its exacerbation leads to an increased susceptibility to infections(6, 8).

So far, the main features of this sepsis-induced immunosuppression are

1. *A decreased antigen presentation ability by antigen presenting cells (APCs).* APCs, mainly Dendritic Cells (DCs) and monocytes, have a central role in the capture, in the processing and in the presentation of antigens to effector lymphocyte T cells. These functions, essential for the establishment of an inflammatory response, are altered for weeks in mice and humans cured from systemic inflammation (9, 10)
2. *Dysregulation of the secretions of cytokines.* During infection, cytokines are messengers which ensure the coordination of all the cellular families. For example, APCs shape the response of effector T cells and innate-lymphoid cells to immunity or tolerance *via* the secretion of pro- or anti-inflammatory cytokines (e.g., Interleukin-12 or TGF-β). In critically ill patients, a decreased production of pro-inflammatory cytokines (such as TNF-α and IL-12) associated with a blunt release of anti-inflammatory cytokines (IL-10, TGF- β) have been associated with altered levels of pattern recognition receptors (11) epigenetic modifications (12) and post-transcriptional regulations.
3. *T cell exhaustion and apoptosis:* Exhaustion corresponds to the progressive loss of effector functions of T cells in the presence of a high antigenic load (13), while excessive inflammation results in caspase-3-dependent apoptosis (14, 15).

The capacity of DCs to detect environmental changes, to produce cytokines and present antigens to T cells suggests that they are a corner-stone of the physiopathology of the susceptibility to secondary pneumonia. Indeed, type 1 DCs (cDC1s) which are a highly potent cytokines secretion subtype of DCs, are a major source of IL-12 and hence promote NK and NKT cell IFN-γ production during systemic bacterial or viral infections (16). Mouse models of primary pneumonia (e.g., due to pneumococcal infection) have demonstrated a critical role for the activation of NK and iNKT in mediating the innate immune response to pulmonary infection (17) and especially in post-influenza bacterial secondary pneumonia (18, 19). In this review, we will thus focus on the fate of bona fide DCs (i.e., DCs not derived from monocytes) during and after sepsis, and will highlight the effects of glucocorticoids which are the first efficient immunotherapy in severe sepsis (20).

DENDRITIC CELLS LIFE-CYCLE BEFORE, DURING AND AFTER ACUTE INFLAMMATION

Dendritic cells are bone marrow derived cells which play an essential interface between innate and adaptive immunity. DCs, which are the most potent antigen presenting cells (APCs), are involved in the initiation and the regulation of T cell-dependent immune response (21). According to the microenvironment and the signaling, DCs can secret pro-inflammatory cytokines to fight against infection or anti-inflammatory cytokines to maintain tolerance to self-tissue.

Before acute inflammation, DC precursors (pre-DCs) continuously leave the bone marrow as precursors and colonize peripheral tissues and lymphoid organs (e.g., spleen) where they develop into fully functional immature DCs (22). DCs are classified in different subsets: "plasmacytoid DCs" (pDCs) are the main source of type 1 interferons during many viral infections; the "conventional DCs" (cDCs), including mouse CD8+ cDCs and CD11b cDCs, have high antigen-presentation capacity and mainly produce other pro-inflammatory cytokines. In mice and human, two lineages of cDCs are clearly identified by differential expression of Xcr1 and Sirpa (23, 24) which recently allowed proposing a unified nomenclature of DCs across tissues and species, namely cDC1s and cDC2s, respectively (25). Indeed, the expression of CD141 (thrombomoduline) and CD1c (BDCA1) enable the distinction of two populations of Human DCs (26). The gene-expression profiles and functions of CD141+ cDCs and of CD1c+ cDCs resemble those of mouse cDC1 and cDC2 respectively (27, 28). cDC subsets are functionally well characterized: both cDC1s and cDC2s efficiently present extrinsic antigens on the MHC-II complex to CD4 T cells, although cDC2s appear to be more efficient for that function, cDC1s excel in antigen cross-presentation (presentation of extrinsic antigens to CD8 T cells on the MHC-I complex), although the other DC subsets can also exert this functions under specific conditions (29).

DCs can be further classified according to their organ localization and their migratory capacity: (1) the migratory DCs (including cDCs) are localized in peripheral tissues and migrate to the draining lymph nodes upon activation where they can exert their function of antigen presentation (for example Langerhans cells and dermal DCs), whereas (2) the resident DCs (including pDCs and cDCs) which remain in lymphoid organs where they

locally collect Ag, (including from migratory DCs) to act as an amplificatory signal for T cell priming (for example thymic cDCs and splenic cDCs) (30). The classification proposed by Guilliams et al can integrate multiple layers of information in the denomination of DC subsets while still preserving a unifying nomenclature for their lineage belonging: for example, mouse spleen resident CD8a+ cDCs can be called "spleen resident CD8a+ cDC1s," and the mouse CD103+ cDCs that have migrated from the skin into the cutaneous lymph node can be called "mouse CLN migratory CD103+ cDC1s."

In steady state, the DCs have low expression of major histocompatibility complex class-II (MHC-II) and of membrane costimulatory molecules (such as CD86). DCs thus have high endocytic function for capturing Pathogen or Danger-associated molecular patterns (PAMPs or DAMPs), but are incompetent to present newly encountered antigens on MHC II molecules and to prime T-cells (31).

In the absence of infection, antigens presented by DCs silence effector T cells either by inducing apoptosis or by expanding regulatory T cells (32). This phenomenon has been recently better understood. In steady state, in contrast with DC maturation during inflammation, the maturation of migratory DCs (involving a novel NF-κB-regulated gene network) is associated with the induction of tolerance rather than T cell priming and activation (33). This process of terminal differentiation of steady state DCs is called "homeostatic maturation." Some authors suggest that the signals triggering homeostatic, tolerogenic, DC maturation are conveyed via multiple pathways, some overlapping in part with those triggering inflammation but also leading to the expression of a specific transcriptional genetic program (34, 35). This homeostatic maturation leads to tolerogenic DC which promote the expansion of regulatory T cells (Treg) and tolerance to self-antigens (36).

During infection, the maturation of DCs is induced by the detection of PAMPs (*direct* activation) and by inflammatory cytokines released by other activated immune cells (*indirect* activation) (37). Direct activation of DCs induces several conformational and functional changes: (1) DCs become efficient at presenting the antigens by transient upregulation of MHC II synthesis (38); (2) they secrete cytokines for T cell polarization. Directly activated DCs are thus competent to prime naive T cells but they lose the ability to process and to present newly encountered antigens (9).

DCs can also be indirectly activated by inflammatory cytokines produced by PAMP-stimulated immune and epithelial cells (e.g., IFN-α/β, TNF-α …) (39). The levels of MHC-II and of co-stimulatory molecules are increased on the surface of indirectly activated DCs. Indirectly activated DCs can prime naive T cells like directly matured DCs, however their cytokine secretion function is altered and they retain the capacity to process new antigens (40, 41). During inflammation, directly and indirectly-activated DCs coexist and could theoretically be selectively targeted by interventions aiming to restore immune competence after inflammation.

Protracted impairment of antigen presentation and of cytokine production in DCs of mice and patients cured from acute inflammation have been reported (42). Yet, new DCs continue to be produced after the onset of sepsis and inflammation with similar rates as in healthy conditions (43). Thus, after a few days, bodies cured from inflammation are seeded by newly formed immature DCs which are supposed to be fully functional. However, the susceptibility to infections last for weeks in critically ill patients cured from SIRS, and paralyzed DCs are still observed weeks after the cure from infections. If the paralysis of DCs lasts for weeks after inflammation, two periods can be distinguished: an early stage corresponding to the inflammatory response, and a later one lasting several weeks, probably months, after resolution of SIRS and characterized by an apparent return to non-inflammatory conditions but persistent dysfunctions of DCs. An important consideration when aiming to restore immune-competence during and after sepsis is to differentiate the mechanisms of DCs alterations during these two stages (**Figure 1**).

DECREASED NUMBER OF IMMATURE DCS

The early decreased ability to present new antigens by the direct activation of DCs is not deleterious during local infections because a small number of DCs encounters the infecting pathogen and becomes activated, while the numerous remaining immature DCs can respond to new challenge. However, systemic circulation of PAMPs and of inflammatory mediators during sepsis causes systemic activation of DC, reducing the number of immature DCs capable of mounting an effective response to new threats, and limiting the ability of innate immunity to prime T cell responses (9, 44). The simultaneous activation of an excessive number of cDCs during systemic inflammation depletes the body from fully functional DCs and is thus immunosuppressive. Moreover, the total number of migratory and resident DCs is decreased following lung inflammation (45). The depletion of circulating DCs is reported in murine models of sepsis by caecal ligation and puncture (46) and the number of splenic DCs is decreased in patients dying from severe sepsis in intensive care units (15). Early after a lung infection by influenza virus, the presence of DCs in the lung was reduced (17, 47). Currently, the mechanisms of these "DC-penia" have not been fully elucidated. Some authors describe a defective *de novo* formation of DCs from common progenitors in the bone marrow (48) when others describe apoptotis mechanisms (46, 49, 50) or lysis by regulatory innate like lymphocytes (51). The mechanism involved in DCs apoptosis after SIRS is still unclear but a study has shown that an enzyme called acid sphingomyelinase (A-SMase), which is activated when DCs are treated with high numbers of *Escherichia coli,* induces apoptosis (52). The clearance of apoptotic DCs by viable DCs induces antigen-specific Tregs cells, and is thus probably beneficial to prevent auto-immune diseases (53). In addition to inducing immunosuppression by reducing the number of DCs, this phase of apoptosis could also induce a tolerogenic microenvironment maintaining this immunosuppressive state (54). The prolonged decrease in the number of circulating cDCs and pDCs has been associated with

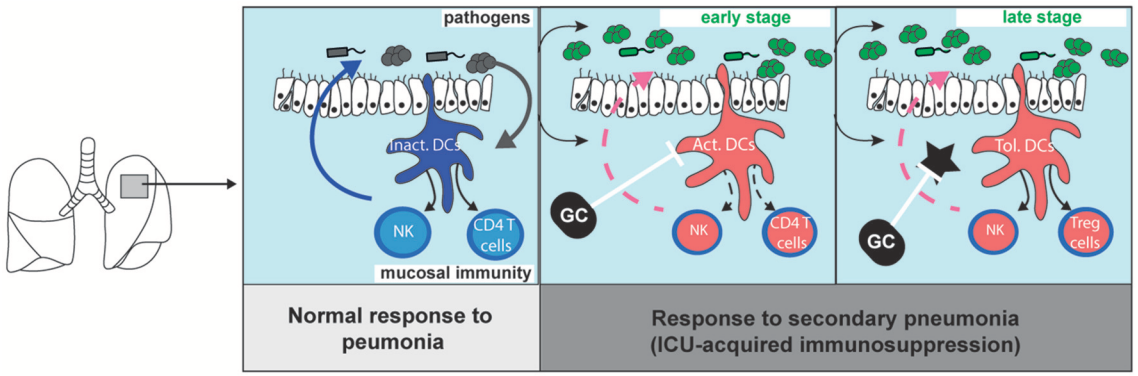

FIGURE 1 | Migratory dendritic cell response during primary pneumonia, and during sepsis-induced immunosuppression (left). The stimulation of immature migratory dendritic cells (im.DCs) by pathogen-associated molecular patterns induces the production of inflammatory cytokines (such as Interleukin-12) which stimulate innate-like lymphocyte and natural killer cell (NK) functions and primes naive CD4 T cells. During sepsis-induced immunosuppression (middle and right panels), bacterial clearance is decreased as compared to what is observed during primary pneumonia. (middle) Early after primary infection, activated DCs (Act.DCs) are unable to respond to subsequent pathogens, and fail to produce cytokines and prime new CD4 T cells. (right) Lately, newly formed DCs locally acquire a tolerogenic programing (Tol. DCs) upon instruction by local tolerogenic mediators (star). Glucocorticoid (GC) inhibits DC activation and limit the SIRS. Upon stimulation by pathogens, Tol-DCs do not activate NK cells but induce the local accumulation of Treg cells. GC inhibits tolerogenic mediators and can restore immunogenic functions of newly formed DCs.

the risk of secondary infection in septic patients (55). This critical loss of DCs, which has also been associated with secondary pneumonia in burned patients (56) and in brain-injured patients (57), is probably a mechanism common to all the critical illness-inducing immunosuppression.

cDCs are continuously renewed from bone marrow pre-DCs and have a dependence for FLT3L/FLT3 (58). In the case of IAV infection, it seems that the drop of cDC number in the lungs is due to a defective FLT3L production (47). One the other hand, some DC-like cells, such as the mo-DCs (monocytes-derived DCs), are derived from monocytes in a GM-CSF dependent mechanism. In case of inflammation, an increase in the proportion of mo-DCs, which are more susceptible to polarization toward immunosuppressive functions by the local microenvironment, is also a cause of "sepsis induced immunosuppression." Indeed, these mo-DCs have also been reported to induce TH2 and TH17 responses (59, 60). Sepsis-induced immunologic dysregulation occurs at every level of the ontogeny of each subset of DCs (61). Considering these results, several teams have hypothesized that the correction of the number of DCs after inflammation, notably by injecting FLT3L which is the DC growth factor, can restore immune-competence and limit the susceptibility to secondary pneumonia (47, 62, 63). To the best of our knowledge, the effects of FLT3L have never been investigated in septic patients, but GM-CSF, which is not specific to DCs but accelerates DC maturation, demonstrates disappointing effects in patients with sepsis (64, 65).

Patients lacking cDC2 due to IRF-8 genetic mutations are susceptible to infections (66). It is thus likely that lack of cDCs participates to the susceptibility to secondary infections, and functional defects of newly formed DCs can be of importance when aiming to restore a DC network after sepsis.

FUNCTIONAL ALTERATIONS OF THE NEWLY FORMED DCS

Bone-marrow released pre-DCs reach peripheral tissue where they receive final differentiation messages and become fully functional. This final tissue maturation process explains the diversity of DC populations observed in the different organs in normal conditions and is called tissue-imprinting. It was recently shown after sepsis that the newly formed DCs are modulated both in the bone-marrow at a progenitor state (67) and locally in peripheral tissue at a final differentiation state by an immunological scare left-over by a primary inflammation response (10, 68). DC-precursors exposed to this new microenvironment are deficient for their capacity to produce IL-12, due to epigenetic alterations (69), impaired antigen (cross)-presentation capacity, and preferentially drive T cellular immunity to tolerogenic functions (10).

Several mediators of this suppressive-microenvironment left-over by primary sepsis have be demonstrated to be important as will be detailed below.

Blimp-1

B lymphocyte-induced maturation protein-1 (Blimp-1) is a pleiotropic transcriptional factor which represses the IFN-β promoter and regulates functions of many immune cells, especially in lymphocytes (B and T cells). Blimp-1 is also expressed and functionally important for the myeloid lineage cells such as DCs and macrophages (70). The tolerogenic functions of Blimp-1 on DCs are well demonstrated in systemic autoimmune diseases, such as systemic lupus erythematosus. Mice with a Blimp-1ko phenotype in all CD11c-expressing cells including DCs (Blimp-1flox/flox; CD11c-CRE+) present an increased secretion of interleukin 6, an increased differentiation of effector T cells and suffer from the development of a lupus-like

syndrome (71). Likewise, Blimp-1 regulates cDC2 homeostasis by preventing the excessive production of pro-inflammatory cytokines and overwhelming expansion of cDC2s after TLR stimulation (72). Blimp-1 could also be involved in SIRS and may be partly responsible for the observed susceptibility of patients to nosocomial pneumonia. We showed that cDC2s from patients suffering of SIRS expressed a high level of Blimp1 compared with healthy donors and thus lose their ability to produce type 1 cytokines (including interleukin-12) (10). Blimp-1 expression is also increased in DCs from patient suffering of post-trauma SIRS whose physiopathology is similar to sepsis and who are also susceptible to secondary pneumonia (73). In trauma patients, the increased expression of Blimp-1 has been correlated with the trauma severity (Glasgow Coma Scale) and with respiratory complications in intensive care unit (10). The overexpression of Blimp1 in cDC2s of critically ill patients recovering from a primary pneumonia might be a marker of the severity of immunosuppression and may thus allow identifying and treating early the patients at high risk of severe secondary infections.

Interleukin (IL)-10

Numerous cell types, including NK cells, B cells, monocytes and DCs, were shown to produce IL-10 during "sepsis induced immunosuppression" (74).

IL-10 induces the apoptosis of mature DCs during chronic viral infections (75) and decreases the number of live DCs during post-traumatic pneumonia (76). In response to IL-12 secretion by mature DCs, NK cells rapidly express IL-10 which inhibits the production of IL-12 by DCs to prevent an overwhelming and deleterious immune response (51). For example, IL-10 neutralization by anti-IL-10 mAb restores the production of inflammatory cytokines, such as IL-12 and TNF-α, by DCs (77). During systemic infection, IL-10 inhibits the maturation of DCs and impairs the ability of cDC1s to prime a T cell response. This autocrine IL-10 regulation limits the development of new mature DCs (78) and limits the capacity of mature DCs to initiate Th1 responses. Immunosuppressive IL-10^{+} DCs induce Th2 response by stimulating cytokine secretion like IL-4 and "regulatory DCs" secreting IL-10 are also associated with up-regulation of T regulatory cells (T-reg). This regulatory mechanism is notably involved in hyper-eosinophilic airway inflammation (79, 80). IL-10 secretion is an essential component for the protective response against airway hyper reactivity and asthma (81) and is involved in development of lung tolerogenic DCs after pneumonia (82).

Tumor Growth Factor-Beta: TGF-β

TGF-β molecules act as cellular switches regulating numerous physiological processes such as immunity, cell renewal and healing. TGF-β is a pleiotropic cytokine involved in the development of Treg lymphocytes by inducing the *Foxp3* transcription factor expression in CD25^{-} naive T cells in order to enforce the transition to Treg cells (61). TGF-β are expressed constitutively by a wide variety of cells in the lung, including myeloid cells (DCs and alveolar macrophages), T cells and fibroblasts (83).

TGF-β are produced as inactive proprotein composed of mature TGF-β bound to latency-associated peptide. TGF-β activation from latency is controlled by numerous pathways that include actions of proteases present in the microenvironment such as plasmin, and/or by thrombospondin 1 or selected integrins expressed at the membrane of cDCs (84, 85). The unusual temporal discontinuity of TGF-β synthesis and action is an original mechanism which allows the TGF-β/LAP complex to behave as a matrix-localized sensor. During sepsis-induced immunosuppression, DCs are thus both a source and an activator of TGF-β in the tissue of mice cured from pneumonia (10). Our previous results indicate that cDCs of mice recovering from lung infection produce TGF-β and induce Treg cell accumulation (10). When they are activated by TGF-β after primary pneumonia, these Treg cell decrease the pro-inflammatory cytokine secretion pattern and the upregulation of CD80 and CD86 costimulatory molecules of immature cDCs, creating a tolerogenic environment (86). This mechanism is also found in intestinal epithelium where intestinal DCs promote a tolerogenic environment via TGF-β secretion to prevent an exacerbated response against the many non-pathogenic antigens in the gut (87). The crucial role of TGF-β in self-tolerance has long been established, with genetic deletion of TGF-β inducing multifocal inflammatory disease (88) or with the TGF-β down-regulation of co-stimulatory molecules expression on the surface of DCs limiting the functions of T cell effectors in the epidermis (89). The DCs-Treg cells-TGF-β loop plays a central role in the susceptibility to hospital-acquired pneumonia observed after severe infections.

GLUCOCORTICOIDS & DENDRITIC CELLS

Lately after a primary lung inflammation, newly formed DCs receive tolerogenic messages during terminal differentiation in the tissue, and local imprinting drives DCs toward a new tolerogenic transcriptional programing (**Figure 1**). Tolerogenic DCs fail to develop immunogenic functions in response to subsequent infectious threats, and bacterial clearance is decreased during secondary pneumonia. Host-targeted approaches aiming to modulate the lung imprinting of DCs have the potential to restore immune competence after sepsis, and to decrease the risk of secondary pneumonia. Yet, specific interventions, such as the injection of blocking anti-IL-10 or anti-TGFβ antibody, have not been tested for the prevention of hospital-acquired infections in patients, probably because of safety concerns.

Glucocorticoids for the modulation of inflammatory-induced immunosuppression have been extensively tested in humans. Recent randomized studies have demonstrated that glucocorticoids decrease the risk of death of patients with septic shock (20) or with community acquired pneumonia (90). Low doses of steroid also prevent hospital-acquired pneumonia in severe trauma patients (91). It can seem counterintuitive to use drugs classically considered as immunosuppressive in patients with severe infections or at high risk of sepsis. Indeed, glucocorticoids are highly anti-inflammatory molecules (92) and steroids have long been indicated for the management of patients suffering from non-septical inflammatory diseases such as rheumatoid arthritis or systemic erythematous lupus (93), and

for the induction of tolerance to graft (94). A reappraisal of the immunological effects of steroids during acute inflammation, and a better comprehension of the impacts of inflammation on the development of immune response to secondary infections, have provided the rational to explain these clinical observations. We propose that steroids prevent the excessive activation of DCs during the severe inflammatory stage (**Figure 1**, middle panel) and limit the alterations of DCs observed during the late stage of sepsis-induced immunosuppression (**Figure 1**, right panel).

It has been long known that the Hypothalamic-Pituitary Adrenal (HPA) axis, and in particular glucocorticoids, is a major component of the response to sepsis (95), as demonstrated by the susceptibility of adrenalectomized mice to septic shock (96). Endogenous glucocorticoid (i.e., cortisol), as well as therapeutic glucocorticoids (i.e., dexamethasone), control many essential metabolic, cardiovascular, and homeostatic functions during inflammation. These numerous effects results from the pleiotropic activity of the glucocorticoid receptor (GR) on multiple gene promotors and on multiple target cells (94). Multiple GR isoforms exist (including the main GRα and β receptors) with distinct tissue distribution patterns and functions. The activated glucocorticoid–glucocorticoid receptor-alpha (GC-GRα) complex acts at the intra-cytoplasmic level to reduce the post-transcriptional expression of pro-inflammatory cytokines and to increase the transcription of anti-inflammatory and tolerogenic genes (94).

Endogenous or synthetic glucocorticoids particularly influence the innate immune cells during the inflammation period. One of the main targets of glucocorticoids are innate lymphoid cells and the neuroendocrine axis is crucial for tolerization of the innate immune system to microbial endotoxin exposure through direct corticosterone-mediated effects on innate cells (97). Glucocorticoids also modulate DCs during and after inflammation (98). *In vitro,* exogenous GCs at therapeutic concentrations inhibit the differentiation of DCs from their precursor cell (99), and limit their activation by PAMPs/DAMPs (100). GCs induce apoptosis of mature migratory DCs *in vivo* and *in vitro* (101). Interestingly, many studies have demonstrated that glucocorticoids suppress mature DCs but spare immature DCs *via* a differential expression of GR translational isoforms (102, 103) and the activation of cell survival pathways (104). Endogenous glucocorticoid elevation following pneumonia participates to the clearance of mature pro-inflammatory cDCs and to the development of tolerogenic DCs (105–107). In humans suffering from septic shock, GC restores MHC-II expression on myeloid cells, suggesting a better antigen presentation by APCs during treatment (108). During viral pneumonia, the initial hypercorticism limits the inflammatory-induced lung injuries and prevents mortality during bacterial superinfection (109). This protective effect (108) is notably mediated by direct effect of GC on the cytokine production by DCs since the conditional deletion of GR in CD11c+ cells prevents mice from death upon LPS stimulation. These results suggest that glucocorticoids are necessary to control the initial inflammatory response, limiting the initial shortage on immature DCs, and limiting the local imprinting which induces the formation of tolerogenic DCs for weeks after the primary pneumonia.

CONCLUSION

Clinical and bacterial cure failures are common in patients treated for pneumonia, and the susceptibility to secondary infection is high. These observations have been linked to the development of sepsis-induced immunosuppression. Acquired alterations in the numbers and functions of respiratory DCs are crucial in this condition. To develop targeted-host approaches, it is necessary to closely consider the timing of the interventions. A loss of immature DCs is the main mechanisms during the early phase, and alterations of the terminal maturation of newly formed DCs participate to the month-long susceptibility to secondary pneumonia. To treat the sepsis-induced immunosuppression, and limit the susceptibility to secondary pneumonia, many therapies have been tested in recent years. They aimed either to limit the initial SIRS (and thus the CARS) in particular by the use of low dose glucocorticoids (20, 91, 110) or to restore or supplement the secretion of pro-inflammatory cytokines by the injection of IFN-γ, GM-CSF (110) or interleukin-12 (10).

Using exogenous glucocorticoid at early phase of sepsis may limit the immune paralysis by decreasing the number of tolerogenic mature DCs and by limiting the development of a tolerogenic trained innate immunity.

AUTHOR CONTRIBUTIONS

MB and AR wrote the draft. KA extensively reviewed the manuscript. All the authors approved the manuscript before the submission

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REVIEW
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Respiratory Viral Infection-Induced Microbiome Alterations and Secondary Bacterial Pneumonia

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Influenza and other respiratory viral infections are the most common type of acute respiratory infection. Viral infections predispose patients to secondary bacterial infections, which often have a more severe clinical course. The mechanisms underlying post-viral bacterial infections are complex, and include multifactorial processes mediated by interactions between viruses, bacteria, and the host immune system. Studies over the past 15 years have demonstrated that unique microbial communities reside on the mucosal surfaces of the gastrointestinal tract and the respiratory tract, which have both direct and indirect effects on host defense against viral infections. In addition, antiviral immune responses induced by acute respiratory infections such as influenza are associated with changes in microbial composition and function ("dysbiosis") in the respiratory and gastrointestinal tract, which in turn may alter subsequent immune function against secondary bacterial infection or alter the dynamics of inter-microbial interactions, thereby enhancing the proliferation of potentially pathogenic bacterial species. In this review, we summarize the literature on the interactions between host microbial communities and host defense, and how influenza, and other acute respiratory viral infections disrupt these interactions, thereby contributing to the pathogenesis of secondary bacterial infections.

Keywords: gut microbiome, respiratory viral infection, bacterial pneumonia, viral-bacterial interaction, influenza, host-microbe interaction

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INTRODUCTION

Influenza and bacterial pneumonia are the leading cause of morbidity and mortality from infectious diseases worldwide. Influenza and other respiratory viral infections predispose patients to secondary bacterial super-infections, which are frequently associated with a more severe clinical course. It is estimated that the so-called "Spanish Flu" pandemic of H1N1 influenza A virus from 1918 to 1919 resulted in more than 50 million deaths, with many caused by bacterial super-infection leading to secondary pneumonia (1–7). Even in the antibiotic era, over half of patients with severe infections in the 1957 H2N2 and 1968 H3N2 pandemics had bacterial complications (8–10). Bacterial co-infection was also detected in ∼30% of cases in the 2009 H1N1 pandemic, with high mortality rates despite administration of appropriate antibiotics (11–18). Thus, it is evident that a better understanding of the pathogenesis of secondary bacterial pneumonia following viral infections is needed in order to make therapeutic strides for this devastating complication.

The mechanisms of post-viral bacterial infection are complex, comprising multifactorial processes mediated by interactions between viruses, bacteria, and the host immune system.

The pathogenesis of super-infection has been attributed to direct mucosal/epithelial damage by influenza virus, increased bacterial colonization of the upper and lower respiratory tracts (URT and LRT, respectively), and dysregulation of immune responses, which all lead to increased susceptibility to secondary bacterial infections. However, emerging evidence suggests that our microbial communities residing on our mucosal surfaces likely shape the rigor of our immune responses and shape the ecological relationships between host and pathogens. Over the past 10 years, intense interest has focused on examining how the microbial communities which inhabit our bodies—which some consider to be a separate "organ system" given the sheer physical bulk, number of genes, and metabolic activities—govern the balance between health and susceptibility to diseases, including infections. This raises the possibility that disruptions in the normal microbial communities by an acute viral infection might contribute to the development of post-viral bacterial pneumonia.

The recent development of culture-independent methods of microbial identification has enabled the study of microbial communities on mucosal surfaces of the human body, referred to as "microbiota." The microbial communities of mammalian hosts are diverse, comprised of bacterial, viruses, archaea, parasites, and fungi. The Human Microbiome Project (HMP) and other similar large-scale sequencing projects worldwide have characterized the distinct microbial communities that have adapted to the unique environmental niches within our bodies, such as the gut, skin, airways, genitourinary tract, and oral cavity. The gut microbiome, in particular, has been shown to play an integral role in shaping the immune system starting early in life, with continued influence on priming the nature and robustness of immune responses throughout one's lifetime. The respiratory tract also harbors distinct communities of microbes, with multiple discrete ecological niches (e.g., nasal cavity, oropharynx, upper airways) that vary in terms of temperature, pH, oxygen tension, mucus production, and other factors.

The effects of viral infections on both the gut and respiratory microbiome have recently undergone examination. Surprisingly, influenza infection has been found to result in significant changes in the gut microbiome, despite the lack of detectable virions in the GI tract. By comparison, the effects of viral infection on the respiratory microbiome appear to be relatively modest, but detectable. While the effects of these alterations on risk of secondary bacterial pneumonia have not been studied, potential mechanisms by which these changes might modulate susceptibility to secondary bacterial infections include alterations in the nature and magnitude of the immune response in the host (microbiome on host effects) and facilitating growth of pathogens in the absence of normal commensals (inter-microbial effects). In this article, we review the current understanding of how alterations in the microbiome following viral infection might alter host immune responses and increase susceptibility to secondary bacterial infections. Although the term "microbiome" encompasses all microbial communities, there is currently a paucity of studies on how the mycobiome (fungal microbiome) and the virome (viral microbiome) affect host defense against respiratory infections and vice-versa; thus, this review will focus on the bacterial microbiome literature.

THE GUT MICROBIOME AND RESPIRATORY INFECTIONS

Of the niches in the body, the gut microbial community has been the most intensively studied, with over 20,000 publications to date. While the virome and mycobiome (fungi) are also being analyzed, the bulk of the literature has focused on the bacterial component of the microbiome, and thus most of our understanding of the relation of the gut microbiome to host immunity and pathogenesis of chronic diseases comes largely from studies of the bacterial community. During health, the human gut bacterial community is diverse, with each individual harboring over 100 trillion bacteria, comprised of over 150 different species. The gastrointestinal microbiota is dominated by Firmicutes (e.g., *Lactobacillus*, *Bacillus*, and *Clostridium*) and Bacteroidetes (e.g., *Bacteroides*), with lower abundances of Proteobacteria (e.g., *Escherichia*) and Actinobacteria (e.g., *Bifidobacterium*) (19, 20). Wild-living mice exhibit more diverse microbiomes, with significant abundance of Proteobacteria as well as Firmicutes and Bacteroidetes (21). The gut microbiome, in addition to its metabolic functions in the host, plays an integral role in the development, instruction, and priming of the immune system. Germ-free (GF) mice (which lack microbiota) have markedly underdeveloped gut-associated lymphoid tissues, decreased number and smaller-sized Peyer's patches and mesenteric lymph nodes, and defects in antibody production, compared to specific pathogen free (SPF) mice. Not surprisingly, germ-free animals exhibit increased susceptibility to multiple types of infections, including viruses, bacteria, and parasites (22–27). However, compared to free-living mice or laboratory animals exposed to gut flora from wild mice, SPF animals have a more limited microbial community and are also more susceptible to inflammatory diseases, with a reduced immune repertoire including deficits in memory responses (21, 28, 29).

Although an extensive discussion of the healthy gut microbiome and its impact on host immunity is beyond the scope of this review, we will highlight a few important aspects of how the intestinal bacterial community microbiome maintains a healthy host immune environment. First, bacterial metabolites generated by gut commensals contribute to the maintenance of intact epithelial integrity, regulatory T-cell development, and a relatively anti-inflammatory immune state. In particular, short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate are fermentation products of dietary fiber and carbohydrates by large intestinal bacteria (30). In addition to being a major energy source for intestinal epithelial cells, SCFAs promote the development of naive $CD4^+$ T cells into regulatory T cells (31, 32), induce "tolerogenic" dendritic cells in the intestinal mucosa (33), and limit autoimmity (34, 35). At the same time, microbial metabolites are integral for promoting immune responses in the gut against pathogens, including inducing secretion of IL-18 (36) and defensins (37, 38). Thus, the products of microbiome metabolism are integral to the appropriate regulation of mucosal barrier integrity and immune homeostasis. In addition, specific members of the bacterial community have been shown to foster the proper maturation and development of the immune system. While this is still an

area undergoing intense investigation, one notable example is the discovery that segmented filamentous bacteria are critical promoters of intestinal mucosal IgA production (39, 40)and Th17 cell induction (41, 42).

Dysbiosis, or an imbalance in the normal composition of the microbiome, is associated with a variety of chronic diseases, many of which are characterized by chronic inflammation or abnormal metabolism, including inflammatory bowel disease, cardiovascular disease, and diabetes. Thus, fostering appropriate levels of diversity and composition of the gut microbial community is critical for promoting health and immune homeostasis. During health, the composition of the microbiome is governed by a number of selective pressures unique to each anatomic niche, including temperature, nutrient availability, pH, oxygen tension, and the local immune environment. Short-term perturbations in the gut microenvironment caused by illness, antibiotic usage, or dietary changes (e.g., starvation) can alter the gut microbiome and subsequently lead to transient alterations in immune responses. Thus, investigating whether influenza and other respiratory viruses alter the gastrointestinal microbiome could have mechanistic implications for viral-mediated suppression of antibacterial immune responses.

Effects of Acute Respiratory Viral Infection on Gut Microbiome

Although the composition of the gastrointestinal microbiome is largely influenced by dietary patterns, respiratory viral infections could also contribute, along with other stress inducers such as broad-spectrum antibiotics exposure and chronic inflammation. Using animal models of pulmonary infections by influenza and respiratory syncytial virus (RSV), multiple groups have shown that the gut microbiome is clearly impacted by respiratory viral infections, despite the lack of detectable respiratory virus in the gut (43–47). In a murine model of influenza infection, the investigators found that although the total numbers of bacteria in the gut did not decrease, there was a reduction in the quantities of segmented filamentous bacteria (SFB) and *Lactobacillus/Lactococcus*, accompanied by increases in *Enterobacteriaceae*. Interestingly, although SFB have previously been shown to induce Th17 cells (41, 48), flu-infected mice had increased IL-17A levels and numbers of Th17 cells in the small intestine and colon, which appeared to contribute to intestinal injury (43). In this study, antibiotic treatment prior to influenza infection ameliorated the degree of intestinal injury, but not lung injury, suggesting that gut dysbiosis contributed to local but not systemic inflammation. Other groups have similarly reported increased Proteobacteria (the phylum of which *Enterobacteriaceae* are members) (44, 45), decreased Firmicutes (which include SFB, *Lactobacillus* and *Lactococcus* species), and increased Bacteroidetes (47) following infection by flu or RSVs but not after administration of live attenuated influenza vaccine (LAIV), indicating that live viral infection is required for these changes (47). The increase in Proteobacteria appears to be mediated by type I interferons (IFNs) (18), which not only depleted anaerobic bacteria but also increased susceptibility to secondary *Salmonella* colitis. However, caloric restriction also results in increased relative abundance of Proteobacteria and increased Bacteroidetes to Firmicutes ratio, raising the possibility that decreased oral intake during influenza may contribute to changes in the microbiome (45, 47, 49, 50). It has also been shown that influenza infection alters intestinal microbiota composition through type II IFN produced by lung-derived T cells recruited to the intestine (43). Thus, changes in the gut microbiome appear to result not from direct viral effects but from systemic inflammatory signals that travel from the lung and trigger local inflammatory responses in the gut (**Figure 1**).

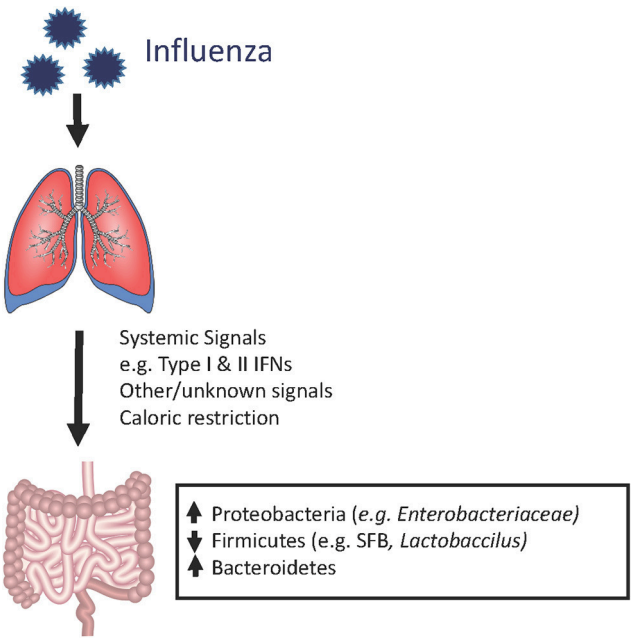

FIGURE 1 | Shifts in the mouse gut microbiome in the setting of influenza infection. During an acute respiratory viral infection, changes in the bacterial composition of the gut microbiome can be observed despite the absence of detectable virus in the gastrointestinal compartment. This suggests that systemic immune signals, physiologic changes (e.g., weight loss), and other still unknown factors are disrupting the normal ecology of the gut, thereby leading to dysbiosis. However, the majority of these studies have been conducted in laboratory animals housed under SPF conditions. It remains to be determined whether human patients and mammalian hosts with more diverse baseline gut microbiota (i.e., mice in the wild), exhibit similar qualitative or quantitative changes.

Effects of Gut Microbiome on Host Immune Responses

Interactions between respiratory tract infections and the gut microbiome are bidirectional. While respiratory viral infections can change the gut microbiome, the gut microbiome also shapes the adaptive immune responses against respiratory pathogens. Mice pretreated with an antibiotic cocktail showed increased morbidity and mortality during influenza infection (51, 52). The severity of infection was associated with reductions in dendritic cell migration rate and the number of local T cells. Mice given a 4 week oral course of broad-spectrum antibiotics

before respiratory viral infection mounted an attenuated anti-PR8 antibody response, were incapable of inducing $CD4^+$ T cell-mediated IFN-γ response to PR8 antigen, and had fewer influenza-specific $CD8^+$T cells (51, 52). These mice also had higher viral titers in their lungs (51). Germ-free mice and antibiotic-treated mice also exhibit impaired antibody responses to seasonal influenza vaccination, which was restored by oral administration of flagellated *E. coli*, demonstrating a dependence on TLR5-mediated sensing of the host microbiota (53).

The gut microbiome is essential for priming innate immune responses against pulmonary infections as well. During viral infections, the degree of macrophage response to respiratory viruses depends on the presence of gut microbes. Macrophages from animals treated with antibiotics exhibited defective responses to type I and II IFNs and impaired capacity to limit viral replication, suggesting that intestinal microbiota provide immune stimulation that establishes an "activation threshold" for innate antiviral immune responses (52). A comparison of C57BL/6 mice from The Jackson Laboratory (which lack SFB in the stool) and Taconic Biosciences (which are SFB positive) revealed that SFB-deficient animals have increased lung bacterial burdens and more severe pneumonia when challenged with methicillin-resistant *Staphylococcus aureus* (MRSA) (54), which was associated with decreased IL-17-mediated responses in the lung. Another study using broad-spectrum antibiotic treatment followed by intranasal administration of *S. pneumoniae* in mice demonstrated that microbiome depletion led to decreased survival, increased lung bacterial burden, and increased systemic dissemination of bacteria (55). Antibiotic-pretreated animals displayed altered cytokine profiles in the lung compared to untreated controls following *S. pneumoniae* infection, including significantly decreased TNF-α levels at 6 and 24 h after infection. Additionally, in the microbiota-depleted group, alveolar macrophages and blood neutrophils exhibited decreased phagocytic activity, and decreased inflammatory cytokine production following *ex vivo* stimulation by Toll-like receptor (TLR) ligands such as lipoteichoic acid (LTA) (55). These effects might be mediated in part by decreased Nod1 sensing of meso-DAP (diaminopimelic acid)-containing peptidoglycan found in gut microbiota, which previously was shown to be essential for priming innate immune responses to *S. pneumoniae* (56). Thus, antibiotic-induced disruptions in the normal gut microbial community alter multiple aspects of normal host defense against acute respiratory pathogens (**Figure 2**).

Gut Microbiome: Therapeutic Avenues for Acute Respiratory Infections

Collectively, the studies above suggest that modulation of the gastrointestinal tract microbiome plays an important role in acute respiratory infections, but precisely how the microbiome should be manipulated to promote appropriate immune responses during acute respiratory infections is unclear. Currently, clinical studies have shown that although probiotics do not influence the incidence of respiratory tract infection, they do reduce the severity of symptoms and duration of the illness (57, 58). Pinpointing which members of the gut microbial community are essential for proper immune priming is challenging, but necessary for guiding further microbiome-based therapies. *Clostridium orbiscindens*, a member of the human gut microbiome, has been found to produce desaminotyrosine (DAT) from metabolism of flavonoids and amino acids. Antibiotic-treated mice exhibited markedly decreased fecal and serum DAT levels, which was associated with attenuated type I IFN responses to influenza infection and increased mortality (59). Thus, identification of DAT-producing microbiota might serve as a modality for priming type I IFN responses against viral infections. Another group demonstrated that oral administration of *Lactobacillus plantarum* enhanced the type I IFN response and lowered viral titers in the lungs in a murine model of influenza infection (60). Other *Lactobacillus* strains are known to enhance TNF-α and IFN-γ production by nasal lymphocytes upon influenza infection (61). Oral administration of a probiotic cocktail containing *Lactobacillus* restored the immune response and enhanced the activation of signaling pathways associated with recognition of single-stranded RNA virus (62). An alternative approach to administering probiotics is to alter the local metabolic environment to regulate immune responses. A recent report demonstrated that animals fed a high fiber diet had increased generation of SCFAs, leading to enhanced antiviral $CD8^+$ T cell immune responses and attenuated neutrophil-mediated lung injury during influenza infection, resulting in improved survival (63). Thus, one strategy for decreasing the incidence of post-viral bacterial infections is to limit the severity of the primary viral infection.

However, activation of antiviral immune responses, including type I and type II IFNs, have been associated with increased susceptibility to secondary bacterial pneumonia (64, 65). Thus, another strategy is to enhance immune responses against common bacterial causes of pneumonia. One group re-colonized antibiotic-treated or germ-free mice with groups of cultivatable commensal bacteria, and found that administration of *Lactobacillus reuteri*, *Enterococcus faecalis*, *Lactobacillus crispatus*, and *Clostridium orbiscindens*, which are strong stimulators of NOD2 (i.e., cytosolic receptor for muramyl dipeptide, which is found in cell walls of certain bacteria), are able to protect against bacterial pneumonia by enhancing GM-CSF production (66). Whether viral-induced changes in the gut microbiome is associated with immune defects that promote secondary bacterial pneumonia, or whether the impaired antibacterial defenses observed in virally-infected hosts can be restored by augmenting certain components of the microbiome are important areas to be investigated.

THE RESPIRATORY TRACT MICROBIOME

The microbiome of the respiratory tract has also been investigated in the context of viral infections. Its role in the development of secondary bacterial pneumonia following influenza and other acute respiratory viral infections is unclear. The respiratory tract is the main site of continuous contact with

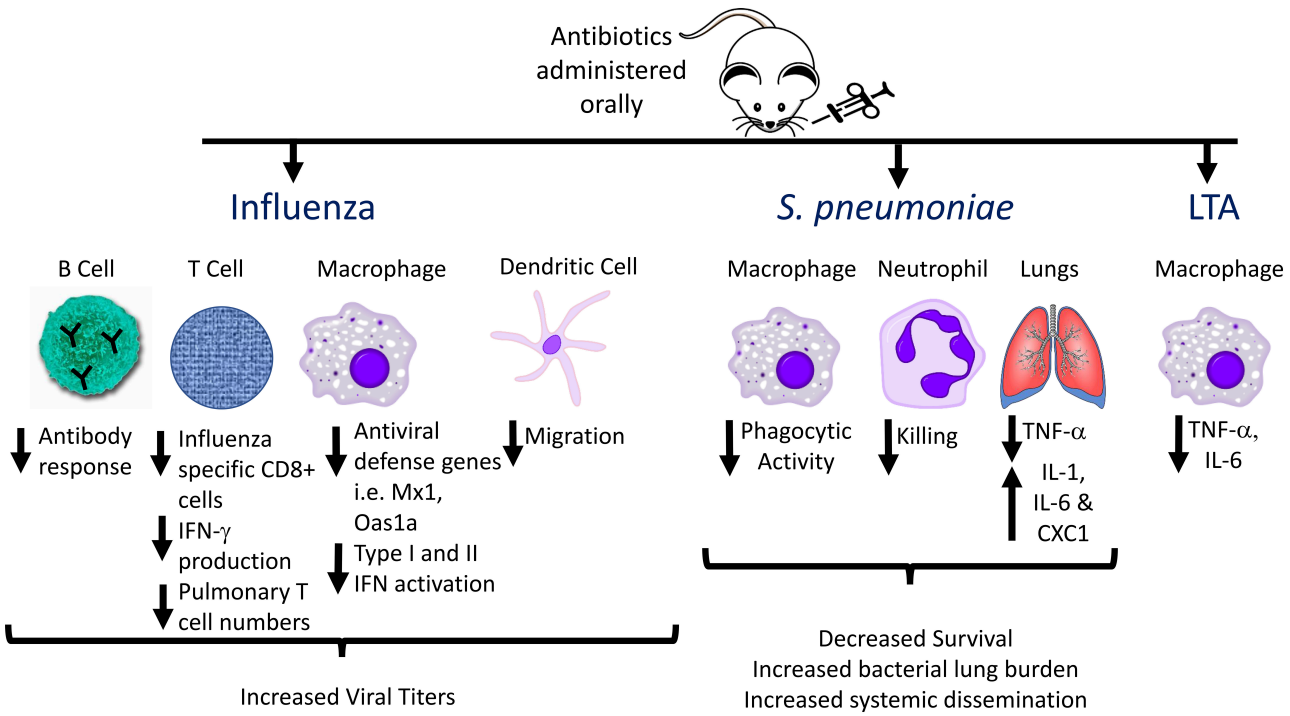

FIGURE 2 | Effects of antibiotic pre-treatment on immune responses to influenza, *Streptococcus pneumoniae*, and Lipoteichoic acid (LTA). The effects of the gut microbiome on immune responses to respiratory pathogens have been investigated by administration of oral antibiotics to generate alterations in the gut flora, followed by acute infection, and analyzing host immune responses compared to non-antibiotic-pretreated animals. Multiple aspects of innate and adaptive immune responses are altered in antibiotic treated animals, including decreased antibody production, decreased phagocytic activity, and decreased inflammatory cytokine production by innate immune cells (e.g., alveolar and peritoneal macrophages) following *ex vivo* stimulation with TLR ligands.

exogenous microbes. As is the case with the gut, immunity at the mucosal interface of the respiratory tract is a constant balance of tolerance of commensal and non-invasive microbes and immune activation against pathogens. The URT and LRT have similar microbial community compositions, although microbe densities are much higher in the former in healthy hosts. Several factors are known to influence airway microbiome composition including infection history, age, genetics, and structural lung disease.

The URT is an interconnected system consisting of the anterior nares, nasal cavity, nasopharynx, sinuses, Eustachian tube, middle ear cavity, oral cavity, oropharynx, and larynx, each of which serve as distinct niches with their own microbial communities. In healthy adults, bacteria present in the nasal cavity are typically those associated with skin, predominantly members of the Actinobacteria (e.g., *Corynebacterium* spp., *Propionibacterium* spp.), followed by Firmicutes (e.g., *Staphylococcus* spp.), and Proteobacteria (67–69). The oropharynx contains members of Firmicutes, Proteobacteria, and Bacteroidetes, including *Streptococcus*, *Neisseria*, *Haemophilus*, and *Lachnospira* spp. (68, 70, 71). Skin and oral cavity lineages are represented in the nasopharynx—e.g., *Streptococcus*, *Staphylococcus*, *Corynebacterium*, and *Prevotella* (70, 72, 73). A limited number of pathogens including *Streptococcus pneumoniae, Neisseria meningitides,* and *Haemophilus influenzae* are commensal bacteria of the URT.

In healthy individuals, the microbial community richness (i.e., the total number of bacterial taxa) is lower in the LRT than that in the URT (70, 74–76). Contrary to dogma that normal healthy lungs are a sterile environment, a distinct, and somewhat dynamic lung microbiome can be identified using sequencing technology, with microaspiration serving as the primary route of microbial immigration from the URT to the LRT (76, 77). The major phyla in healthy lungs are Bacteroidetes and Firmicutes, which mainly include *Prevotella*, *Veillonella*, and *Streptococcus* (78–80). Individuals with chronic airway diseases (e.g., cystic fibrosis, COPD) have increased bacterial populations in the lungs (77) and differences in the relative abundance of certain species (81). Impaired airway clearance due to intrinsic or extrinsic factors leads to the proliferation of bacterial species that can exploit this growth opportunity (82). How respiratory viral infection affects the diversity of microbial communities and whether viral-induced dysbiosis influences immune functions is being examined. Nonetheless, bacterial colonization of the URT is generally considered as the first step in the development of invasive bacterial infections (83, 84), including secondary bacterial infections following respiratory viral infection. Bacterial abundance, species diversity, and factors that shape the immune

response to subsequent infections are discussed in greater detail below.

Studies of the URT Microbiome During Respiratory Viral Infection

Respiratory viruses enter the human body through the URT and are the most common type of acute infections of the respiratory tract. One possible mechanism by which influenza and other viral infections might predispose infected hosts to secondary bacterial pneumonia is by altering the microbial composition of the upper respiratory tract, fostering enhanced growth of pathogens, and facilitating the subsequent entry of large bacterial loads into the LRT (85). This section will examine recent literature on how acute respiratory viral infections have changed the URT microbiome.

Cross-Sectional Studies

Given the effects of viruses on enhancing bacterial adherence to the epithelium (86–88), it is perhaps not surprising that multiple studies of human subjects as well as in animal models have shown that viral infections are associated with increased colonization by potentially pathogenic bacteria (known as "pathobionts"). A comparative analysis using qPCR to detect specific bacteria in adult patients with or without influenza A infection showed that *Staphylococcus aureus*, *S. pneumoniae*, and *H. influenzae* were present in 12, 24, and 32% of infected patients, respectively as compared to 5, 11, and 10% of uninfected patients (89). In experimental *in vitro* models, viral infections increase the colonization rates of various bacteria in the URT (90–95), including *S. pneumoniae* and *H. influenzae* (86–88). In children, influenza is associated with a 15-fold increase in nasopharyngeal titer of *S. pneumoniae* (96). Animal models have similarly confirmed that viral infection, particularly influenza, increases bacterial colonization rates in the URT, enhancing the risk of secondary bacterial infections (97–99). Higher pneumococcal colonization density has been linked to respiratory virus co-infection and invasive pneumococcal pneumonia, after adjusting for age and sex (85). Another case-control study comparing nasopharyngeal bacteria with and without pneumonia also found an association between nasopharyngeal load of *S. pneumoniae*—but not of *H. influenza* and *M. catarrhalis*—and viral co-infection and pneumonia (96). In addition, viral infections potentially may enhance transmission of bacteria. In a study of mice colonized with *S. pneumoniae* and then infected with influenza A virus 3 days after, *S. pneumoniae* transmission occurred only when all mice were infected with influenza and was blocked by an influenza-neutralizing antibody (95). However, while specific bacteria might gain a competitive advantage during viral infections, this does not universally translate to all bacterial taxa. A recent study of subjects with and without respiratory viral infections demonstrated lower overall bacterial reads from nasopharyngeal samples in virally-infected subjects compared with uninfected controls (100).

The relationship between acute viral infections and bacterial colonization appears to be bidirectional. Bacterial carriage or their ligands can increase or decrease viral infectivity rate, thereby positively or negatively influencing the subsequent host immune response to viral infection. Viral replication in the respiratory tract can be enhanced by exposure to *S. pneumoniae* (101). Patients harboring *S. pneumoniae* are more likely to experience subsequent acute respiratory illness episodes than those without colonization (102). In addition, bacteria present in the airways can modulate host responses against viral infection. The presence of a nasopharyngeal commensal protected mice against RSV-induced airway hyperresponsiveness. RSV-infected mice who underwent antibiotic-mediated depletion of *Streptococcus viridans* in the nasopharynx exhibited increases in number of inflammatory lymphocytes and airway hyperresponsiveness, and decreases in regulatory T cell number and transforming growth factor-β production (103). Others have shown that colonization of the URT with *S. aureus* drastically reduced influenza-induced acute lung injury and mortality in mice by recruiting a C-C chemokine receptor type 2^+ cluster of differentiation (CD)$11b^+$ monocyte subset to the lungs and inducing an M2 macrophage phenotype (104).

With the availability of next-generation 16S rRNA sequencing, microbiome-based studies have attempted to discern global patterns of change in the bacterial community of each anatomic niche during viral infections, such as changes in diversity. Diversity can be assessed using a variety of indices, such as total number of unique species of the microbiome (i.e., richness) or other measures that account for both richness and the evenness of relative abundance of the members of the community (e.g., Shannon index). Results from microbiome analyses have not demonstrated consistent changes in diversity when comparing virally infected subjects with healthy controls. This is not surprising given the variability of the subjects sampled, differences in type and severity of viral infections, type and timing of sample collection, and analysis methodology. In some studies, increased bacterial diversity appeared to be associated with influenza severity. A French study of children admitted to the hospital with influenza revealed increased diversity of the nasopharyngeal microflora with increased influenza severity (105). Children with severe influenza showed decreased relative abundance of *S. aureus* and increased abundance of *Prevotella*, *Streptobacillus*, *Porphyromonas*, *Granulicatella*, *Veillonella*, *Fusobacterium*, and *Haemophilus.* A recent Chinese study in patients with H7N9 avian influenza demonstrated significantly increased diversity in the oropharyngeal microbiome of H7N9-infected patients compared to healthy controls, particularly H7N9 patients with secondary bacterial pneumonia (106). Conversely, a French study of nasopharyngeal samples and a South Korean study of oropharyngeal samples from patients with acute respiratory viral infections both displayed decreases in diversity indices during viral infections compared to healthy controls (71, 100). Both studies included subjects ranging from infants to adults >80 years of age, limiting conclusions about age-related effects. Longitudinal studies conducted in healthy volunteers who underwent experimental self-innoculation with rhinovirus also failed to demonstrate significant changes in diversity of the URT microbiome, while administration of LAIV vaccine to healthy adults led to increases in diversity measures following viral

challenge (73, 107). Thus, unlike other diseases where decreased diversity is considered deleterious to the host, the effects of viral infections on diversity *per se* are variable and not presently considered a good indicator of risk for complications, including secondary bacterial pneumonias.

Microbiome sequencing studies also enable investigators to identify changes in abundance among multiple bacterial taxa simultaneously, beyond just what can be cultured individually. This allows investigators to determine what groups of bacteria are changing in unison during viral infection and which are existing in competition with one another. This information may have implications for the development of probiotic therapies (as discussed below). A recent metagenomics-based study in France reported enrichment of *S. aureus*, *S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis* and *Klebsiella pneumoniae* in nasopharyngeal samples of subjects with confirmed respiratory viral infections compared to healthy controls (100). An examination of the oropharyngeal microbiome of pneumonia patients with and without 2009 influenza A H1N1 pandemic viral infection showed that Firmicutes (which include *Staphylococcus* and *Streptococcus* spp.) and Proteobacteria (mainly *Pseudomonas amygdali*, *P. fluorescens*, *Pseudomonas* sp. UK4, *Acinetobacter baumanii* and *A. junii*)—were significantly enriched in patients with influenza (108). Another study of patients with 2009 pandemic H1N1 influenza infection revealed that the predominant phyla of the upper respiratory tract (nasal and nasopharyngeal samples) in patients harboring pandemic H1N1 were Actinobacteria, Firmicutes, and Proteobacteria although normal controls were not included; however, the authors suggested that flu is associated with an expansion of Proteobacteria (109) which is generally less abundant in healthy hosts. These findings are supported by another group who found that *Moraxella* and *Enterobacter* spp. (which are classified as Proteobacteria) were the most highly represented bacteria in nasopharyngeal samples obtained from patients with pandemic H1N1 influenza (110). However, these studies demonstrated that there was considerable inter-subject variability, highlighting the need for longitudinal studies to decipher changes following viral infection.

Investigators have also sought to determine whether specific viruses are consistently linked to enrichment of certain bacterial taxa. In the nasopharyngeal compartment of Aboriginal and non-Aboriginal children in Australia, positive associations were detected between hRV and *S. pneumoniae*, *H. influenza,* and *Moraxella catarrhalis* carriage as well as between adenovirus and *M. catarrhalis* (111). Another study examining the presence of 20 respiratory viruses by PCR panel and prevalence of bacterial carriage in the nasopharynx of children found a strong positive association between *S. aureus* colonization and influenza virus (112). Moreover, *S. pneumoniae* colonization was positively associated with the presence of hRV and enteroviruses; *H. influenzae* was positively associated with hRV and RSV; and *M. catarrhalis* colonization was positively associated with coronaviruses and adenoviruses. A 16s rRNA sequencing-based study conducted in infants with acute RSV or hRV respiratory infections reported that infants with RSV had significantly higher abundance of *Staphylococcus* spp. compared to hRV-infected infants (113). An analysis of the URT bacterial content of 57 healthy asymptomatic individuals and 59 patients with influenza virus, parainfluenza, hRV, RSV, coronavirus, adenovirus, or metapneumovirus by culture-independent pyrosequencing revealed six distinct bacterial profiles—i.e., *Streptococcus* + *Prevotella* + *Veillonella*, *Streptococcus* + *Haemophilus* + *Neisseria*, *Streptococcus*, *Moraxella*, *Haemophilus*, and *Klebsiella*. These profiles, however, were not associated with virus type but were linked to the age of subjects (71).

Given that many human studies are cross-sectional in nature, it remains unclear whether post-viral bacterial pneumonias might be the result of viral infections enhancing bacterial colonization or acquisition, colonizing bacteria influencing host susceptibility to respiratory viral infections, or a combination of both. Another complicating factor particularly in cross-sectional studies examining the microbiome during viral infections is that the groups are not well-controlled and the sample numbers are relatively small considering the number of variables that could affect the respiratory tract microbiome–such as age, gender, oral hygiene and nose-picking habits, healthcare-based employment status, smoking status, medication use, exposure to small children, etc. The underlying type of viral infection, sampling timepoint after onset of infection, severity of infection, and concomittant antimicrobial usage are other confounding factors. This may underlie the highly variable and sometimes discrepant observations from microbiome studies in patients with viral infections.

Longitudinal Studies

There have been few clinical studies comparing baseline pre- and post-infection microbiomes in otherwise healthy individuals with acute viral infections due to the difficulty of sampling before infection. However, the relatively few studies available provide insights into the dynamicity and stability of bacteria colonization patterns over time, and whether and how perturbations brought on by acute viral infections alter these patterns. In healthy children, the major phyla among nasopharyngeal microbiotas are Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria, with *Moraxella*, *Haemophilus*, *Streptococcus*, *Flavobacteria*, *Dolosigranulum*, *Corynebacterium*, and *Neisseria* as predominant genera. Changes in nasopharyngeal microbiome diversity were observed across seasons, with a predominance of *Proteobacteria* and *Fusobacteria* in fall-winter and *Bacteroidetes* and *Firmicutes* in spring; these differences were independent of recent antibiotics and viral co-infection (114). However, another analysis of two nasopharyngeal washes collected 5.5–6.5 months apart from 40 children and adolescents with asthma showed no significant differences in nasopharyngeal microbiome diversity across seasons, although mean relative abundances of *Haemophilus*, *Moraxella*, *Staphylococcus*, and *Corynebacterium* varied significantly between summer and fall samples and between age groups. Moreover, in 87.5% of patients, operational taxonomic units (OTUs) in patients varied significantly between time points (115). An investigation of the frequency and seasonal variation in bacterial and viral load in asymptomatic healthcare professionals during the winter and summer months showed that of the 100 subjects

tested during the winter, 34 were colonized with at least one bacterial species and 11 tested positive for at least one virus. The most frequently detected pathogens were methicillin-resistant *Staphylococcus aureus* (MRSA), *M. catarrhalis*, and coronavirus. In contrast, of the 100 subjects tested during the summer, 37 harbored at least one bacterium (mainly MRSA and *K. pneumoniae*) and four tested positive for one virus (116).

Several larger scale surveillance studies of mainly pediatric populations have examined the natural temporal patterns in bacterial colonization during viral infections. One clinical investigation assessed the presence and density of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in the nasopharynx of children during URT infection and in the healthy state, and reported that the proportion of children colonized with these bacteria was higher during infection than during asymptomatic surveillance visits. Mean density of all bacterial species was significantly higher at each visit when a virus was detected. Interestingly, the percentage of colonized children and bacterial density were also higher at asymptomatic visits in which virus was detected than at those in which virus was not detected (117). Another study of 31 families with small children using longitudinal nasal swab sampling demonstrated that rhinovirus infection was associated with increased acquisition of *S. pneumoniae* from the community as well as increased transmission of *S. pneumoniae* within the family (118).

Other groups have examined the effects of experimental innoculation of hRV into the URT (nares) (**Figure 3**). These studies reported no significant changes in total read counts or of the main phyla (e.g., Actinobacteria, Firmicutes, and Proteobacteria) over time in nasopharyngeal samples (73) or throat swabs (119). In the oropharyngeal compartment, rhinovirus infection was associated with a strong trend toward transient increases in the relative abundances of *H. parainfluenzae*, *Neisseria subflava* and a weak trend toward an increase in *S. aureus* (119). By 60 days, abundance of these bacteria had returned to baseline. Nasopharyngeal sampling showed completely opposite results, with decreased relative abundance of *Haemophilus* and *Neisseria* spp., but an increase in the normal nasal commensal, *Propionibacterium*, in subjects following hRV infection (73). No differences in *Staphylococcus* were observed. However, the number of subjects were small in both studies, limiting the power to detect changes over time.

Nasopharyngeal microbiota composition has been shown to be altered by influenza vaccination (**Figure 3**). Administration of live attenuated influenza vaccine (LAIV), which is nasally instilled, to healthy children increased the nasal colonization density of *S. pneumoniae* in subjects who harbored this bacterium at the time of vaccination, and transiently increased rates of colonization by *H. influenza* (120). In healthy adult volunteers, it was demonstrated that intranasal LAIV administration induced an increase in the diversity of the nasopharyngeal microbiome,

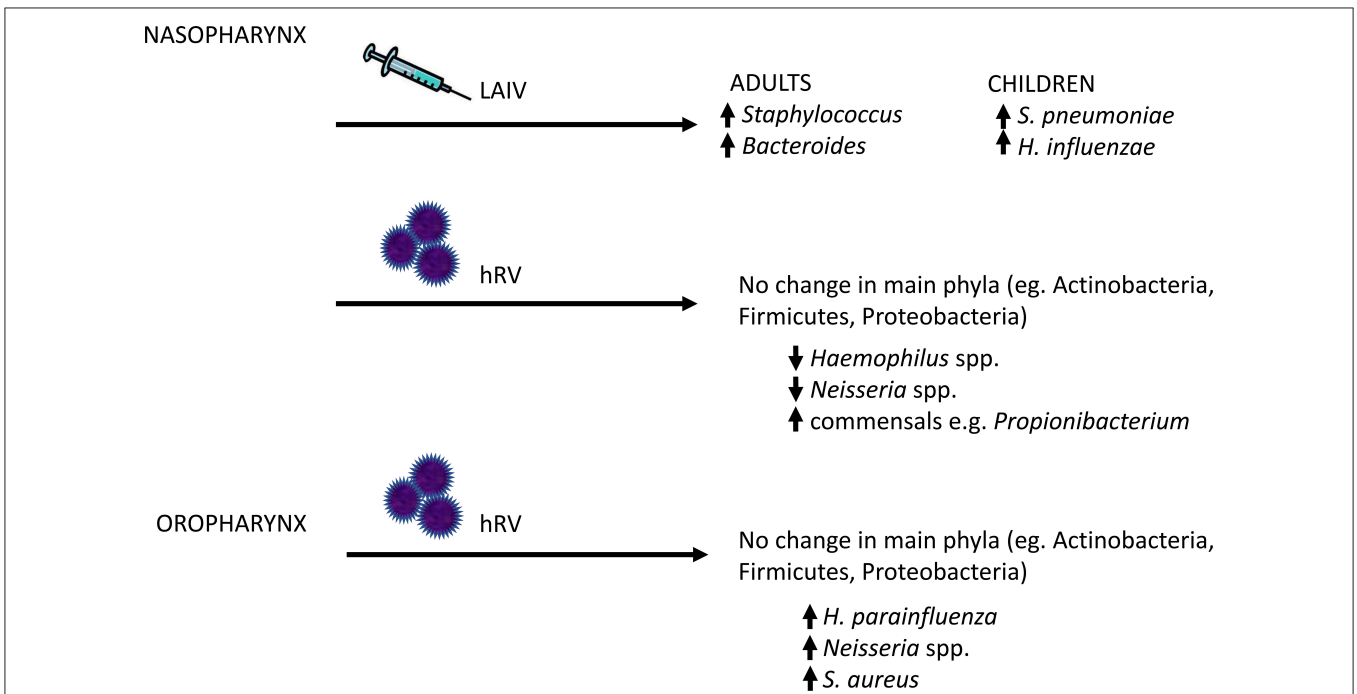

FIGURE 3 | Changes in the human upper respiratory tract microbiome following viral exposure. Given that bacterial pneumonia frequently arises as a result of aspirated bacterial pathogens, a potential mechanism by which viral infections might increase the risk of secondary bacterial infections is through increased colonization of the upper respiratory tract by bacterial pathogens. In human subjects, live attenuated influenza vaccine (LAIV) and human rhinovirus (hRV) have been shown to disrupt the local host bacterial community, with increased relative abundance of potential pathogens (or pathobionts), such as Staphylococcal and Neisseria species. The major changes in the upper respiratory tract microbiome are highlighted here.

as well as relative abundances of *Staphylococcus* and *Bacteroides* (107). These changes were not observed in subjects given saline nasal spray. In a mouse model, bacterial density in the nasopharynx after LAIV administration was increased as much as 100,000 times compared to influenza-naive hosts, and the duration of carriage of *S. pneumoniae* or *S. aureus* was also increased 2 to 5-fold (121). However, systemic vaccination can also alter the URT microbiome. A longitudinal study of healthy subjects found a significant association between the presence of *Lactobacillus helveticus*, *Prevotella melaninogenica*, *Streptococcus infantis*, *Veillonella dispar*, and *Bacteroides ovatus* and influenza-specific H1 and H3 IgA antibody response (122). Thus, it is remarkable that a relatively mild viral stimulus such as flu vaccine can lead to detectable changes in the URT microbiome.

Although the data are still preliminary, animal studies have suggested that antiviral immune activation contributes to changes in the URT microbiome and facilitate colonization by potential pathogens, such as *S. aureus*. In a mouse model of *S. aureus* nasal colonization, the absence of type I IFN receptor was associated with decreased persistence of bacteria (107). Type III IFN, which is also induced during influenza infections, led to changes in the nasal microbiome, including increased numbers of culturable bacteria. Increased upper respiratory tract persistence of *S. aureus* as well as increased risk of *S. aureus* pneumonia was observed in flu-infected wildtype mice compared to mice lacking the type III IFN receptor (123). Currently, however, is it unclear to what extent viral-induced changes in the URT microbiome alter subsequent immune responses against secondary bacterial infections.

Studies of the LRT Microbiome During Respiratory Viral Infection

Compared to studies of the URT microbiome, studies of the LRT microbiome following viral infections are relatively scarce due to the difficulty of obtaining uncontaminated samples from the lung. Samples of convenience, such as sputum, suffer from oral contamination, but bronchoscopic samples are invasive and expensive to obtain on a regular basis. Moreover, it is unclear whether outside of patients with chronic lung disease (e.g., COPD), the lung microbial burden is of sufficient magnitude to exert robust effects on immune responses and risk of secondary bacterial infection during viral infection. Data from a mouse model of influenza infection seem to indicate that flu infection has only a modest effect on bacterial counts, diversity and composition of the lung microbiome (46). In subjects with chronic obstructive pulmonary disease (COPD) after hRV infection but not in healthy individuals, there was an increase in bacterial burden and growth of bacteria present at baseline, particularly *H. influenzae* (124). The researchers observed that the growth of bacteria seemed to arise from the existing community. *S. pneumoniae* intranasally inoculated into mice pre-infected with influenza virus first colonized the nose, followed by the trachea and lungs several days later with purulent inflammation. However, this effect was not observed in uninfected animals. This suggests that pneumococcal infection may sequentially develop from the URT to the LRT in influenza virus-infected subjects (97). Thus, it is possible that some individuals with influenza infection might develop changes in their lung microbiome as a result of changes in their URT microbial communities.

Respiratory viruses not only alter the bacterial community in the URT, but also promote bacterial colonization of the LRT by a variety of mechanisms that impair bacterial clearance. First, mucus production in the respiratory tract is increased to facilitate viral clearance during infections. However, excessive mucus production can lead to airway obstruction by impeding mucociliary clearance (125). Second, viral infections can also reduce ciliary beat frequency and the number of ciliated cells, disrupt the coordinated movement of cilia, and impede the repair of respiratory epithelial cells, further leading to reduced mucociliary clearance (126, 127). Third, respiratory viral infections impair innate immune responses against bacteria (128–130). Innate immune cells including macrophages and neutrophils are recruited to the lung by cytokines and chemokines for phagocytosis and bactericidal activity. Prior viral infections dysregulate both alveolar macrophages (64, 131–136) and neutrophils (65, 128, 129), thereby inhibiting bactericidal activity. Thus, with multiple aspects of pulmonary host defense impaired, it would not be entirely surprising if a subset of influenza infected patients developed secondary bacterial pneumonia as a result of being unable to clear aspirated pathobionts from the URT.

INTERMICROBIAL INTERACTIONS AND POSTVIRAL SECONDARY INFECTIONS

In addition to enabling us to determine what is present during states of health, large-scale sequencing-based microbiome analyses have also revealed who is not present during disease. It has long been appreciated that mechanisms have evolved in bacteria that confer competitive advantages, permitting them to survive in an otherwise inhospitable host environment. However, interspecies competition also maintains homeostasis of the microbial community, either through their abilities to capture scare resources (e.g., iron), or targeted killing of other bacteria (e.g., bacteriocins), preventing one microbe from dominating the community. Thus, it is possible that the immune response incited by acute viral infections, changes in the host epithelial surface caused by the virus, or the virus itself might lead to elimination of a host commensal that is responsible for keeping pathobionts in check. For example, *S. epidermidis* and *Propionibacterium acnes* abundance in the nares has been shown to be negatively associated with *S. aureus* carriage (67). Understanding these interactions may create new avenues for therapeutic interventions aimed at reducing colonization by pathogenic bacteria during influenza epidemics or pandemics.

One group of commensals that has been examined for its role in inhibiting nasal carriage by *S. aureus* and *S. pneumoniae* is *Corynebacterium* spp. An early study in Japan reported on the effects of introducing a *Corynebacterium* strain into the nares of healthy adult hospital workers who were persistent carriers of *S. aureus*, with successful eradication in 71% of subjects

(137). The mechanism appeared to be bacteriocin-independent. In comparison, *S. epidermidis* implantation did not have an effect. Whether the *S. epidermidis* strain used expressed the serine protease Esp, which inhibits biofilm formation by *S. aureus* and nasal colonization (138), is unknown. Subsequent studies by another group reported that *C. pseudodiphtheriticum* inhibited *S. aureus* growth, whereas *C. accolens* and *S. aureus* appeared to support each other's growth (139). Conversely, other investigators observed that *Corynebacterium* spp. were enriched in children who were not nasally colonized with pneumococcus, and demonstrated that *C. accolens* inhibit *S. pneumoniae* growth *in vitro* by expressing a lipase that releases free fatty acids from skin surface triacylglycerols, which inhibit pneumococcal growth. Thus, painstaking identification and mechanistic interrogation of interspecies competition between commensals might lead to novel insights as to how viral infections might confer competitive advantage to pathobionts, and how to exploit natural strategies employed by commensals to restore homeostasis to the host microbial niche. Interestingly, a recent preclinical study using a murine model of RSV and *S. pneumoniae* superinfection employed nasal priming by a *C. pseudodiphtheriticum* strain to augment host defense against the viral infection, which enhanced clearance of secondary bacterial challenge and reduced lung injury measures (140).

Finally, direct effects of the infecting virus on bacteria that comprise the microbiome may facilitate the transition from pathobiont to pathogen. A metagenomic analysis showed that pH1N1-associated airway microbiotas were enriched in genes associated with cell motility, transcriptional regulation, metabolism, and response to chemotaxis compared to the same bacteria in non-infected patients (108). These data imply that influenza infection perturbs the respiratory microbiome, leading to the production of secondary metabolites including immune-modulating molecules. Viruses have also been found to impair bacterial biofilm formation and disrupt existing biofilm (141–144). Influenza has been shown to affect the *S. pneumoniae* transcriptome in terms of downregulating expression of genes associated with the colonizer state and upregulations of bacteriocins (142). Thus, direct effects of viruses on bacterial transcriptional patterns might be a mechanism by which colonizing bacteria acquire invasive potential, thereby leading to bacterial superinfections.

FUTURE DIRECTIONS

There are several areas that must be addressed by future respiratory microbiome research. First, it is necessary to standardize protocols used to analyze the respiratory microbiome, including sampling, processing, and bioinformatics methodologies. For example, sputum may be an appropriate material for investigations of respiratory diseases since it contains components of the LRT and can be obtained easily. However, more reliable information on the LRT requires invasive samples such as BAL or protected specimen brush

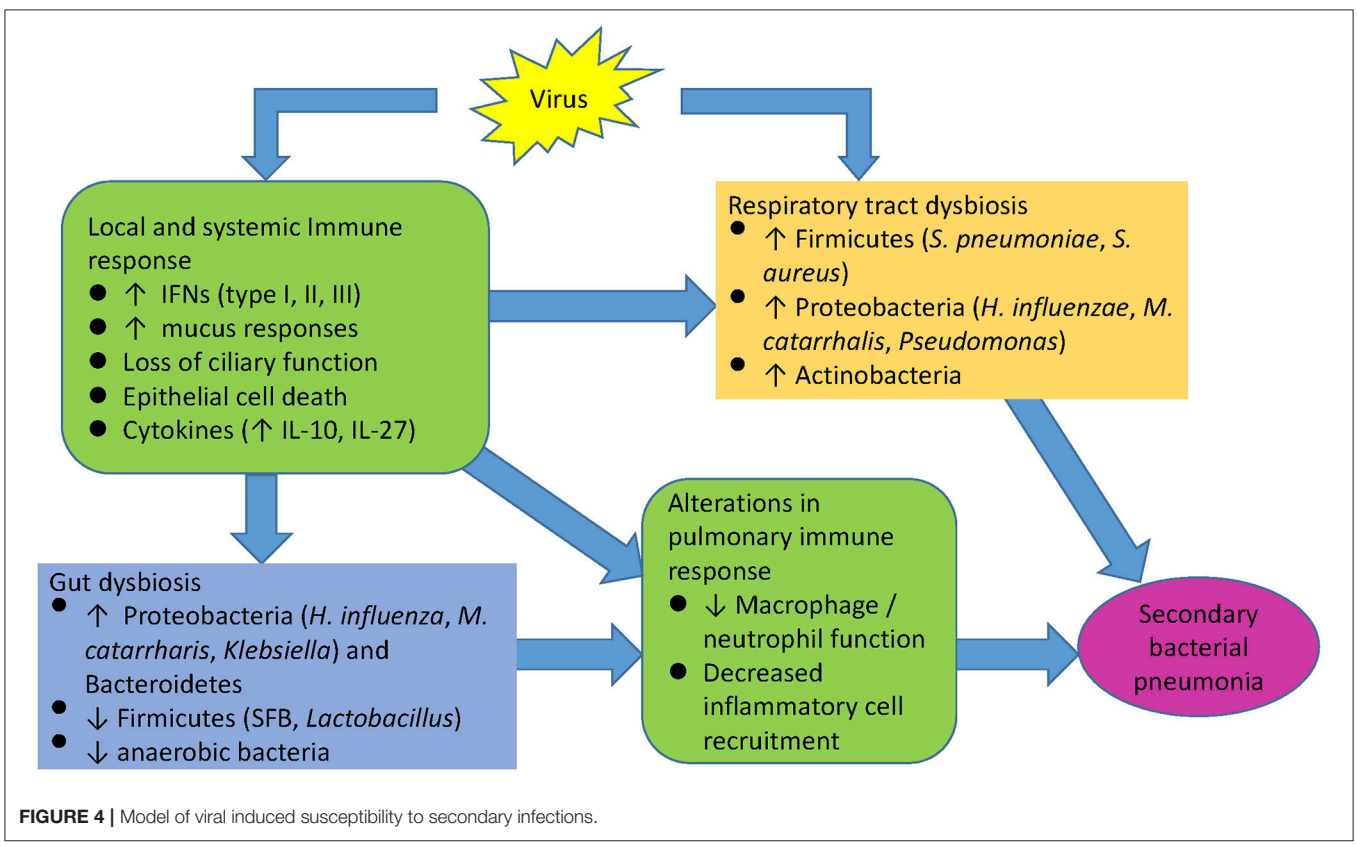

FIGURE 4 | Model of viral induced susceptibility to secondary infections.

or bronchial/lung biopsies. Second, most studies are limited to experiments conducted in animal models. Even in human studies, most analyses have been performed in a small number of patients and have described bacterial communities in the URT. The role of microbial communities outside of the lungs including gut, sinus, and skin should be considered in the context of airway diseases. Third, most studies on the microbiome have focused on the bacterial component, and have largely omitted fungi and viruses. The role of viruses—including the vast number of phages that infect bacteria—and fungi in respiratory diseases cannot be examined through 16S rRNA gene analyses, and there are no studies describing the composition and role of the respiratory virome due to the difficulty of comprehensive analyses for viruses. Fourth, it is not sufficient to study microbial communities based on species composition; a functional characterization through transcriptome and proteasome analyses is necessary to understand mechanistic role of microbiome on outcomes of infection. Finally, mucosal microbiome manipulations by vaccines, antibiotics, and probiotics in the gastrointestinal and respiratory tract niches represent novel approaches for the prevention, treatment, and management of acute and chronic lung diseases. However, given that antibiotic therapy could affect commensal bacteria and hasten the emergence of drug-resistant bacteria, more research is needed on the long-term effects of this therapy. Animal models should be developed to study the influence of the URT and LRT microbiomes on immune responses to respiratory viral infections; only then will it be possible to consider the clinical application of microbiome modulation strategies.

CONCLUSION

Respiratory viral infections can initiate a cascade of host immune responses that alter microbial growth conditions in the URT, LRT, and the gut (**Supplemental Table 1**). Activation of influenza-induced antiviral interferon pathways can lead to inadequate innate immune cell responses during host defense against secondary bacterial infections, resulting in the proliferation of potentially pathogenic bacterial species. Concomitant changes in the gut microbiome caused by the initial viral infection may also alter immune cell priming against secondary bacterial challenge, although this has not been examined to date. Although the picture is incomplete, recent microbiome literature provides additional insights into the pathogenesis of dysregulated immune responses following acute viral infections, that may promote the development of secondary bacterial pneumonias (**Figure 4**). Clarifying the differences and dynamics of respiratory microbiota in healthy subjects and chronic lung diseases during acute respiratory viral infections can elucidate pathogenesis of viral-bacterial interactions and provide a basis for developing novel approaches for the prevention, treatment, or management of acute respiratory infection and exacerbation of chronic lung diseases.

AUTHOR CONTRIBUTIONS

SH and JD co-wrote the manuscript. SH, JD, and MP designed the figures and table. KC and MP edited and provided critical revisions of the manuscript. All authors approve the final version and agree to be accountable for the content of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02640/full#supplementary-material

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Innate Immune Cell Suppression and the Link With Secondary Lung Bacterial Pneumonia

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Secondary infections arise as a consequence of previous or concurrent conditions and occur in the community or in the hospital setting. The events allowing secondary infections to gain a foothold have been studied for many years and include poor nutrition, anxiety, mental health issues, underlying chronic diseases, resolution of acute inflammation, primary immune deficiencies, and immune suppression by infection or medication. Children, the elderly and the ill are particularly susceptible. This review is concerned with secondary bacterial infections of the lung that occur following viral infection. Using influenza virus infection as an example, with comparisons to rhinovirus and respiratory syncytial virus infection, we will update and review defective bacterial innate immunity and also highlight areas for potential new investigation. It is currently estimated that one in 16 National Health Service (NHS) hospital patients develop an infection, the most common being pneumonia, lower respiratory tract infections, urinary tract infections and infection of surgical sites. The continued drive to understand the mechanisms of why secondary infections arise is therefore of key importance.

Keywords: lung, macrophage, innate immunity, bacteria, virus, matrix, apoptotic cells, training

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INTRODUCTION

It has been appreciated for a long time that infections following surgical cases are caused by a breach of skin barrier integrity. This breach of barrier tissue (e.g. the skin or epithelial surfaces lining the lung, gastro-intestinal or urogenital tract) however, is common during non-surgical infection and was one of the first causes identified to enhance bacterial outgrowth in the lung, by providing different substrates for adhesion and access to additional proteins for bacteria to metabolize. In this review we will discuss the changes in immunity that lead to dysregulation of responses and how prior viral infection in the lung suppresses cellular innate immunity facilitating bacterial outgrowth to occur. Though we assume that cellular innate immunity is adequate before viral infection, it is important to consider that patients most at risk of developing secondary bacterial complications may have a complex inflammatory history, medications, co-morbidities or mental-health history that has already influenced innate immunity. We will not cover the more soluble innate elements such as anti-microbial peptides or surfactant proteins, as these have been covered extensively elsewhere (1).

Innate Immunity in the Healthy Lung

Innate immunity in the lung is important since it can facilitate the elimination of many pathogens in the absence of adaptive immunity and without immunopathological side effects. The actual location of some innate immune cell subsets is unclear due to the changing environment within the branching structure of the lung. A general rule however is that the density of immune cells gets lower the further down the respiratory tract you look, which facilitates optimal gaseous exchange.

As will be described for macrophages later, the immune components present in a healthy lung are specialized and sparse. Innate lymphoid cells exist in the naïve mouse lung at a low frequency of 0.4–1%. Their precise lung location in health however, has not been determined (2, 3), though they do expand during lung inflammation [for a review see (4)]. Gamma delta (γδ) T cells are also present and rare, accounting for ∼1–5% of blood (5) and 8–10% of lung lymphocytes. They display a restricted profile of variable genes (V**γ**4, V**δ**1, and V**δ**6) (6) in their T cell receptor, which changes with age to become predominantly V$\boldsymbol{\gamma}4^{+}$ (7, 8). NK cells constitute 10% of resident lymphocytes in the lung (9) and it is thought their survival depends on IL-15 production by bronchial epithelial cells (10). NK cells detect an absence of MHC class I molecules using a variety of cell surface receptors and are induced to kill target cells by an activating receptor that binds stress ligands (11). In this way, NK cells present in the interstitial compartment are poised to recognize abnormality.

Dendritic cells (DCs) are present in the lung interstitial spaces (12) and the pulmonary epithelium (13), but are absent from the airspaces. In mice, DCs in the epithelium ($CD103^{+}$ $CD11b^{lo}$) require Batf3, IRF8, and Flt3 ligand for development, whereas those in the lung parenchyma require M-CSF (14). Either population may derive from bone marrow or a local precursor cell population (15). In the steady state, the DCs present in the epithelium may be important for sampling luminal content and/or clearing apoptotic cell turnover (16). As with other innate immune cells, the density of dendritic cells will depend on the position in the respiratory tree with more being present in bronchi than alveoli. Dendritic cells and follicular dendritic cells are also located in sparse B cell follicles. Though typically absent in naïve mice and humans, aggregates of B and T cells may be located next to the major bronchi and include follicular dendritic, dendritic, and stromal, cells (17).

Macrophage Subsets in the Lung

Generally, an absence in any of the innate immune cells described above has little affect in healthy lungs. However, lung macrophages have a unique role in health by performing general housekeeping duties, as exemplified by the build-up of proteinaceous material due to an absence of macrophages in mice lacking granulocyte macrophage-colony-stimulating factor (GM-CSF) (18). In rodents and humans, the lungs are home to two distinct macrophage subsets: airway macrophages and interstitial macrophages (19). We refer to airway, rather than alveolar macrophages since bronchoalveolar lavage (BAL) samples the whole airway. This procedure typically elutes 90–95% macrophages in health, the majority of which will be derived from the alveoli, in addition to a small number of lymphocytes (20, 21). Alveolar macrophages are remarkably long-lived and self-renewing and therefore do not require continuous replenishment from bone marrow-derived precursors in health (22–24). In contrast, interstitial macrophages have a higher turnover rate and are shorter lived in the steady state (25). Interstitial macrophages are located in the interstitial space between the alveoli and capillaries and are less abundant than alveolar macrophages (26).

Alveolar macrophages are initially derived from fetal monocytes and their development is reliant on GM-CSF, of which there is an abundance of in the airspaces shortly after birth (27, 28). GM-CSF drives production of alveolar macrophages through induction of peroxisome proliferator-activated receptor-γ (PPARγ) expression (27, 29, 30). Mice lacking GM-CSF (or its receptor), and patients with defects in GM-CSF signaling, develop pulmonary alveolar proteinosis due to a build-up of surfactant in the airways because of a lack of clearance by macrophages (31, 32). Following irradiation (27) or influenza infection (22) airway macrophages become depleted and are replenished from the periphery or the interstitial lung macrophage pool, respectively. On the other hand, interstitial macrophages originate from bone marrow derived-monocytes and are preferentially replenished by this population during inflammation (33). A recent study has identified 3 populations of interstitial macrophages based on phenotypic and transcriptomic studies, which are different to airway macrophages (34).

The Function of Airway Macrophages

The mechanisms leading to bacterial outgrowth following lung viral infection are, to a large extent, driven by the attempt to return the lung to health. Understanding the role of innate immune cells in lung health therefore, may provide clues to why complications can occur. Due to their location, macrophages in the airways display phenotypic and functional differences to other macrophage populations. Alveolar macrophages reside in the alveolar lumen and are surrounded by surfactant, which contains proteins that dampen macrophage activity (35). This allows alveolar macrophages to be tolerant to cellular debris and innocuous antigens, thereby preventing excessive tissue damage, while setting an activation threshold that needs to be overcome to efficiently clear more pathogenic microorganisms (21). On the other hand, interstitial macrophages are in close contact with the extracellular matrix (ECM) and, as such, have a more prominent role in modulating tissue fibrosis, as well as being better equipped for antigen presentation (36, 37). Moreover, alveolar macrophages have reduced phagocytic activity and respiratory burst in comparison to interstitial macrophages (38, 39). Both subsets of macrophages inhibit T cell activation and subsequent onset of adaptive immunity via the suppression of DC activation; a process dependent on the anti-inflammatory cytokine interleukin-10 (IL-10), transforming growth factor-β (TGFβ) and prostaglandins (40, 41). Alveolar macrophages are poor at presenting antigen to T cells (42), although they are capable of transporting antigens to the lung-draining lymph nodes (43). Likewise, human alveolar macrophages induce T cell antigen-specific unresponsiveness as a result of poor antigen presentation and a lack of expression of co-stimulatory

molecules, such as CD86 (44); which in itself promotes tolerance to innocuous antigens.

Regulation of Alveolar Macrophages by the Airway Epithelium

With respect to bacterial complications following viral infection, it is important to appreciate the role of the epithelium in regulating airway macrophage activity. Due to their direct exposure to environmental challenges in the alveolar lumen, strategies need to be in place for alveolar macrophages to discern a harmless antigen from a serious pathogenic threat. For this reason, alveolar macrophages are tightly regulated in order to prevent an inflammatory response against cellular debris and innocuous antigens, whilst still providing protection against harmful pathogens by propelling an inflammatory response (35). For example, alveolar macrophages are hypo-responsive to low levels of endotoxins, which are present in ambient air (21), thereby preventing an inappropriate innate immune response to innocuous antigens. A number of mechanisms are in place to suppress the activity of alveolar macrophages, including their interaction with the airway epithelium. The airway epithelium, through both direct contact and secreted products, negatively regulates alveolar macrophage activity. These factors include CD200, TGF-β, IL-10 and surfactant proteins (SP-A and SP-D), which act to suppress macrophage phagocytic ability and production of pro-inflammatory cytokines (45–47) (**Figure 1**). Though beneficial in some instances, these pathways can slow immediate immune activity. For example, knockout of IL-10 is beneficial as it allows immediate protection against acute influenza with better survival at lethal infection levels (48, 49). However, inhibiting IL-10 after acute influenza infection results in tissue inflammation and damage, with decreased survival (49, 50), similar to IL-10 knockout *S.pneumoniae* bacterial models (51). For an in-depth discussion on this see (48–51).

In addition, these mechanisms set a threshold of activation that needs to be overcome in order for an inflammatory response to be triggered. Activation of toll-like receptor (TLR) signaling, through recognition of an invading pathogen, elicits a strong enough immune response to exceed the inhibitory regulation of alveolar macrophages and causes up-regulation of TLR co-receptors including CD14 and triggering receptor expressed on myeloid cells 1 (TREM1) (52). Furthermore, loss of epithelial integrity during inflammation reduces the level of regulatory factors, releasing alveolar macrophages from epithelial-induced inhibition. This increases their phagocytic capabilities and initiates the production of pro-inflammatory cytokines (37, 53). The inhibitory factors that are important in maintaining airway homeostasis are also crucial in resolving inflammation after elimination of the microbial pathogen. Both CD200 and TGF-β assist in the suppression of inflammation, promote resolution and restore homeostasis (47).

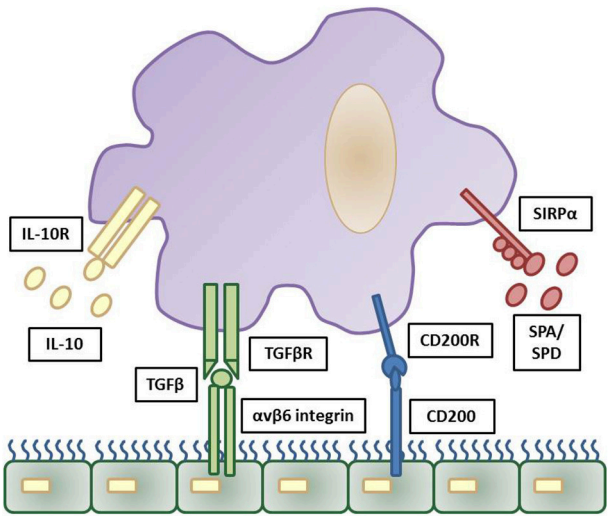

FIGURE 1 | Inhibitory regulation of alveolar macrophages by the airway epithelium. Strict regulation of macrophage activation is required for homeostatic control of the general lung environment. As alveolar macrophages are under constant exposure to airborne endotoxins hypo-responsiveness is required for normal airway macrophage function. This is contributed through a number of downstream pathways triggered by airway epithelial cells production of IL-10, TGFβ, CD200, and surfactant proteins (SPA and SPD) and these reduce pro-inflammatory signaling and phagocytosis in airway macrophages via their respective cell surface receptors. The cascade of downstream inhibitory pathways to suppress macrophage activation are summarized elsewhere. Adapted from (35).

Dominant Viral Infections in the Lung

Human respiratory syncytial virus (hRSV), human rhinovirus (hRV), human parainfluenza virus (hPIV) and human metapneumovirus (hMPV), are the major types of viruses responsible for acute infections of the upper and lower respiratory tract (54). These respiratory viruses represent a significant burden on global public health, with acute respiratory tract infections (ARTIs) being the fourth highest cause of global mortality (55).

Influenza virus is a member of the orthomyxovirus family and a negative sense, single stranded RNA virus (56). The viral envelope of influenza virus is composed of haemagglutinin (HA) and neuraminidase (NA) (57), which are used as identifiers of virus subtypes (58, 59). There are four genera of influenza virus; A, B, C, and D, with the influenza A subtypes H1N1 and H3N2 causing the largest proportion of influenza cases (60). Influenza virus infection is one of the leading causes of respiratory tract infections worldwide, with ∼5–20% of the global population infected and a mortality rate of up to 650,000 patients annually[1] (61). The influenza virus predominantly invades human upper airway epithelial cells by binding to α-2,6 or α-2,3-linked sialy glycans expressed on their surface (62–64). The influenza virus can effectively evade detection by the host immune system. Genetic changes due to the error-prone nature of the viral RNA polymerase, that result in antigenic drift or recombination events between influenza viruses, can give rise to new subtypes of influenza that can lead to epidemic or pandemic outbreaks (65–67). Currently, our best options to combat influenza are by prevention using vaccines and treatment with antiviral medications. However, the variable nature of the virus limits the

[1]https://www.who.int/influenza/en/

efficacy of both approaches as they need to be updated annually to keep up with the evolution of new subtypes (68).

hRSV is the main cause of acute lower respiratory tract infection (ALTRI) in infants, young children and older adults (aged ≥65 years) (69). hRSV is an enveloped negative-sense single-stranded RNA virus belonging to the Pneumoviridae family, Orthopneumovirus genus (69, 70). There are 2 major antigenic groups of hRSV, A and B, which can be further subdivided into 10 A genotypes and 13 B genotypes (71). The highly contagious nature of the virus means nearly all children will have been infected with hRSV by the age of 2 years old (72). Bronchiolitis or pneumonia caused by hRSV infection is the major cause of hospitalisations in children under the age of 2 years old. Additionally, hRSV infection has been implicated in the development of childhood asthma and recurrent wheezing (72–75). The global public health burden of hRSV is significant, with ∼10% of all hospital admissions for severe bronchiolitis or pneumonia due to the virus, representing an annual cost of about 394 million USD (76–78). The severity of hRSV infection and associated clinical symptoms can be controlled by the use of palivizumab, a neutralizing monoclonal antibody to the fusion glycoprotein (F protein), which is a transmembrane surface protein in the viral envelope of hRSV (79–82). However, an effective vaccine against hRSV has yet to be developed.

The development of childhood asthma and recurrent wheezing is not only closely linked with infant hRSV-induced bronchiolitis, but is also associated with wheezing illnesses due to hRV infection in infancy (83–86). A member of the Picornaviridae, genus Enterovirus, hRV is a non-enveloped positive single-stranded virus (87, 88). hRVs can be classified into three species, with RV-A and RV-C, causing more severe respiratory illness, when compared to RV-B (88, 89). The species can be further categorized into genotypes, of which there are over 100 (87, 90). hRVs circulate throughout the year, are transmitted through direct contact or aerosol particles and are capable of infecting both the lower and upper respiratory tracts (87, 91, 92). Symptoms following infection are generally that of the common cold, including sore throat, cough, nasal congestion, sneezing and rhinorrhoea. However, in infants, the elderly, immunocompromised adults or those suffering from chronic respiratory illnesses, infection with hRV can be more severe. For example, hRV is responsible for 20–40% of all hospitalisations due to wheezing in infants aged 12 months or less (93, 94). Development of a vaccine and antivirals against hRV has been hindered by the vast quantity of genetically distinct genotypes (90, 95).

hPIV is second most common cause of ALTRI in children, after hRSV (96). hPIV, like hRSV, is an enveloped negative-sense single-stranded RNA virus of the Paramyxoviridae family (97–99). hPIV consists of four major serotypes—hPIV-1 and hPIV-3, genus Respirovirus and hPIV-2 and hPIV-4, genus Rubulavirus (100). By the age of 2 years old 60% of children have been infected by hPIV-3 and at the age of 5 years the majority have been infected by hPIV-1, hPIV-2 and hPIV-3 (97, 101). Although hPIV has been predominantly viewed as a cause of respiratory illness in pediatric patients, both immunocompromised and older adults are also susceptible to infection (97, 100). Clinical manifestations of infection by hPIV include the common cold, croup (laryngotracheobronchitis), tracheobronchitis, bronchiolitis and pneumonia (100). However, as of yet there is no effective antiviral treatment or vaccine available for hPIV.

Since its discovery in 2001, hMPV has been identified as one of the major causes of upper and lower respiratory tract infection in children, immunocompromised patients and the elderly, being detected in 4–16% of patients with ARTIs (102–108). hMPV, a negative-sense single stranded RNA virus, is a member of the Paramyxoviridae family, genus Metapneumovirus, and is closely related to hRSV and parainfluenza (108). Most infections with hMPV elicit mild to moderate clinical symptoms, although 5–10% of cases result in admission to pediatric intensive care (102, 107, 109).

Bacterial Outgrowths in the Lung Following Viral Infection

A significant contributor to morbidity and mortality in respiratory viral infections is bacterial invasion. Given the colonization of the upper respiratory tract with common pathogens including *Streptococcus (S) pneumoniae, Haemophilus (H) influenzae* and most of the Staphylococcus species, a shift in immunological balance and the airway environment can undoubtedly cause severe secondary bacterial infection in the host. The most famous reports of bacterial colonization after lung viral infection stem from the 1918 influenza pandemic where between 20 and 60 million deaths were due to bacterial co-infection (110). It is estimated that ∼25% of all influenza-related deaths are associated with co-infections, particularly during seasonal outbreaks (111, 112). Viral respiratory infections elevate nasopharyngeal bacterial density (113, 114), which may promote their colonization in the lower airways, though the precise mechanisms are unclear.

Bacterial co-infection is not limited to influenza virus. A retrospective cohort study of 6,000 hospitalized neonates in China showed that 94% had RSV infection, with the remainder having parainfluenza, influenza virus or adenovirus. The dominant co-infections in RSV infected neonates were *E. coli*, *Klebsiella (K) pneumoniae*, *S. aureus*, and *Enterobacter cloacae* (115). The high frequency of RSV and pneumococci co-infection in hospitalized children is reduced by prior pneumococcal conjugate vaccination and has led to the suggestion that treatment for secondary bacterial infections should be considered for pneumonia cases even if a child tests positive for RSV (116). The choice of antibacterial strategy may be critical since RSV can increase *S. pneumoniae* virulence by binding to penicillin binding protein 1a (117) and so penicillin derivatives may be ineffective. Experimental studies on human Rhinovirus 16 infection enhances *H. parainfluenzae*, *Neisseria subflava*, and to a lesser extent *S. aureus* in throat swabs (118). One study in adults revealed that rhinovirus was the most common (23.6%), then parainfluenza virus (20.8%), hMPV (18.1%), influenza (16.7%), and RSV (13.9%). However, virus strain occurrence may also be influenced by co-infections as RSV was significantly

more common in those that also had community-associated pneumonia (119).

Bacterial and viral infections co-exist, and the post-viral bacterial outgrowths are often co-infections made up of different species of bacteria. In a recent meta-analysis, 28–35% of patients demonstrated positive laboratory culture with the co-infective species, *S. aureus* and *S. pneumoniae*, respectively (120). *S. pneumoniae* is the most common pathogen that causes community-acquired pneumonia and potential overwhelming sepsis, and is associated with high mortality and morbidity during influenza epidemics and pandemics (121, 122).

S. aureus, a gram-positive cocci and a common commensal in the nose and skin, is a major cause of bacteraemia (123). It is unclear why *S. aureus* has become a major cause of concern particularly in the pediatric population, of which a study of the 2003–4 season in the USA found that this organism not only dominated influenza-associated childhood mortalities, but was also found to be the most common causative bacterial agent in 46% of isolates, whereby more than 50% were methicillin-resistant strains (111). A rare and severe complication of community-acquired pneumonia is necrotising pneumonia, characterized by pulmonary consolidation, inflammation, necrosis, and ultimately gangrene, which is caused by methicillin-resistant *S. aureus*, a major public health concern due to its resistance to antimicrobials. Prior or co-infection with influenza infection and the presence of Panton-Valentine leukocidin (PVL) are both significantly associated with the necrotising pneumonia (124).

Mechanisms of Bacterial Susceptibility After Lung Viral Infection

Other than a breach of the epithelial barrier, there are a number of modifications to cellular innate immunity in the lung that contribute to secondary bacterial infection.

The Role of Apoptotic Cell Clearance Following Viral Infection in Susceptibility to Secondary Bacterial Infections

Cellular turnover by apoptosis features in health and inflammation. Airway macrophages play an important function in clearing apoptotic cells, a process known as efferocytosis, which is essential in maintaining airway homeostasis (125). Inefficient clearance of apoptotic cells leads to secondary necrosis and the release of damage associated molecular patterns (DAMPs) that subsequently promote an inflammatory response (126). Efferocytosis is mediated by a plethora of receptors that recognize externalized proteins on the cell surface of apoptotic cells. One of the most commonly studied proteins mediating efferocytosis is phosphatidylserine (PtdSer). PtdSer is present on the inner plasma membrane in living cells, but is externalized upon induction of apoptosis (127) by caspase inactivation of flippase (ATP11C) that is required to "flip" PtdSer back into the plasma membrane (128). Caspases also activate scramblases that "scramble" phospholipids in the plasma membrane; promoting exposure of PtdSer on apoptotic cells (129). Other proteins that flag up the presence of an apoptotic cell include oxidized low-density lipoprotein, calreticulin, annexin A1, ICAM-3, C1q, and thrombospondin (130). In parallel there are a number of receptors that recognize these proteins on apoptotic cells, including many that bind PtdSer: Triggering receptor expressed by myeloid cells-2 (TREM2) (131), CD300 (132), receptor for advanced glycation end products (RAGE) (133), Stabilin-2 (134), brain-specific angiogenesis inhibitor-1 (BAI1) (135) and TIM family members (T cell/transmembrane, immunoglobulin, and mucin) (136, 137) (**Figure 2**). For a review of other receptors recognizing externalized molecules on apoptotic cells see (130).

One PtdSer recognizing receptor family pertinent to the lung and its susceptibility to bacterial complications is the TAM receptor family (Tyro3, Axl, and Mertk receptors). These engulfment receptors require bridging molecules to link them to externalized PtdSer; protein S or growth arrest specific 6 (Gas6) (138). MerTK is ubiquitously expressed on macrophages, and even used as a defining marker for them. Axl, however, shows a more restricted distribution and is constitutively expressed on airway macrophages driven by GM-CSF and up-regulated during viral infection (139). Ligation of TAM receptors, in the presence of type 1 interferons (IFNs) enhances the expression of suppressor of cytokine signaling (SOCS) 1 and SOCS3, which reduce TLR and cytokine receptor signaling pathways (140–142). Furthermore, signaling via TAM receptors also induces TGFβ, IL-10 and prostaglandin production (143–146). This anti-inflammatory airway macrophage state is important to tolerate self-cells (125) but also reduces responses to subsequent coinfections (see **Figure 2**). Expression of IL-10 is raised following secondary bacterial coinfection after influenza virus exposure (147, 148). This is likely designed to prevent further tissue damage and to allow a return to homeostasis.

Lung viral infection enhances the apoptotic load due to cytopathology of infected cells and also the requirement to clear the large recruited immune cell infiltrate (149, 150). An absence of the TAM receptor Axl leads to excessive weight loss upon influenza infection in mice (139) that is likely linked to heightened secondary necrosis, which liberates DAMPS (151) recognized by pattern recognition receptors (PRRs), such as RAGE and ST2 (151–153). Axl knockout mice display increased nucleosome release in the airways corroborating the idea of enhanced secondary necrosis. This propagation of severe inflammation is likely to damage the lungs further and enhance the likelihood of secondary bacterial infections. Supporting this idea, prior exposure of mouse airway macrophages to apoptotic cells results in suppression of FcR-mediated phagocytosis and killing of bacteria. Furthermore, intrapulmonary administration of apoptotic cells impairs *S. pneumoniae* clearance from the infected lung (154). Also, suppression of antimicrobial responses of airway macrophages is enhanced by glucocorticoids, which promote efferocytosis, and treatment of mice with apoptotic cells in the presence of glucocorticoids is associated with elevated bacterial burden in the infected lungs (155).

Therefore, the normal process of clearing dying cells can have long term consequences and is particularly evident in chronic lung diseases (156) [for a review see: (157)]. However, further studies are required to determine the importance of this process, including analysis of the redundancy

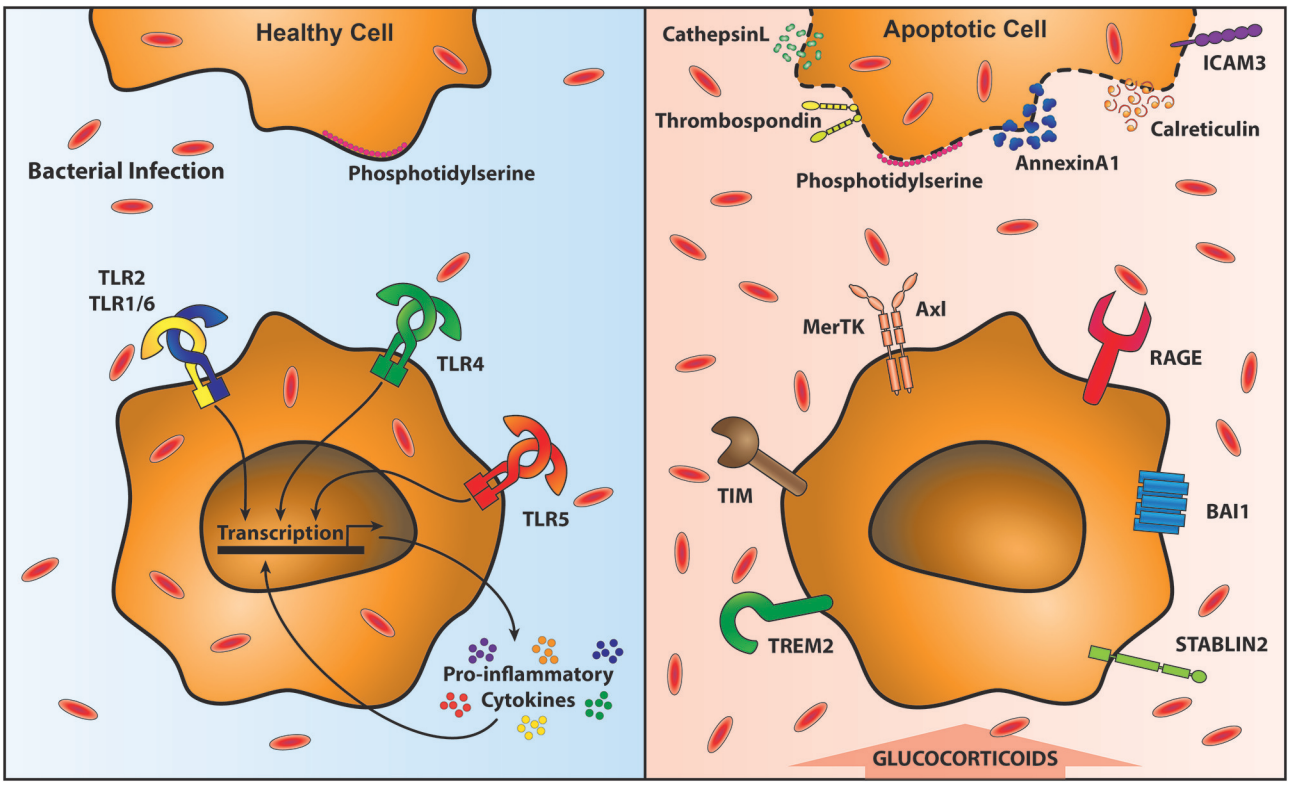

FIGURE 2 | Clearance of apoptotic cells impairs anti-bacterial immunity. Removal of apoptotic cells requires their recognition by specialized receptors on phagocytic cells, including macrophages. In the presence of healthy cells (top left) Phosphatidylserine (PtdSer) is on the inner leaflet of the membrane. Local macrophages do not recognize them and therefore are able to signal through Toll-like receptors (TLR) unimpeded, resulting in the proinflammatory cytokine response. This optimal response is able to contain and clear bacterial infections (shown in red ovals). However, upon programmed cell death, PtdSer and a variety of other proteins are translocated to the outside of the cell membrane (top right). Macrophages recognize these exposed proteins via specific receptors (bottom right). These receptors facilitate apoptotic cell recognition and engulfment (known as efferocytosis) however, during efferocytosis macrophages are unable to respond to bacteria leading to their outgrowth (bottom right).

between apoptotic recognition receptors and the long term outcome of their manipulation. Efficient clearance of apoptotic cells may therefore provide an opportunity for therapeutic manipulation to lessen the severity of lung viral infections and prevent bacterial complications. In addition to the clearance of apoptotic cells by phagocytes, the phagocytes themselves (neutrophils and macrophages) may also undergo apoptosis.

Reduced Responsiveness of PRRs Following Viral Infection

Another natural process that occurs following viral infection is the cessation of inflammation. This is particularly important to allow efficient repair. Therefore, a prolonged inhibition of innate immunity is a common occurrence. However, a timely response to bacterial infection is critical to limit the pathogen load. Any delay in early immunity results in logarithmically higher bacterial loads that are difficult to clear and cause extensive bystander tissue damage. PRRs are important in this regard, but may be impaired by previous or concurrent inflammatory conditions. PRRs are widely expressed in the lungs on airway epithelial cells, alveolar macrophages and DCs and their ligation leads to the release of cytokines, chemokines, eicosanoids and type I IFNs into the airspaces (158, 159). The kinetics of this initial inflammatory wave limits early pathogen replication (159) by recruitment of monocytes, neutrophils and natural killer (NK) cells. NK cells target infected airway epithelial cells that have lost or reduced MHC class I expression (160), whereas monocytes and neutrophils aid alveolar macrophages in removing infected dead cells (161) and co-existing bacteria. Furthermore, type I IFNs stimulate the production of interferon-stimulated genes (ISGs), leading to cell-intrinsic and extrinsic antiviral activity (162). However, many studies have observed that subsequent stimulation via PRRs is defective following lung viral infection. This effect is not restricted to PRRs as defects in multiple processes employed by the mononuclear phagocyte system have been observed (69).

Following an acute viral infection, mouse airway macrophages display a similar phenotype to those in health (CD11c, CD11b, F4/80, and Siglec F). However, their responsiveness to TLR agonists is significantly dampened (163). We called this "innate imprinting" in 2004 (164), which is similar to the concept of "trained immunity" described by others in which monocytes acquire a tolerant phenotype after stimulation (165–167). This

un-responsive state has recently been described as "immune paralysis" (168). In addition to influenza virus, human rhinovirus infection also predisposes to bacterial infection via degradation of IRAK-1 (interleukin 1 receptor associated kinase) leading to enhanced infection of respiratory epithelial cells by *H. influenza* (169). Defective TLR signaling would clearly lead to a reduction in many aspects of inflammation. With respect to subsequent bacterial infections, however, the most damaging consequences are the IFNγ induced impairment of macrophage phagocytosis (170, 171) and the reduction in neutrophil recruitment due to suppressed IL-8 production. In addition to reduced recruitment, neutrophil function also appears impaired following viral infection with reported reductions in myeloperoxidase, reactive oxygen species and the bactericidal properties of neutrophil extracellular traps (NETs) [for a review see (172)]. Reduced recruitment of neutrophils would also impact on airway macrophage NLRP3 inflammasome activation that is important for the production of IL-1β (173–176).

Reduced TLR signaling during viral infection may contribute to the impairment of the IL-17 response required for bacterial containment. Th17 cells produce IL-17 and IL-22 and are regulated by IL-23 (177, 178). These cytokines are crucial for lung epithelial production of neutrophil recruiting chemokines and anti-microbial peptides (179). Influenza virus induced type 1 IFNs reduce IL-17, IL-22, and IL-23 and impair the clearance of *S. aureus*; an outcome that can be rescued by adenoviral delivery of IL-23 (180). Type 1 IFNs also impair IL-17 production from γδ T cells (180).

The anti-inflammatory state that occurs following lung viral infection creates some confusion as patients and mice that succumb to secondary bacterial infection ultimately display enhanced inflammation (147, 181–183). However, a sluggish immune response will ultimately lead to enhanced inflammation due to an exponentially higher bacterial load.

The Impact of Viral Infection on Other Airway Innate Immune Cells

In addition to viral induced modification of airway macrophages, other innate immune cells are also affected. Type-2 innate lymphoid cells (ILCs) increase during influenza virus infection and secrete IL-13 (3), which although important for wound repair, are not useful during bacterial infection. A similar population of Lin^{-} $CD127^{+}$ $ST2^{+}$ $CRTH2^{+}$ ILC2s have also been identified in human lung tissue and BAL and are known to produce IL-13. In mice, methicillin-resistant *Staphylococcus aureus* induces IL-13 up to 3 days after influenza virus infection and impairs viral clearance. Later infection of MRSA after influenza however, exacerbates bacterial replication due to inhibition of IL-13 and an upregulation of IFNγ (184). A detrimental impact of IL-13 is also evident following chlamydia (185) and tuberculosis (186). IL-13 also promotes *Mycoplasma pneumoniae* and non-typeable *H. influenza* adhesion in cultured bronchial epithelial cells by increasing MUC18 (187) and overcomes the enhanced bactericidal effects on epithelial cells of beta-2 agonists (188). Collectively, these studies suggest that ILC2s can be beneficial or harmful depending on their kinetics.

Viral infection induces the early recruitment of NK cells to the lungs where they promote anti-viral immune cells through the release of cytokines and limit viral replication by removing infected cells that have down-regulated MHC class I. If NK cells are depleted, adaptive immunity is not optimal, which could lead to prolonged viral infection (189, 190). NK cells also influence dendritic cells to support Th17 and Th1 cells that are important in anti-bacterial immunity (191) and NK cell production of IL-22 is protective against Klebsiella lung infection (192). However, NK cells appear early in the antiviral response to lung viral infection and so may not be present during secondary bacterial infection. Indeed influenza virus is reported to decrease NK cells, which reduces clearance of *S. aureus* in a process dependent on TNF-mediated enhancement of macrophage phagocytosis (193).

IL-10 is upregulated by viral infection and dampens the activation of invariant natural killer T (iNKT) cells by inhibiting the production of IL-12 by lung monocyte-derived dendritic cells, which contributes to *S. pneumoniae* outgrowth (194). IFNγ increases susceptibility to secondary bacterial infection by promoting inflammation and damage in the upper respiratory tract through both the ligand IFNγ and IFNγ receptor (245). Though IFNγ stimulates a pro-inflammatory phenotype in alveolar macrophages, it inhibits bacterial phagocytosis (49)

Neutrophils are critical components of anti-bacterial immunity. In addition to their reduced recruitment due to impaired chemokine production, influenza virus also inhibits their activity by inhibiting Th17 cell induction of anti-microbial peptides (195). Viral induction of Setdb2 (a protein lysine methyltransferase) also represses the expression of the CXCL1 gene that recruits neutrophils (196) and defective G-CSF production impairs neutrophil digestion and/or killing of phagocytized bacteria via myeloperoxidase (MPO) activity (197).

γδ T cells are also important in susceptibility to secondary bacterial infections. These rare T cells directly recognize pathogen-associated molecular patterns (PAMPs), express a range of cytokine receptors that modulate their function, mediate cell cytolysis via FAS and TRAIL and release anti-microbial peptides and cytotoxic molecules. They also produce IFN-γ, TNF-α, and IL-17. γδ T cell IL-17 production is impaired during influenza infection by type I IFNs causing susceptibility to *S. pneumoniae* infection (198). The role of γδ T cells in the extent of lung inflammation during viral infection depends, however, on whether other underlying conditions are present. For example, γδ T cell depletion in murine models of rhinovirus infection in asthmatic mice enhances airway hyper-reactivity (199).

Mucosal-associated invariant T (MAIT) cells (200, 201) are a recently studied population that are important in mucosal tissues for anti-bacterial immunity. They express cytotoxic markers such as CD107a and granzyme B via synergistic actions of IL-12 and IL-7 (202) and produce IFN-γ, TNF-α, and IL-17A (203). Their role in the lung is beginning to emerge. Lower numbers of peripheral blood CD161(+)Vα7.2(+) MAIT cells are associated with fatality in hospitalized patients with avian H7N9 influenza (204). However, it is not currently known whether defects in this population may predispose to bacteria following virus infections in the lung.

Wound Repair and Bacterial Susceptibility in the Airways

In addition to reduced neutrophil chemoattractants, the post-viral lung may be skewed toward wound repair that will not be conducive for bacterial recognition and clearance. The molecules mediating wound repair are often immune suppressive. IL-10 is enhanced following influenza infection and promotes bacterial replication in the post-influenza virus infected lung (148) by inhibiting multiple facets of immunity; a process that may be driven by the upregulation of indoleamine 2,3-dioxygenase (IDO) (205). Furthermore, regulatory T cells and TGFβ are raised post-viral infection to dampen inflammation and facilitate processes of wound repair; for example by inducing the synthesis of collagen (206). However, TGFβ is also anti-inflammatory and is required to limit the activity of dendritic cells (168). A recent study by the Schulz-Cherry group showed that knockout of the β6 integrin prevents the activation of latent TGFβ leading to the presence of constitutively activated airway macrophages (207). Wound repair therefore represents a double edged sword where anti-inflammatory components limit inflammation and promote repair, but at the same time leave hosts susceptible to bacterial infection.

A few studies have described that epithelial cell proliferation and the expression of lung repair genes are reduced following respiratory viral infection (208, 209). This implies that barrier repair is delayed, which may prolong the access to alternative adhesion and nutrition sources for bacteria.

The importance of the repair process in the outcome of viral and bacterial infection of the respiratory tract is elegantly illustrated by the administration of amphiregulin, which decreases inflammation and lung damage to influenza virus (3) and prevents mortality to a secondary bacterial infection in the absence of any discernible influence on bacterial load (208).

Matrix, Innate Immunity, and Bacterial Adhesion in the Lung

Extracellular matrix is a highly organized structure containing precise patterning of 43 different types of collagen, 200 glycoproteins and 40 proteoglycans (210). These components combine to form the interstitial matrix and the basement membrane. Alterations in both of these impacts on the cellular content of the lung and airways, and the adhesion, growth and location of bacterial species.

The basement membrane contributes to tissue architecture and is a highly organized structure made up of collagen IV, laminins, proteoglycans (decorin, biglycan, aggrecan and versican), heparan sulfate proteoglycans (perlecan and agrin), and nidogen (211). Some of these components can bind to other proteins that have immune modulatory properties. Decorin and biglycan for example, bind TGF-β1 (212) and so any alteration of their density or position will impact on lung inflammation and tissue repair. Similarly, fibrillar collagens type I and III of the interstitial matrix, in addition to binding other collagens and ECM components, also interact with inflammatory cell surface receptors particularly integrins. VLA-1, for example, is expressed on influenza-specific lung CD8+ T cells and binds α1β1 on interstitial matrix facilitating retention of memory CD8+ T cells in the lung (213). It is not hard to imagine that matrix re-modeling due to viral infection will have numerous consequences, such as the retention of a higher immune cell burden (214). Those retained immune cells, however, may not be optimal for subsequent bacterial infections and may even hinder the early migration of anti-bacterial immunity. For a recent review on immune cell:matrix interactions in the lung see (215).

The degradation of matrix can also liberate bioactive fragments now called matrikines which have immune modulatory properties. For example, the proteolytic processing by matrix metalloproteinases, MMP8 and MMP9, of interstitial collagens liberates the bioactive fragment, acetylated tripeptide Pro-Gly-Pro (acetyl-PGP) which promotes lung neutrophil recruitment (216, 217).

Accumulation of extracellular matrix components requires additional effort from interstitial and alveolar macrophages to clear them. This renders them hypo-responsive to subsequent bacteria. Recently we have found that excess hyaluronan induces adverse events in this way (218). Hyaluronan is a glycosaminoglycan that is abundant in the lung interstitial matrix. It is extruded from cells by hyaluronan synthases forming long cable-like polysaccharide structures. Degradation of hyaluronan is mediated by hyaluronidases. High- and -low molecular weight hyaluronan is reported to be anti-inflammatory and pro-inflammatory, respectively (219). Furthermore, hyaluronan can be sampled in sputum and by bronchoalveolar lavage, suggesting accumulation in the airways (220, 221). We have recently reported that hyauronan continues to accumulate in the lung and airway long after resolution of acute influenza virus infection in mice due to excess production via HA synthase 2. Furthermore, this excess hyaluronan is cross-linked with inter-α-inhibitor heavy chains due to elevated TNF-stimulated gene 6 expression. IαI is a proteoglycan containing two heavy chains of ∼80 kDa, and a light chain (bikunin) of ∼25 kDa that confers protease inhibitory properties (222, 223). Circulating IαI leaks into tissues during inflammation. Its synthesis has also been described in lung epithelia where it mediates repair after lung injury (224). In our study, administration of intranasal hyaluronidase completely restored lung function without any deleterious side effects (218).

There are other examples of matrix alterations contributing to the pathogenesis of lung viral infections (225). Influenza infection induces the recruitment of myeloid cells expressing membrane type I matrix metalloprotease (MT1-MMP/MMP-14) that is important in lung development and homeostasis (226). MT1-MMP inhibition rescues tissue damage and mortality in influenza-infected mice and combined with the anti-viral, oseltamivir, affords complete recovery. Furthermore, MT1-MMP inhibition also prevents outgrowth of *S. pneumoniae* following influenza infection (227). The modulation of extracellular matrix may depend on the viral strain. Analysis of RNA datasets from patients infected with pandemic associated influenza strains shows that H5N1 and H7N9 infection are enriched for genes involved with the extracellular matrix pathway (228). The importance of lung recovery and resilience is also demonstrated in mice lacking endophilin B2 that display

improved mechanosensing and collagen and elastin ECM remodeling compared to wild-type mice (229). There are many other examples where matrix and associated components impact on lung immunity, which have been comprehensively reviewed elsewhere (230).

In addition to viruses directly promoting bacterial adherence (e.g., the neuraminidase in influenza virus exposes bacterial attachment sites by cleaving sialic acids, which are also metabolized by bacteria as a food source (231)), viral induced changes in extracellular matrix will change the lung microbiome. Dysbiosis of microbial commensalism can significantly impact on the overall health and progression of disease. Bacteria and bacterial products induce phenotypic and functional changes in immune pro-inflammatory gene expression, cellular adhesion and migration, and cell death (232). Binding to the ECM allows bacteria to adhere to, and colonize, host tissue. In addition, bacteria demonstrate affinity for different matrix substrates and changes in ECM components may increase host-pathogen accessibility and increase of bacterial virulence (233).

A number of microbes have elastase activity and/or express binding proteins for elastin that aid their pathogenicity (234). *S aureus* binds to elastin rich sites and expresses elastin binding proteins (EbpS) which bind to soluble, but not structurally intact chains of elastin (234). The expression of EbpS is also associated with greater bacterial cell growth, promoting cell proliferation and colonization (234, 235) and evasion of phagocytosis (234). In addition, elastin proteolytic products induce MMP activity and a number of bacteria express elastases (234, 235) further promoting elastin availability and consequently bacterial binding.

S. aureus encodes the fibronectin binding proteins (FnBPs), MSCRAMM (microbial surface component recognizing adhesive matrix molecule) that adhere to fibronectin and fibrinogen (236). Since components of fibronectin influence TLR4 receptor signaling, FnBPs may also promote immune regulation (237). Bacteria express collagen receptors and their binding appears to depend on collagen fiber tensile strength, conformation and structural dynamics. In an *in vitro* model, applying increasing high tensile forces to collagen peptides restricts receptor binding, suggesting that structurally normal collagen fibers decrease available sites for bacterial adhesion. Injured states, where collagen fibers are cleaved by high MMP activity, may increase susceptibility toward bacterial colonization with reduced structural strength and increased accessibility for more bacterial binding capacity (238).

Von Willebrand factor (vWF) is a large multimeric adhesion molecule and stimulates adhesion of bacteria. In bacteria such as *S aureus*, adherence to host can also be mediated via vWF and bacterial binding protein staphylococcal protein A (SPA). SPA binds to soluble and insoluble forms of vWF, promoting bacterial attachment and enhancing virulence in the absence of immune cell detection and clearance (239).

Glycosaminoglycan (GAG) interactions are ubiquitously used for cellular and extracellular signaling in all biological processes. Microbes utilize this universal process of the host for binding, and colonization of the host environment. Bacteria express GAG species and different binding domains across their entire surface. Studies blocking, removing or decreasing expression of these GAG binding domains decrease bacterial virulence (attachment, colonization and infection) in a number of bacterial strains (240). Bacterial communities have different affinities for GAG species (240). A large study manipulating GAG binding domains showed that the removal of heparin sulfate in *S. aureus* and *S. pneumoniae* decreases bacterial attachment to lung epithelial cells and fibroblasts and the inhibition of synthesis produced the same effect (240). The normal GAG interactions of the host are also used by microbes to prevent immune detection and clearance. Bacteria such as Streptococcus coat their surface with soluble high molecular weight hyaluronan, inhibiting detection and clearance by macrophages (241). Degradation of hyaluronan from the host tissues or bacteria into the low molecular weight protein stimulates phagocytosis, demonstrating bacterial colonization and infection can be influenced by the processing of GAGs from both the bacterium and host (241).

Fast Inflammation Is Good

Interestingly, a time limited burst of inflammation from the outset is beneficial during influenza infection in mice, which results in faster clearance and less collateral damage (**Figure 3**). The evidence to support this comes from detailed studies on IL-10 knockout mice and the response to pathogen clearance discussed earlier, and our studies with CD200 or CD200R knockout mice. CD200R signaling on myeloid cells limits inflammatory activity (242). Mice lacking CD200 or CD200R show heightened weight loss during influenza infection due to raised levels of inflammation (47). However, when these mice are next exposed to *S. pneumoniae*, they do not show susceptibility, because the first inflammatory event to influenza was quicker, thus causing less collateral damage (243). The benefit of a short

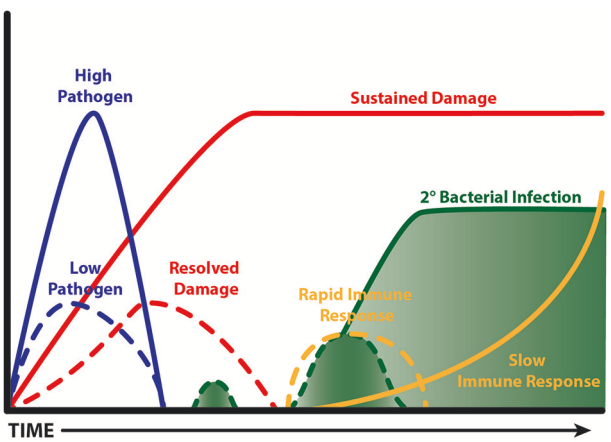

FIGURE 3 | Fast and limited immunity is good. A time limited burst of inflammation limits bystander tissue damage, which in turn limits the extent of tissue repair. This leads to less impairment of anti-bacterial immunity and so a secondary bacterial infection is cleared. A virulent pathogen, or one that isn't cleared quickly, causes prolonged bystander tissue damage leading to a lengthy period of repair; the processes of which are anti-inflammatory. A subsequent bacterial infection is ignored and grows exponentially. Ultimately, innate immunity is activated when the bacterial load is excessive causing deleterious consequences.

burst of inflammation has recently been supported by data from the Metzger group where mice lacking SOCS-1 or IFNγ cleared influenza virus faster than littermate controls due to a rapid induction of immunity. By contrast, in the presence of SOCS-1, inflammation was prolonged and collateral damage increased (171). It would be interesting to test the impact of subsequent respiratory bacterial infection in the SOCS1 and IFNγ deficient model. Such studies might suggest that patients experiencing severe disease do so because their immune system is too sluggish. However, upon presentation at care facilities it would be too late to consider boosting immunity. The speed of immunity could possibly be specifically tackled in patients with other underlying conditions that render their innate immune system suppressed, as in the case of chronic obstructive pulmonary disease, or of the wrong phenotype to limit viral replication, as in the case of asthma. These patient groups are known to be at risk of severe viral infections [for example see (244, 245)].

The benefits of rapid induction of immunity to viral infection are also supported by research on IL-22. IL-22 is an interesting cytokine that is produced by innate immune cells and is critical for host protective immunity to lung *K. pneumoniae* (246), and *S. aureus* (183), but not to Mycobacterium tuberculosis or *M. avium* infection (164). IL-22 is upregulated during lung infection, but its neutralization has no effect on the kinetics of the disease or viral clearance. Rather it seems to function by promoting epithelial integrity and limiting lung damage (3, 247–249), which in turn prevents secondary lung infections by *S. pneumoniae* in mice (250). Interestingly, progesterone treatment of female mice also induces heightened IL-22 (and TGFβ and IL-6) and promotes faster recovery from influenza infection in female mice via epithelial production of amphiregulin. The resultant improvement of pulmonary function and reduced protein leakage is likely to diminish the risk of bacterial outgrowth, though this was not tested (251). In murine models of influenza infection, administration of GM-CSF promotes resistance to *S. pneumoniae* by promoting neutrophil recruitment and reactive oxygen species production from macrophages (252).

Another study that supports stimulation of immunity to prevent bacterial super-infections showed that the TLR-2 agonist, macrophage-activating lipopeptide 2 (MALP-2), reduces pneumococcal outgrowth in influenza virus infected mice (253). Also administration of nanoparticles containing the coat protein of a plant virus (papaya mosaic virus) and a single-stranded RNA causes the rapid recruitment of neutrophils, monocytes/macrophages and lymphocytes with beneficial effects on influenza virus and subsequent *S. pneumoniae* infection (254).

Creating a Debate in Matrix Modulation

Matrix modulation research is a field with great potential in restoring immune function via alternative key mechanisms. Extracellular matrix production is elevated following severe acute viral infection, which could have consequences on cell retention, immune paralysis of phagocytic cells and the physical properties of the airspaces into which it leaks. Respiratory fluids from COPD patients for example, contain higher levels of hyaluronan (HA) than healthy controls (59, 218) and we have recently shown this is exacerbated further by viral infection in COPD patients. Hyaluronidase treatment of mice after resolution of influenza virus infection restores lung function suggesting that the consequences of increased airway and lung hyaluronan is an impaired lung physiology (218). Airway hyper-reactivity is also improved during ozone-induced airway disease in CD44 or IαI deficient mice (60, 255) that bind HA or cross-link it, respectively. TNF-stimulated gene 6 catalyzes the transfer of IαI heavy chains onto HA (256) and TSG-6 null mice are resistant to airway hyporesponsiveness (257). Also TSG-6 promotes anti-inflammatory macrophages, (258) and inhibits neutrophil recruitment (259–262) and NFκB nuclear translocation. Just considering one matrix protein such as hyaluronan, the method of its production and degradation and the proteins that cross link it, provides multiple avenues for modulation. Therapeutic development in this area, to our knowledge, is poor with most focus on neutralizing enzymes that degrade matrix to prevent the liberation of small chemotactic matrix products. However, recombinant human hyaluronidase is licensed for therapeutic use in humans to increase barrier permeability, and although it is currently approved to enhance delivery and absorption of subcutaneous anesthetics, increase uptake of fluids, and to improve resorption of radiopaque agents (263, 264), it has the potential to be used to improve inflammatory diseases by immune-matrix modulation.

CONCLUSION

Bacterial susceptibility following lung viral infection has been recognized for over a century and yet treatment options have not really altered since the introduction of antibiotics. It is now clear that long term suppression of innate immune mechanisms occurs following severe acute or chronic inflammation. In contrast to the clinical susceptibility toward bacterial infection that can occur in the 7 days following a viral infection, there are multiple long term modifications in immune mechanisms long after severe viral infections. These changes re-set the inflammatory tone of various immune cells by processes now known as trained immunity, innate imprinting or immune paralysis (164–166, 168). These molecular changes are evident during peak infection, but not in naïve un-infected lungs. This modified, tardy innate immunity immune response contributes to dysregulation of immune mechanisms to secondary bacterial exposure, rather than the clearance of the initial pathogen, and hence may explain the higher risk of long term bacterial outgrowth and chronic infection that cumulatively leads to excessive inflammatory disease. The majority of pathways leading to bacterial complications following viral infection have been discovered in single mouse strain studies. A recent report from the Metzger group shows that different mouse strains (BALB/C and C57BL/6) react differently to alveolar macrophage depletion following acute influenza infection. BALB/c mice respond to an acute influenza insult via IFNγ dependent alveolar macrophage depletion, whereas C57BL/6 mice do not. However, both are susceptible to post-viral bacterial coinfection (265). The precise combination of changes leading to bacterial super-infection may therefore be slightly different depending on genetic background.

Another area that requires development is that the known "at risk" patient groups currently identified for priority influenza vaccination (the elderly, asthmatic, pregnant etc.) do not account for the vast hospitalization numbers over the winter seasons. This suggests there may be other "at risk" groups.

This as exemplified by the rise of bacterial pneumonias in those experiencing low mood, stress, anxiety or mental health issues (266, 267). A mucosal barrier breach cannot explain all infectious complications. A population-based Danish study of 976,398 individuals, including 142,169 with a history of depression, onset of depression was associated with increased respiratory viral or bacterial complications ($IRR = 1.58$; $CI = 1.36–1.85$; $p = 0.000$) (268). Depression and stress are linked to suppression of multiple arms of innate and adaptive immunity [see (269) and references within], including a reduction of neutrophils (270) that are important for bacterial clearance. The link between mental health and infection is an area that will gain momentum in the next few years. Another area of concern that will likely garner research effort in the future is the influence of polypharmacy on respiratory infectious risk. In elderly patients hospitalized for pneumonia in Canada, 45% were taking 5 or more medications prior to hospital admission (271). A number of these medications may also modulate the immune system, though research in this area is sparse.

There is a window of opportunity between recovery from viral infection and the onset of bacterial outgrowth where innate immunity could be primed to react quicker. This may involve removal of immune suppressive pathways (CD200R, IL-10, and TGFβ), facilitation of apoptotic cell clearance (as apoptotic cell recognition receptors switch off innate immunity) or timely removal of high molecular weight matrix components from the airways. To identify these, studies are required that take into account other comorbidities, mental health status and the impact of polypharmacy on outcome.

AUTHOR CONTRIBUTIONS

EC, DM, CC, OB, SL, and TH drafted the manuscript. SL, OB, CJ, JC, and RR provided critical revisions and conceptual diagrams. All the authors made substantial contributions to the conception and design of the work, approved the submitted version of the manuscript, and agreed to be accountable for all aspects of the work.

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HRV16 Impairs Macrophages Cytokine Response to a Secondary Bacterial Trigger

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Human rhinovirus is frequently seen as an upper respiratory tract infection but growing evidence proves the virus can cause lower respiratory tract infections in patients with chronic inflammatory lung diseases including chronic obstructive pulmonary disease (COPD). In addition to airway epithelial cells, macrophages are crucial for regulating inflammatory responses to viral infections. However, the response of macrophages to HRV has not been analyzed in detail. We used *in vitro* monocyte-derived human macrophages to study the cytokine secretion of macrophages in response to the virus. Our results showed that macrophages were competent at responding to HRV, as a robust cytokine response was detected. However, after subsequent exposure to non-typeable *Haemophilus influenzae* (NTHi) or to LPS, HRV-treated macrophages secreted reduced levels of pro-inflammatory or regulatory cytokines. This "paralyzed" phenotype was not mimicked if the macrophages were pre-treated with LPS or CpG instead of the virus. These results begin to deepen our understanding into why patients with COPD show HRV-induced exacerbations and why they mount a defective response toward NTHi.

Keywords: macrophage, rhinovirus, phagocytosis, cytokine, bacteria

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INTRODUCTION

In chronic airway inflammatory diseases such as chronic obstructive pulmonary disease (COPD) viral infections are considered a key driver for disease exacerbations. Human rhinovirus (HRV) is frequently isolated from COPD patients during exacerbations (1, 2). Although exacerbations are likely multifactorial (3), experimental rhinoviral infections in patients with COPD have been successfully utilized to understand the impact of experimental "single" infections to clinical outcomes. Patients with inflammatory airway diseases experience increased lower respiratory tract symptoms and associated fall in lung function parameters in comparison to similarly infected healthy volunteers (4, 5). Recent work in COPD patients has highlighted an increase in infections, bacterial burden and outgrowth of pathogenic bacterial species in viral infected patients (6, 7). More recently, a 2-year longitudinal follow up study (AERIS) of well-characterized COPD patients at stable state and at exacerbations reported a large increase in bacterial and viral coinfections during exacerbations (2), suggesting a possible role for viruses in regulating host defense response to bacterial infections.

Epithelial cells and innate immune cells resident in the airway lumen are key regulators of inflammation and clearance following infections. Viral infection of these cells results in abundant cytokine and chemokine release (8–13). Although epithelial cells are the primary site for HRV infections, airway macrophages are also permissive to rhinoviruses (14, 15) and regulate inflammatory responses to HRV. Furthermore, there are numerous reports of dysfunction of airway macrophages in COPD, as reviewed by Jubrail et al. (16). The emerging clinical literature of co-infections coupled with the reported bacterial clearance defects highlight the importance of dissecting cellular responses in macrophages to multi-pathogen infections and

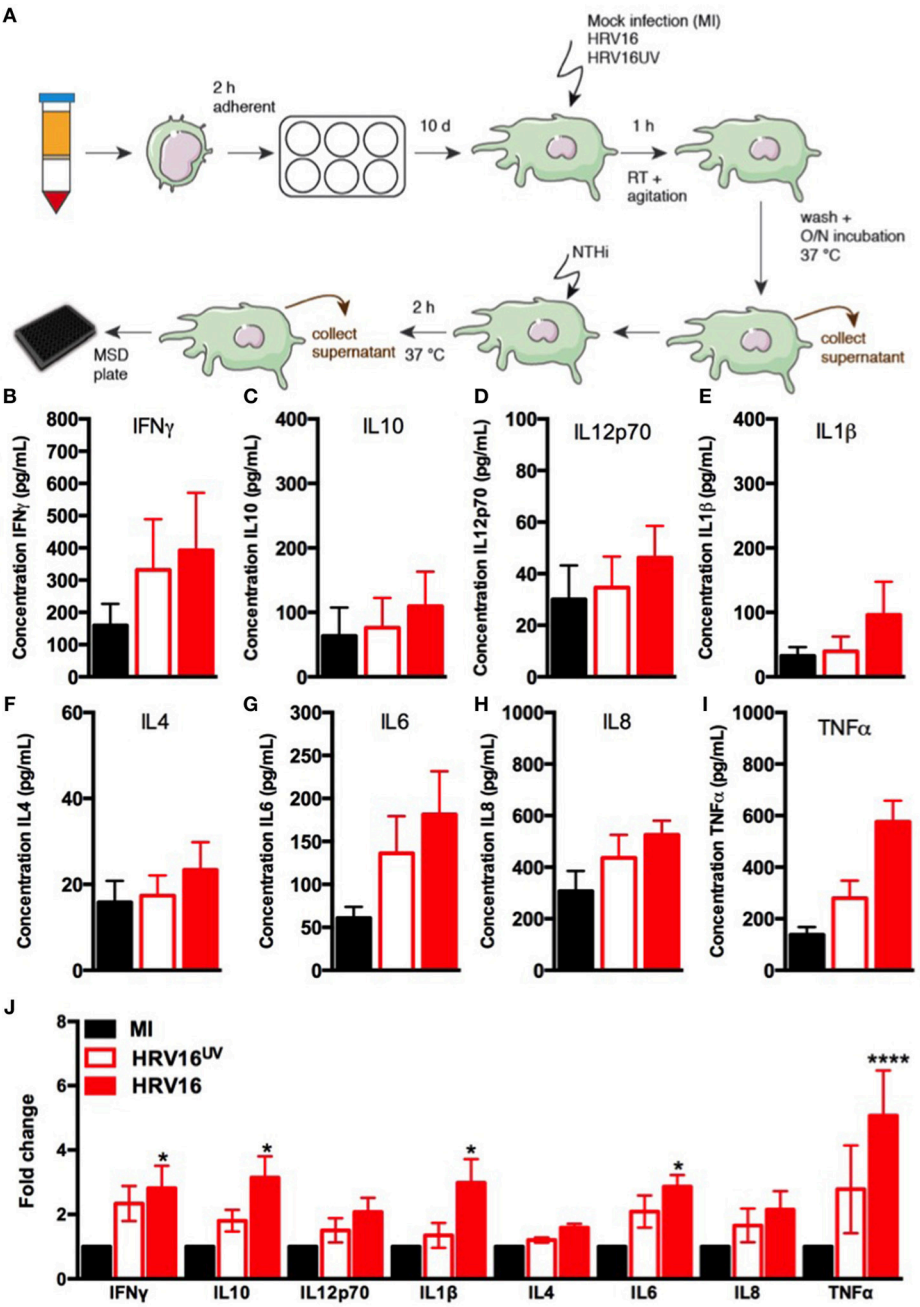

FIGURE 1 | Human macrophages release pro-inflammatory and regulatory cytokines in response to HRV16. **(A)** Experimental protocol: human macrophages differentiated from blood monocytes were exposed to HRV16 (red bars), HRV16^{UV} (open bars) or MI (black bars) for 1 h and rested overnight. Supernatants were collected and analyzed by MSD. MSD results for **(B)** IFNγ, **(C)** IL10, **(D)** IL12p70, **(E)** IL1β, **(F)** IL4, **(G)** IL6, **(H)** IL8, **(I)** TNFα, **(J)** Relative fold changes for cytokine production in HRV16 and HRV16^{UV} exposed human macrophages vs. MI. $n = 5$ independent experiments on different donors. Error bars represent standard error of the mean (SEM). *$p < 0.05$, ****$p < 0.0001$ Two Way Anova with Dunnett's Post Test vs. MI.

the regulation of inflammatory responses in these cells. This work therefore addresses the hypothesis that viral infections can regulate inflammatory cytokine release on subsequent bacterial infections in human macrophages.

MATERIALS AND METHODS

Cell Culture

Human primary monocytes were isolated from the blood of healthy donors (Etablissement Français du Sang, Ile de France, Site Trinité) with the appropriate ethics prior approval as stated in the EFS/ Inserm agreement #15/EFS/012 and #18/EFS/030, ensuring that all donors gave a written informed consent, and providing anonymized samples. Density gradient sedimentation in Ficoll (GE Healthcare) was followed by adhesion on plastic at 37°C for 2 h and culture in the presence of macrophage medium (RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum (FCS) (Eurobio), 100 µg/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen/Gibco). Monocyte-derived macrophages were then obtained as described previously (17). HeLa Ohio cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and were cultured in DMEM GlutaMax containing 25 mM D-glucose and 1 mM sodium pyruvate (Life Technologies) supplemented with 10% FCS, 100 µg/ml penicillin/streptomycin and 2 mM L-glutamine. They were passaged every 3 days.

Preparation of Human Rhinovirus 16 and Non-typeable *Haemophilus Influenzae*

Human rhinovirus 16 (HRV16) (VR-283, strain 11757, lot 62342987) was purchased from the American Type Culture Collection (ATCC) and stocks were produced by infecting HeLa Ohio cells as described previously (18). Briefly, supernatants from infected or mock-infected (MI) cells were collected after 48 h and clarified. In certain experimental conditions, HRV16 was inactivated with UV light (1000 mJ/cm^2) for 20 min. Inactivation was confirmed by adding the inactivated virus to HeLa Ohio cells and checking for cytopathic effects.

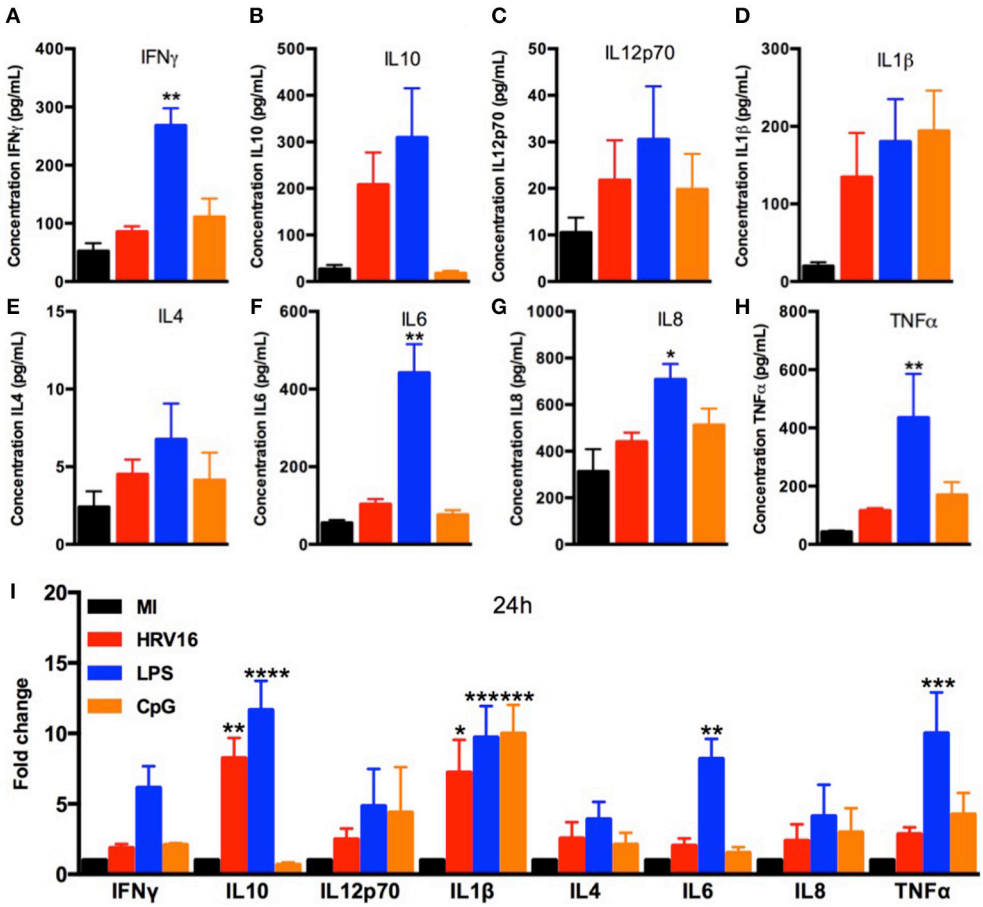

FIGURE 2 | Human macrophages secrete pro-inflammatory and regulatory cytokines in response to LPS and CpG. Human macrophages were exposed to HRV16 (red bars), LPS (blue bars), CpG (orange bars) or MI (black bars) for 1 h and rested overnight. Supernatants were collected and analyzed by MSD. MSD results for **(A)** IFNγ, **(B)** IL10, **(C)** IL12p70, **(D)** IL1β, **(E)** IL4, **(F)** IL6, **(G)** IL8, **(H)** TNFα. *$p < 0.5$, **$p < 0.01$ Kruskal Wallis Test with Dunn's Post Test vs. MI. **(I)** Relative fold changes for cytokine production in HRV16, LPS and CpG exposed human macrophages vs. MI. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ Two Way Anova with Dunnett's Post Test vs. MI. Error bars represent standard error of the mean (SEM). $n = 4$ independent experiments on different donors.

NTHi strain RdKW20 (19) was purchased from the ATCC (51907). It was grown on chocolate agar plates (Biomerieux) at 37°C overnight. Bacteria were grown in LB medium supplemented with 10 μg/ml hemoglobin and 2 μg/ml β-NAD.

Quantification of the Tissue Culture Infective Dose 50 ($TCID_{50}$) of HRV16

HeLa Ohio cells were cultivated in 96 well plates at 1×10^5 cells/well for 24 h. HRV16 was diluted 10-fold from undiluted to 10^{-9} in virus medium (DMEM GlutaMax containing 25 mM D-glucose and 1 mM sodium pyruvate supplemented with 10% FCS and 2 mM L-glutamine). Fifty microliter of each dilution was added to the cells in 8 replicate wells. Fifty microliter of virus medium was added to 2 groups of control wells in 8 replicate wells per group. Cultures were incubated for 4 days at 37°C until cytopathic effect was observed in 50% of wells. $TCID_{50}$ was calculated using the Spearman-Karber formula as previously outlined (18).

HRV16 and NTHi/LPS Infection of Human Macrophages

Macrophages were washed once in PBS and rested in virus medium. HRV16, $HRV16^{UV}$ or MI supernatants were added to the macrophages and placed at room temperature for 1 h with agitation to achieve a $TCID_{50}$ of 1×10^7/ml. Cultures were then washed with virus medium and rested in macrophage medium for 24, 48 or 72 h. Prior to bacterial infection or LPS (Sigma) treatment, culture supernatants were collected and stored at −80°C for further analysis.

NTHi was grown until mid-log growth phase, centrifuged at 1692 *x g* for 5 min and resuspended in 1 ml phagocytosis medium (RPMI supplemented with 2 mM L-glutamine). NTHi was added to macrophages pre-treated with HRV16, $HRV16^{UV}$ or MI to achieve a multiplicity of infection (MOI) of 10/cell. Cultures were then centrifuged at 602 *x g* for 2 min and placed at 37°C, 5% CO_2 for 2 h. Alternatively, LPS was added to

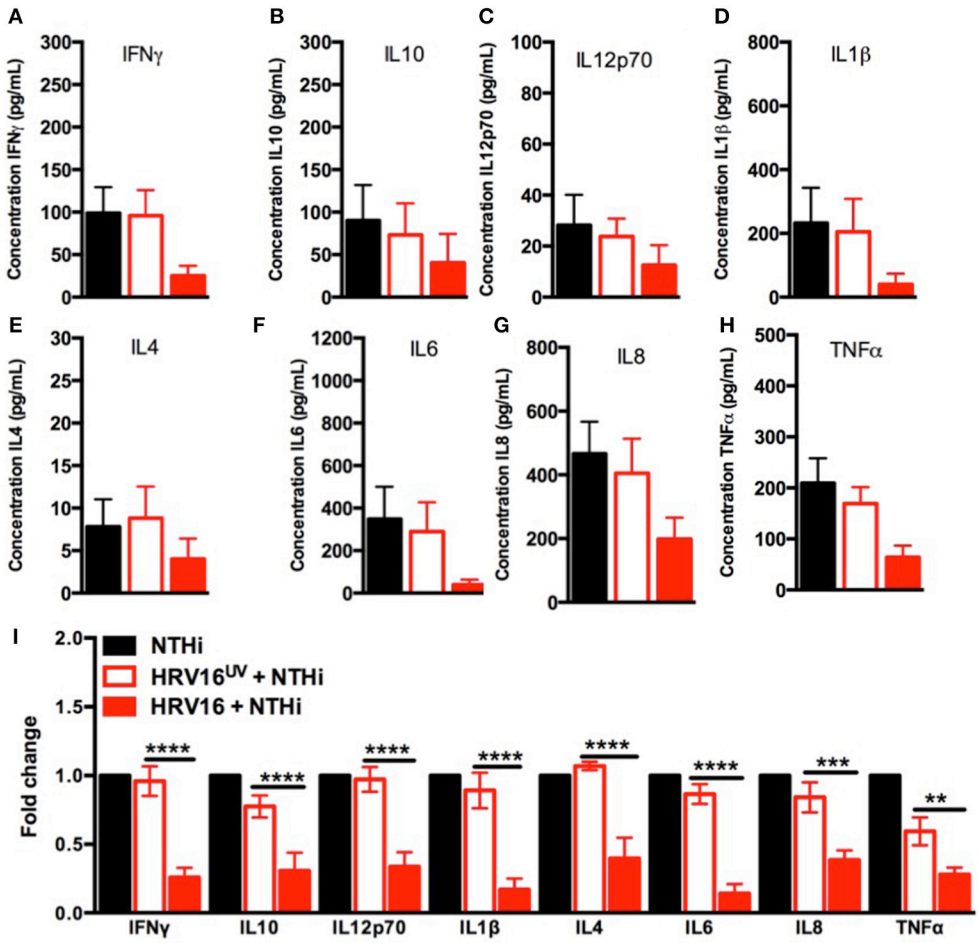

FIGURE 3 | Human macrophages exposed to HRV16 cannot secrete elevated levels of pro-inflammatory and regulatory cytokines in response to NTHi. Human macrophages were exposed to HRV16 (red bars), $HRV16^{UV}$ (open bars) or MI (black bars) for 1 h and rested overnight before being exposed to NTHi for 2 h. Supernatants were collected and analyzed by MSD. MSD results for **(A)** IFNγ, **(B)** IL10, **(C)** IL12p70, **(D)** IL1β, **(E)** IL4, **(F)** IL6, **(G)** IL8, **(H)** TNFα. **(I)** Relative fold changes for cytokine production in HRV16 and $HRV16^{UV}$ exposed human macrophages *vs* NTHi. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ Two Way Anova with Dunnett's Post Test vs. $HRV16^{UV}$. Error bars represent standard error of the mean (SEM). $n = 5$ independent experiments on different donors.

macrophages at a concentration of 10 ng/ml. After centrifugation, cultures were placed at 37°C, 5% CO_2 for 2 h. At this time point, supernatants were collected and stored at −80°C for further analysis.

Lipopolysaccharide and CpG Stimulation of Human Macrophages

Macrophages were washed once in PBS and stimulated with 10 ng/ml LPS or 0.6 μM CpG in macrophage complete medium for 24, 48 or 72 h. At each time point cultures were washed with PBS and stimulated with NTHi as listed above.

Cytotoxicity Assay

Cytotoxicity has been measured by detection of Lactate Dehydrogenase (LDH) released in the cell supernatant with the Cytotoxicity Assay Kit according to the manufacturer's instructions (Pierce).

Analysis of Cytokine Production Using Meso Scale Discovery®

Cytokine production by macrophages was analyzed using the Meso Scale Discovery® technology according to the manufacturer's instructions.

Statistical Analysis

Statistical tests were performed using Graphpad prism® version 6 software. All statistical tests are listed in the figure legends and significance was determined if $p < 0.05$.

RESULTS

HRV16 Infection Induces Robust Cytokine Production From Human Macrophages

In order to assess the ability of macrophages to respond to HRV, we challenged them with HRV16, $HRV16^{UV}$ or MI as controls, for 1 h at room temperature followed by an overnight rest. Supernatants from virus-treated or control macrophages were analyzed by MSD to detect cytokine secretion (**Figure 1A**).

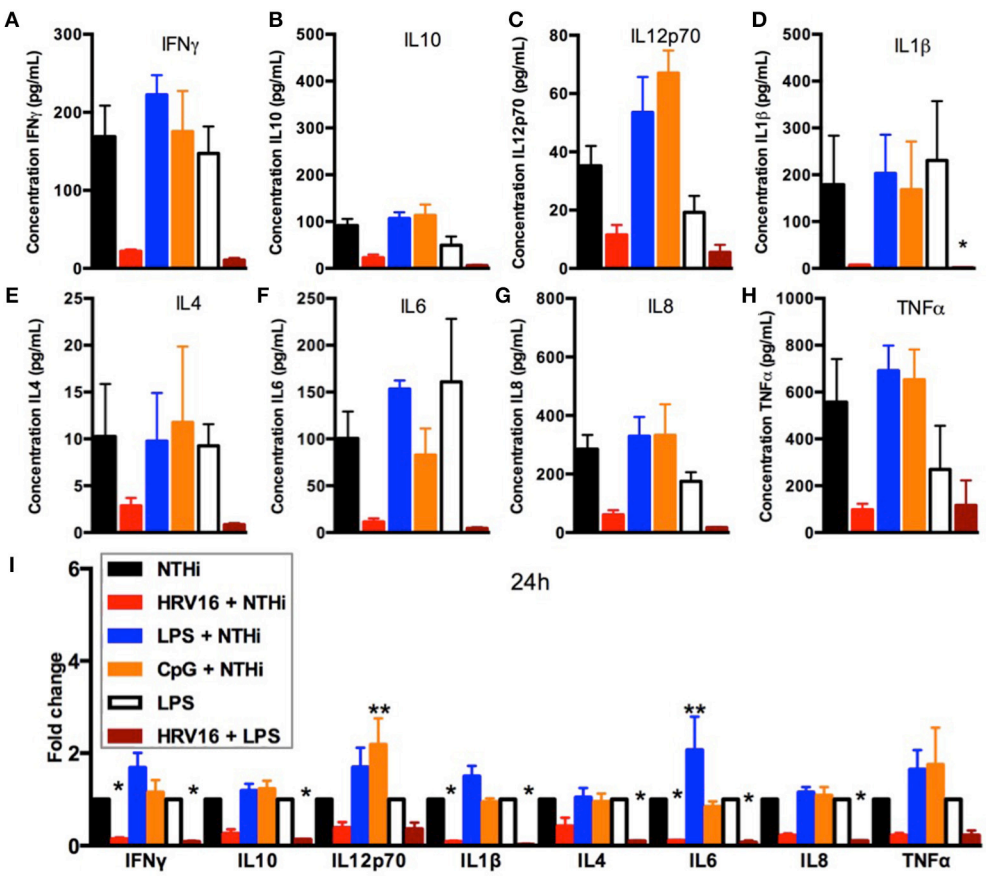

FIGURE 4 | Cytokine response of human macrophages exposed to HRV16 or TLR agonists for 24 h and then challenged with NTHi or LPS. Human macrophages were exposed to HRV16 (red bars), LPS (blue bars), CpG (orange bars) or MI (black bars) for 1 h and rested overnight. Then they were exposed to NTHi or LPS for 2 h and supernatants were collected and analyzed by MSD. MSD results for **(A)** IFNγ, **(B)** IL10, **(C)** IL12p70, **(D)** IL1β, **(E)** IL4, **(F)** IL6, **(G)** IL8, **(H)** TNFα. *$p < 0.05$ Kruskal Wallis Test with Dunn's Post Test vs. NTHi or MI + LPS. **(I)** Relative fold changes for cytokine production in HRV16, LPS or CpG exposed human macrophages. *$p < 0.05$, **$p < 0.01$ Two Way Anova with Dunnett's Post Test vs. NTHi or MI + LPS. Error bars represent standard error of the mean (SEM). $n = 4$ independent experiments on different donors.

We found that HRV16 infected macrophages produced pro-inflammatory and regulatory cytokines at 24 h (**Figures 1B–J**). When we analyzed the fold changes in comparison to the MI control (**Figure 1J**), we observed that IFNγ, IL12p70, IL4, IL6, and IL8 were produced to a similar level by HRV16 and $HRV16^{UV}$. For IL10, IL1β, and TNFα, however, there was a trend toward more secretion after treatment with HRV16 vs. $HRV16^{UV}$ and significantly more secretion vs. MI (**Figure 1J**). These results demonstrate that macrophages are competent to respond to HRV16 and effectively secrete cytokines in response to HRV.

We next wanted to compare cytokine secretion in response to HRV16 to other known stimuli such as the TLR agonists LPS and CpG. For this, macrophages were treated with HRV16, LPS or CpG for 1 h and then rested overnight. We found that LPS stimulation led to increased production of all cytokines tested (**Figures 2A–I**), with a significant difference for IL10, IL1β, IL6, and TNFα compared to control (MI) macrophages (**Figure 2I**). CpG stimulation also led to cytokine production with the exception of IL10 and IL6 (**Figure 2A–I**). These results demonstrate that the HRV16 induced cytokine responses are similar to potent TLR macrophages activators.

HRV16 Infection Impairs Cytokine Secretion From Human Macrophages in Response to NTHi

We next assessed the ability of macrophages to respond to a secondary bacterial trigger. Macrophages were first treated with HRV16 or controls and were challenged 24 h later with NTHi for 2 h. Supernatants were collected and analyzed for cytokine secretion by the MSD technology (**Figure 1A**). We found that HRV16 exposed macrophages were unable to secrete pro-inflammatory and regulatory cytokines in response to NTHi (**Figures 3A–I**). There was a diminished production of all cytokines analyzed in macrophages exposed to HRV16 (**Figure 3A–I**) as compared with $HRV16^{UV}$ or MI, with the greatest decreases seen for IL1β and IL6 (**Figures 3D,F,I**). It is interesting to note that for all cytokines analyzed, $HRV16^{UV}$ + NTHi and MI + NTHi exposed macrophages had a similar response (**Figures 3A–I**). Analysis of the fold changes as compared with MI in these experiments more clearly demonstrated that HRV16 infected macrophages showed significantly diminished cytokine production in response to NTHi (**Figure 3I**). In contrast, despite HRV16 and $HRV16^{UV}$

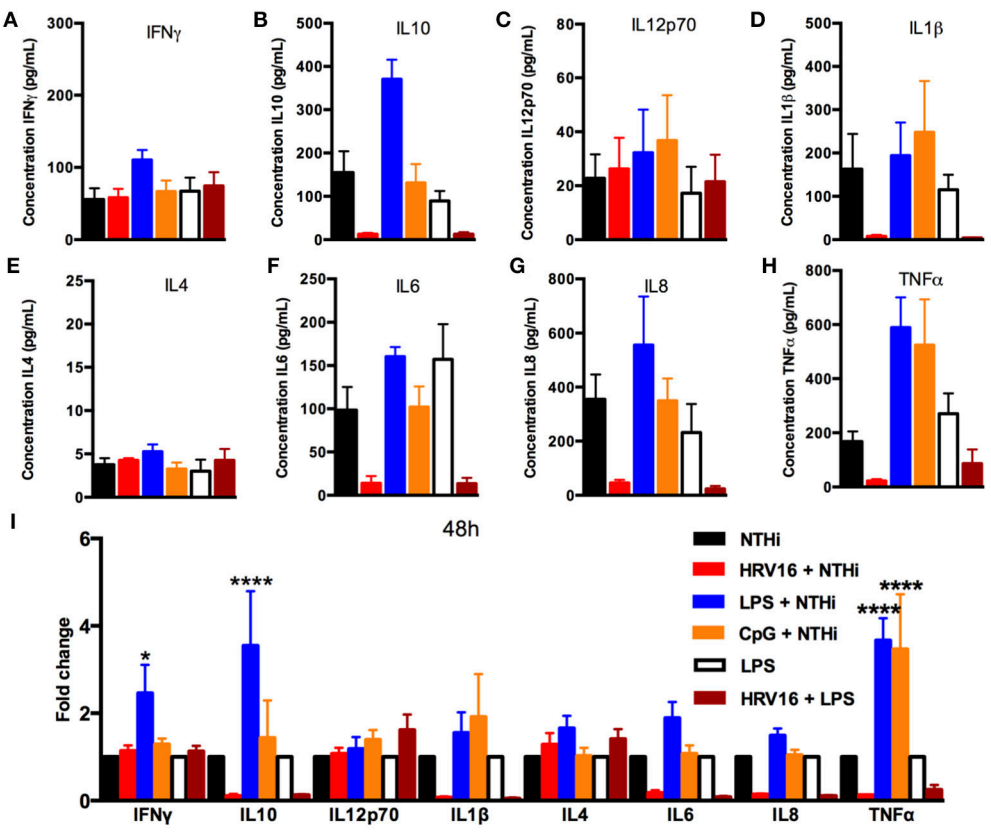

FIGURE 5 | Cytokine response of human macrophages exposed to HRV16 or TLR agonists for 48 h and then challenged with NTHi or LPS. Human macrophages were exposed to HRV16 (red bars), LPS (blue bars), CpG (orange bars) or MI (black bars) for 1 h and rested for 48 h. Then they were exposed to NTHi or LPS for 2 h and supernatants were collected and analyzed by MSD. MSD results for **(A)** IFNγ, **(B)** IL10, **(C)** IL12p70, **(D)** IL1β, **(E)** IL4, **(F)** IL6, **(G)** IL8, **(H)** TNFα. **(I)** Relative fold changes for cytokine production in HRV16, LPS or CpG exposed human macrophages. $^{*}p < 0.05$, $^{****}p < 0.0001$ Two Way Anova with Dunnett's Post Test vs. NTHi or MI + LPS. Error bars represent standard error of the mean (SEM). $n = 4$ independent experiments on different donors.

exposed macrophages showing slightly similar results for some cytokines in **Figure 1** (IFNγ, IL12p70, IL6, and IL8) (**Figure 1I**), HRV16 exposed macrophages showed a significant reduction in all cytokine secretion in response to the bacteria (**Figure 3I**). This suggests that there is some regulation by the live virus and that HRV16 exposed macrophages are unable to mount an efficient response toward secondary bacterial targets.

LPS and CpG Stimulation of Human Macrophages Does Not Impair Secondary Responses to NTHi

We next assessed if the inability to secrete cytokines in response to NTHi after HRV16 treatment was limited to viral infection or could be observed with different pre-activation triggers. Macrophages were first treated with HRV16, LPS, CpG or MI supernatants and then challenged 24 h later with NTHi for 2 h. Supernatants were collected and analyzed for cytokine secretion by the MSD technology (**Figure 4**). We found that HRV16 exposed macrophages were unable to secrete pro-inflammatory and regulatory cytokines in response to NTHi as seen above (**Figures 4A–I**). This was not observed if the cells were pre-activated with LPS or CpG (**Figures 4A–I**), demonstrating that the defective response to a second trigger was specific to viral pre-treatment.

HRV16 Infection Impairs Cytokine Secretion From Human Macrophages in Response to LPS

We next analyzed if the diminished secondary response was restricted to NTHi or extended to bacterial products such as LPS that is a potent stimulator of macrophages. Macrophages were first treated with HRV16 or MI supernatants and were challenged 24 h later with LPS for 2 h. Supernatants were collected and analyzed for cytokine secretion by the MSD technology (**Figure 4**). There was a diminished production of all cytokines analyzed in macrophages exposed to LPS when

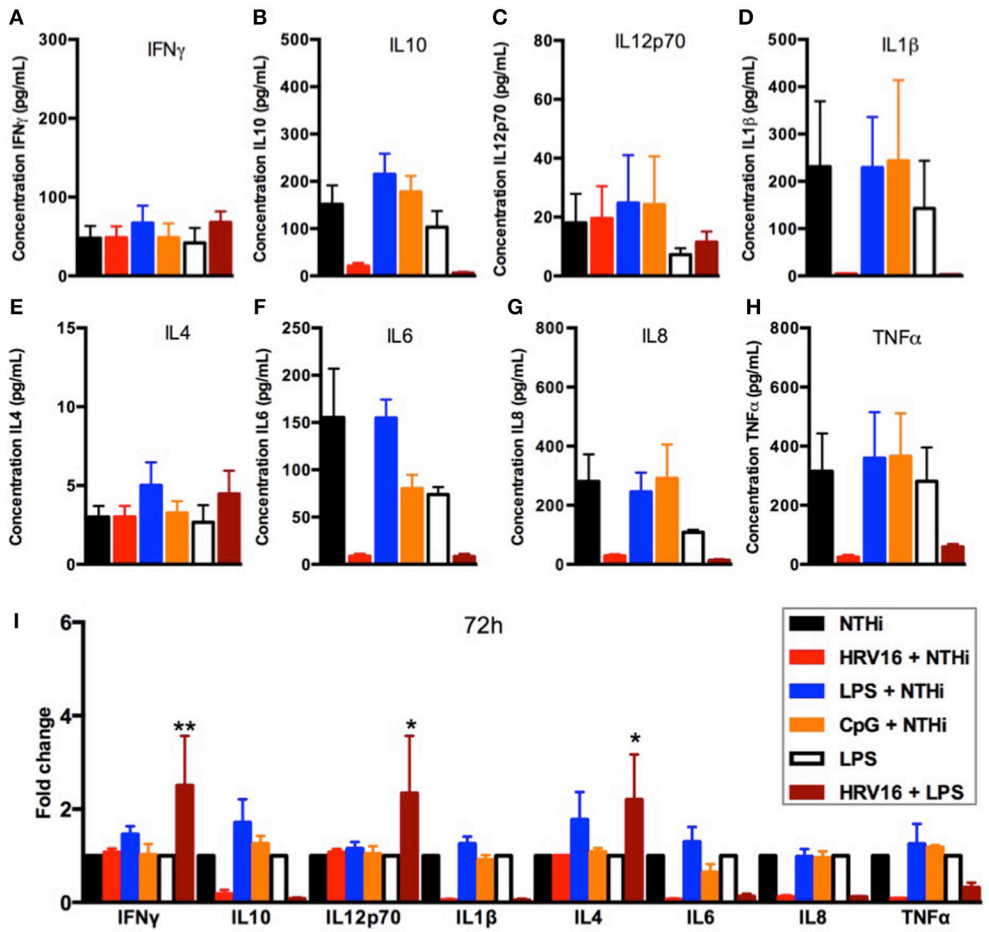

FIGURE 6 | Cytokine response of human macrophages exposed to HRV16 or TLR agonists for 72 h and then challenged with NTHi or LPS. Human macrophages were exposed to HRV16 (red bars), LPS (blue bars), CpG (orange bars) or MI (black bars) for 1 h and rested for 72 h. Then they were exposed to NTHi or LPS for 2 h and supernatants were collected and analyzed by MSD. MSD results for **(A)** IFNγ, **(B)** IL10, **(C)** IL12p70, **(D)** IL1β, **(E)** IL4, **(F)** IL6, **(G)** IL8, **(H)** TNFα. **(I)** Relative fold changes for cytokine production in HRV16, LPS or CpG exposed human macrophages. $^{*}p < 0.05$, $^{**}p < 0.01$ Two Way Anova with Dunnett's Post Test vs. NTHi or MI + LPS. Error bars represent standard error of the mean (SEM). $n = 4$ independent experiments on different donors.

the cells had been pre-treated with HRV16 compared to MI (**Figures 4A–I**). This shows that the defect caused by HRV16 extends beyond NTHi infection.

HRV16 Impairment of Secondary Responses to NTHi Is Still Present at 48 and 72 h

To address whether the inhibitory effect of HRV16 would last more than 24 h, macrophages were first treated with HRV16, LPS, CpG or MI supernatants and then challenged 48 h or 72 h later with NTHi for 2 h (**Figures 5**, **6**, respectively). We found that the diminished secretory response remained at 48 and 72 h for IL10, IL6, IL8, IL1β, and TNFα (**Figures 5**, **6B,D,F–I**). The response triggering IL4, IL12p70 and IFNγ secretion appeared to be restored at these later time points (**Figures 5**, **6A,C,E**), but we noted that the MI controls were secreting progressively less of these cytokines at later time points (**Figures 5**, **6A,I**). Further, if we challenged macrophages with LPS 48 or 72 h after HRV16 exposure, we found that the production of IL10, IL6, IL8, IL1β, and TNFα were still diminished (**Figures 5**, **6B,D,F–I**). This demonstrates that macrophages still present inhibited responses toward second triggers following HRV16 exposure beyond 24 h.

Finally, to confirm that the failure of HRV16 exposed macrophages to secrete cytokines was not due to enhanced cell death, we performed a lactate dehydrogenase (LDH) using the presence of this enzyme in cell supernatants to monitor cell permeability and death, as compared with the activity measured after total cell lysis (**Figure 7**). We observed no increase in cytotoxicity in HRV16 exposed macrophages +/− NTHi or LPS, compared to control conditions over 72 h (**Figure 7**).

Together, these results demonstrate that HRV16 exposed macrophages are unable to mount an efficient response toward secondary targets, in this case bacteria or LPS, and that the defective response persists in part for at least 72 h.

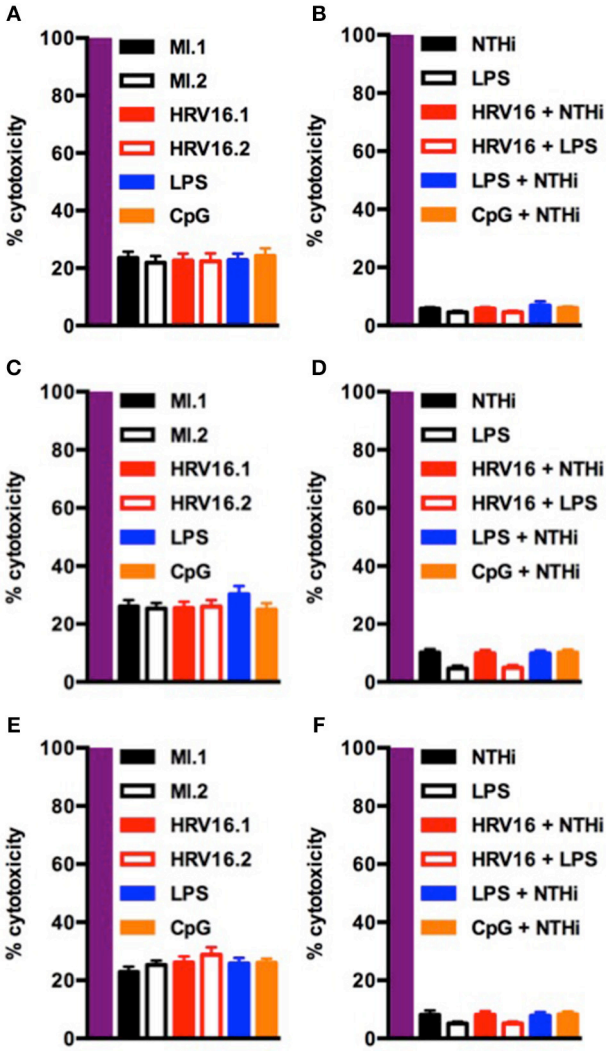

FIGURE 7 | Human macrophages exposed to HRV16 do not display increased cytotoxicity. Human macrophages were exposed to HRV16 (red bars), LPS (blue bars), CpG (orange bars) or MI (black bars) for 1 h and rested overnight, for 48 or 72 h. Then they were exposed to NTHi or LPS for 2 h. Supernatants were collected and analyzed by an LDH assay. LDH results for **(A)** 24 h, **(B)** 24 h + 2 h NTHi or LPS, **(C)** 48 h, **(D)** 48 h + 2 h NTHi or LPS, **(E)** 72 h, **(F)** 72 h + 2 h NTHi or LPS. All results are expressed relative to total lysis (purple bars). $n = 4$ independent experiments on different donors.

DISCUSSION

In this study, we demonstrate that macrophages respond to HRV16 by secreting inflammatory cytokines, but this response is altered upon secondary challenge with live bacteria or bacterial compound such as LPS. This is important, because HRV is routinely isolated at COPD exacerbations (20, 21) and thought to contribute to the dysregulated microbiome seen in these patients (6).

We found that macrophages exposed to HRV16 produced elevated levels of cytokines compared to uninfected control conditions. We detected robust production of TNFα, IL10 and IL1β in response to HRV16. Modest increases in IFNγ, IL4, IL6, and IL12p70 were also observed. Furthermore, in these cells comparable secretion of most cytokines, except IL10, was noted with CpG activation. LPS challenge of these cells also resulted in cytokine secretion in a range higher or similar to that obtained with HRV16 challenge. These results fit with other studies showing that HRV leads to a robust cytokine response (22–28) and specifically in monocytes/macrophages (29–32). HRV is known to cause robust IFNα and IFNβ production (33, 34). In our experiments, we could not detect IFNβ secretion despite elevated mRNA levels (data not shown). In agreement with our observations, other studies have shown no detectable IFNβ secretion in response to HRV (26, 35, 36). It has also been reported that different HRV strains induce different cytokine responses (37, 38) with clinical strains inducing more release of IL6, IP10, IFNγ and IFNβ (28).

Our second major finding was that macrophages infected with HRV16 and subsequently with NTHi or LPS produce less pro-inflammatory and regulatory cytokines compared to control cells. In addition, this phenotype lasts for at least 72 h toward the majority of cytokines tested. Importantly, the diminished

cytokine responses in HRV16 exposed macrophages were not due to increased cytotoxicity. Of note, our observations do not indicate any trend toward a macrophage polarization, but rather a "paralyzed" phenotype that was not mimicked when cells were pre-activated with agonists like LPS or CpG instead of the virus. This was observed even toward those cytokines that showed modest increases in response to HRV16. The same altered response was not seen with $HRV16^{UV}$, suggesting that it is specific to live HRV16. How the virus precisely regulates the cytokine secretion in response to a secondary challenge, however, still requires further investigation. This is critical, because it has been shown that viruses from clade A of the HRV group, including HRV16, are frequently associated with severe COPD exacerbations (39). They are associated with increased possibility of bacterial detection and postulated to be related to secondary effects on the outgrowth of bacteria.

How HRV affects the cytokine response toward bacteria has received limited attention in macrophages. Lung macrophages challenged with HRV showed reduced IL8 and TNFα production in response to LPS and LTA (15). In epithelial cells, HRV and then NTHi exposure led to decreased production of IL8 (40). Combined with our data, these results suggest that HRV can specifically shutdown macrophage responses and cytokine secretion in response to bacterial infection. Our *in vitro* data is not reflective of the entire lung environment where a complete microbiome is present, but our findings could nevertheless contribute to explain how HRV hijacks macrophage functions within the lung and potentially explain why co-infections are increasingly documented in COPD exacerbations.

AUTHOR CONTRIBUTIONS

FN, NK, and GM conceived and designed the study. JJ, KA-G, FH, and EB designed and performed experiments and collected data. GM, DC, NK, and FN contributed to design experiments. All authors analyzed the data. JJ, EB, GM, DC, NK, and FN contributed to writing the manuscript.

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Viral-Bacterial Co-infections in the Cystic Fibrosis Respiratory Tract

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A majority of the morbidity and mortality associated with the genetic disease Cystic Fibrosis (CF) is due to lung disease resulting from chronic respiratory infections. The CF airways become chronically colonized with bacteria in childhood, and over time commensal lung microbes are displaced by bacterial pathogens, leading to a decrease in microbial diversity that correlates with declining patient health. Infection with the pathogen *Pseudomonas aeruginosa* is a major predictor of morbidity and mortality in CF, with CF individuals often becoming chronically colonized with *P. aeruginosa* in early adulthood and thereafter having an increased risk of hospitalization. Progression of CF respiratory disease is also influenced by infection with respiratory viruses. Children and adults with CF experience frequent respiratory viral infections with respiratory syncytial virus (RSV), rhinovirus, influenza, parainfluenza, and adenovirus, with RSV and influenza infection linked to the greatest decreases in lung function. Along with directly causing severe respiratory symptoms in CF populations, the impact of respiratory virus infections may be more far-reaching, indirectly promoting bacterial persistence and pathogenesis in the CF respiratory tract. Acquisition of *P. aeruginosa* in CF patients correlates with seasonal respiratory virus infections, and CF patients colonized with *P. aeruginosa* experience increased severe exacerbations and declines in lung function during respiratory viral co-infection. In light of such observations, efforts to better understand the impact of viral-bacterial co-infections in the CF airways have been a focus of clinical and basic research in recent years. This review summarizes what has been learned about the interactions between viruses and bacteria in the CF upper and lower respiratory tract and how co-infections impact the health of individuals with CF.

Keywords: cystic fibrosis, coinfection, polymicrobial, respiratory infection, biofilm, chronic disease

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FACTORS PROMOTING RESPIRATORY INFECTIONS IN CYSTIC FIBROSIS

Cystic fibrosis (CF) is a lethal genetic disease caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene (1) that result in dysfunction of the CFTR anion channel (2). To date, close to 2,000 individual mutations in the CFTR gene have been identified (3), and these mutations are further sub-divided into five classes based on how they lead to defective production of CFTR protein, resulting in deficiencies in protein folding, intracellular trafficking, and/or gating reviewed in-depth by Rowntree and Harris (4). While CFTR mutations affect most cell types and all mucosal surfaces in the body, manifesting in different types of disease, respiratory disease remains the most heavily-studied pathology of CF.

Chronic respiratory infections and the resulting robust but ineffective inflammatory response, culminating in respiratory failure, are the primary causes of death in CF patients.

In the CF respiratory tract, numerous factors resulting from dysfunctional CFTR combine to create an environment that promotes chronic bacterial and recurring viral infections. A dysfunctional CFTR alters the osmolarity of the airway surface liquid (ASL) layer, resulting in dehydrated ASL, and facilitating the buildup of a thick mucus layer. Diminished ASL hydration and thick mucus at the airway epithelial surface leads to failure of mucociliary clearance in the CF respiratory tract due to collapse of airway cilia, thereby preventing ciliary beat that normally clears debris, and infectious agents from the lungs. This allows microorganisms to repeatedly infect, eliciting robust inflammatory responses dominated by elevated proinflammatory cytokines, and continued accumulation of neutrophils in the CF airway (5). However, these inflammatory responses are ineffective at clearing pathogens in the CF lung, instead creating a hyperinflammatory cycle that leads to host tissue damage, respiratory failure, lung transplant or eventually, death (6). Additionally, the dysregulated conductance of bicarbonate anions by the CFTR channel in CF results in improper mucus formation and an altered ASL pH, which impacts the function of secreted antimicrobial peptides, disrupting a first line of defense against invading bacterial pathogens (7–9). Together, these deficiencies in CF respiratory tract physiology prevent efficient clearance of pathogens from the airways, allowing for the establishment of a robust community of microbes.

THE CF RESPIRATORY MICROBIOME

Identification of Bacterial Species in the CF Microbiome

The microbial community in the CF lung is complex, and lung health is affected by the presence and interactions of bacteria, fungi, and respiratory viruses (10, 11). Identification of bacterial species in the CF airways has traditionally relied on culture of bacteria by clinical microbiology laboratories from expectorated sputum samples, respiratory swabs, or samples obtained through bronchoscopy (referred to as culture-dependent methods) (12). Advances in next-generation sequencing have made it possible to identify populations of bacteria residing in the airways without culturing through the isolation of genomic DNA from CF patient samples and sequencing of the gene encoding the bacterial 16S ribosomal subunit. The 16S ribosomal subunit contains variable regions whose sequences can be assigned to bacteria at the species level (termed culture-independent methods) (13). Because culture-dependent methods require knowledge of which bacterial species to target for identification and how to isolate them, known bacterial pathogens were the main species identified from CF respiratory samples prior to culture-independent methods. With the advent of culture-independent methods, it became appreciated that in addition to traditional pathogens, many other bacterial species often associated with the oral cavity or upper respiratory tract, and considered commensal or colonizing organisms were present in the CF lung at high abundance (14). These newly recognized populations included many species of anaerobic bacteria, which previously were not identified, as clinical laboratories did not use culture methods that would allow anaerobic growth (15). It remains a debated issue in the field as to whether all bacterial species identified via culture-independent methods are truly established in the CF lower airways, or if presence of these species is due to contamination of samples by oral or upper respiratory tract microbes during the collection process (11, 16).

Commensal Microbiome Members

Focusing on bacterial members of the CF microbiome, from culture-independent studies we find that before chronic infections are established by pathogens, the CF lung is colonized by several genera of commensal bacteria. Studies have identified core bacterial airway microbiome members as belonging to the genera *Streptococcus*, *Prevotella*, *Veillonella*, *Rothia*, *Granulicatella*, *Gemella,* and *Fusobacterium* (14, 17, 18). Many factors can impact individual patients' respiratory microbiomes, most notably age and antibiotic exposures. Studies of longitudinal samples collected from patients over time have found distinct bacterial community profiles exist for younger vs. older CF patients, with pediatric CF patients possessing a greater abundance of core/commensal bacterial species, and a more diverse bacterial microbiome than CF adults (19). It is thought that these core species are displaced over time as patients age and pathogens are introduced and become established in the airways (10, 18). Multiple studies have found that a higher diversity of species in the lung correlates with better patient outcomes, and decreased diversity correlates with an over-abundance of CF pathogens and declines in patient health (10, 17, 18). Like microbiomes associated with other organ systems in healthy adults, the microbiome in the CF airways has ultimately been found to change minimally upon exposure to antibiotics, exhibiting an altered community structure or decrease in overall abundance during treatment but rebounding to the original community structure after treatment ends (20, 21). Roles for interspecies interactions occurring between commensal and pathogenic bacteria in the CF airways are just beginning to be elucidated. Species of commensal streptococci have been found to have direct protective effects toward the lung by inhibiting pathogens such as *P. aeruginosa* (22–24). We have only recently begun to recognize the complexity of the microbial ecology in the CF airways and how dynamics of microbial communities, and not solely presence of pathogens, can contribute to disease outcomes (25). It is likely that many as yet unknown interspecies interactions exist that impact bacterial populations in the CF airways at a given time, and future studies will pinpoint specific mechanisms mediating bacterial crosstalk that may be targeted to alter the abundance of distinct species.

Bacterial Respiratory Pathogens in CF

A number of bacterial species have been identified as major respiratory pathogens in CF, including *Staphylococcus aureus*, *Haemophilus influenzae*, *P. aeruginosa*, and *Burkholderia* complex (3, 26). *S. aureus* is the most frequently isolated bacterial

pathogen in CF pediatric populations, whereas *P. aeruginosa* becomes established as CF patients age (3). *S. aureus* is regarded as a commensal species of the nares and upper respiratory tract but is recognized as a pathogen when identified in other body sites, such as the lower airways, and is cultured from over 70% of CF patients (3). *P. aeruginosa* is the dominant pathogen in end-stage CF lung disease, and chronic infection with *P. aeruginosa* is correlated with more severe reductions in pulmonary function measures (27) and mortality in CF patients. Methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *S. aureus* and *P. aeruginosa* that display enhanced antibiotic resistance during chronic infection pose significant challenges to treatment efforts (28). Non-traditional bacterial pathogens, including *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia,* and non-tuberculosis Mycobacterium (NTM), also contribute to respiratory infections in CF patients and have been associated with worsening lung function (26, 29). A mechanism common to most CF bacterial pathogens for evasion of host immune defenses in the CF lung, despite its hyperinflammatory state, is growth in bacterial aggregates or biofilms. During the transition from acute to chronic infection, *P. aeruginosa, S. aureus,* and other bacterial pathogens exhibit altered metabolism, decreased growth rate and up-regulated expression of antibiotic resistance genes, and these changes, together with increased production of polymeric matrix materials, protect organisms within biofilms from the hostile environment in the CF lung (30–32).

RESPIRATORY VIRUSES IN CF

Acute Respiratory Viral Infections in CF

Pediatric and adult CF patients experience frequent acute respiratory virus infections. Specific respiratory viruses responsible for infections are identified when patients present with symptoms indicative of a viral infection, leading physicians to take a viral swab from which genetic material is extracted for PCR, and a viral panel is performed consisting of primer sets specific to common viral culprits. The true incidence of viral infections is likely under-reported for several reasons, including infrequent use of viral swabs and incomplete PCR panels to detect viral infections, as well as the fact that not all patients present with symptoms during a viral infection (33, 34). The most commonly identified viral pathogens in CF populations are respiratory syncytial virus (RSV), human rhinovirus (RV), Influenza types A and B, and parainfluenza, all belonging to families of RNA viruses (34–36). It has been reported that close to 40% of children with CF are hospitalized at some point for severe respiratory infections, and of these hospitalizations, respiratory viruses were identified in 50% of patients, with RSV predominating (33). While in non-CF populations RSV is thought to be almost exclusively a pediatric pathogen, RSV infections are frequent in both adult and pediatric CF patients, and can result in severe symptoms. RSV infection may result in upper respiratory disease, including rhinitis, cough, fever, and acute otitis media, or progress to the lower respiratory tract, resulting in bronchiolitis or pneumonia in children, and exacerbate existing chronic airway disease in adults (37). RSV infection is especially aggressive in young infants with CF, leading to significant respiratory morbidity (38).

Links Between Viral Infections and Exacerbations

CF patients frequently experience periods of rapidly worsening respiratory symptoms, termed pulmonary exacerbations (39). Pulmonary exacerbations are typically defined by a decrease in lung function or increases in patient symptoms, however symptoms and severity of exacerbations vary from patient to patient and can be triggered by a multitude of causes (40). Exacerbations are often treated by initiating courses of additional antibiotics, increasing airway clearance therapies, or hospitalization in severe cases (41). Many clinical studies have now linked viral infections with pulmonary exacerbations (33, 35, 36, 42). Respiratory viral infections account for at least 40% of pulmonary exacerbations of CF adults (38, 43) and are linked to pulmonary function decline, antibiotic use, prolonged hospitalizations, and increased respiratory symptoms in CF patients (44–46). Respiratory viruses most frequently cultured during periods of exacerbation include the major viral pathogens appreciated in CF: influenza A and B, RSV, and RV (47–50).

Severity of Viral Infections in CF

CF patients are known to be pre-disposed to chronic bacterial infections, and several groups have examined whether CF disease also leads to more severe respiratory viral infections. *In vitro* studies evaluating CF vs. non-CF primary human bronchial epithelial cells in culture found that RV replication was increased in CF cells (51). Enhancement of viral infection could be attributed to a diminished innate antiviral response in CF cells, which showed weaker induction of interferon and expression of some interferon-stimulated genes, as compared to non-CF controls (52). A clinical study evaluating severity of RV infections in CF children compared to non-CF pediatric patients with asthma, non-CF bronchiectasis or healthy controls found CF patients had a higher prevalence of RV, and higher viral load in bronchoalveolar lavage (BAL), both when patients were stable and at even higher levels during pulmonary exacerbations (53). Higher RV load correlated with worse lung function scores in CF children, and RV infection in CF resulted in lower levels of inflammatory markers than in non-CF children, again indicating a dysregulated innate immune response in CF patients could be responsible for increased severity of viral infections (53). A longitudinal study reported that RV was identified more frequently in CF children than non-CF subjects, and RV infections in CF children persisted longer (54). These studies suggest inherent properties of CF airway cells may make the CF airway epithelium more prone to viral infection, and together with what is known regarding links between airway physiology and bacterial respiratory infection in CF, these factors could have important implications in cases of viral-bacterial co-infections.

VIRAL-BACTERIAL CO-INFECTIONS IN CF

CF patients are commonly chronically infected with bacterial pathogens and maintain a high abundance of microbes in the respiratory tract, including pathogens, and commensal organisms. These same patient populations also experience frequent acute respiratory viral infections. There are numerous ways in which infection with a viral pathogen can alter the host response, impacting previously existing chronic bacterial infections and microbial communities, potentiating secondary bacterial infections, and/or permitting the acquisition of new bacterial species in the airways (summarized in **Figure 1**). In this section, we evaluate insights from clinical studies of CF patient populations and mechanistic *in vitro* studies that inform us of viral-bacterial interactions occurring in CF during co-infections.

Impact of Virus Infection on the CF Microbiome

Temporal changes in microbiome composition could result from a variety of disturbances that alter the environment in the CF airways, including initiation of antimicrobial therapies, mechanical or airway clearance treatments, or an altered host response. Respiratory viral infections can promote the onset of respiratory symptoms, as well as trigger the innate antiviral response in the CF airway epithelium, resulting in induction of antiviral signaling, and inflammation. From non-CF studies, respiratory viral infection is known to skew the immune status of the respiratory tract to be predisposed to secondary bacterial infection, which has been most studied for influenza (55). The altered host immune status following viral infection reduces antibacterial effector functions, like phagocyte recruitment (56), antimicrobial peptide production (57, 58), and protective adaptive immune responses (59–61), increasing susceptibility to bacterial infections. Specifically, interferon-mediated antiviral responses following influenza infection in mice (62) and vaccination with live attenuated influenza in humans (63) have been shown to shift the composition of the upper respiratory microbiome and increase the potential for emergence of *S. aureus* infections. An altered immune status resulting from respiratory viral infection likely also alters the microbial composition of the CF airways, potentially leading to shifts in bacterial populations comprising the microbiome and promoting infection by specific bacterial pathogens.

How respiratory viral infections impact the CF airway microbiome can be evaluated by observing changes in overall bacterial burden (or bacterial load), community composition, or dominant taxa. In non-viral-associated pulmonary exacerbations, the overall bacterial burden in the CF airways rarely changes in the time leading up to an exacerbation or during exacerbations (64–66). Studies evaluating bacterial burden during acute respiratory viral-associated pulmonary exacerbations have produced conflicting results. In one prospective study of CF adults, *P. aeruginosa* density in sputum was not found to increase during exacerbation, compared to patients' stable states in either the presence or absence of a respiratory virus co-infection (67). A similar study design by another group found the opposite to be true: in adult CF patients evaluated, a significantly higher load of *P. aeruginosa* was observed during respiratory-virus associated exacerbations (48). An observational study of CF children found *P. aeruginosa* density was not significantly different between patients experiencing viral- or non-viral-associated exacerbations (47). Additional prospective studies with larger patient cohorts are needed to gain a more definitive understanding of the effects of virus co-infection on overall bacterial burden and burden of specific organisms, like *P. aeruginosa*, in pediatric and adult CF populations.

While the relationship between respiratory virus co-infection and bacterial burden remains unclear, more efforts have been made toward investigating associations between viral infection and culture of specific bacterial pathogens from the CF airways. Findings from multiple studies indicate approximately 15–25% of CF patients undergoing a respiratory viral infection also culture positive for a known CF bacterial pathogen (68–70). Previously uncolonized CF patients have been reported to undergo new acquisition of *P. aeruginosa* following seasonal respiratory virus infections (44). Regarding roles for specific respiratory viruses, both RSV and RV are linked in clinical studies to development of *P. aeruginosa* co-infections and conversion from intermittent to chronic *P. aeruginosa* colonization in CF patients (33, 35, 43, 44, 71). Similarly, other known bacterial respiratory pathogens, including *H. influenzae*, *Moraxella catarrhalis,* and *Streptococcus pneumoniae*, were cultured more frequently from CF patients experiencing a RV co-infection (68). A separate pediatric study also found that in a cohort of CF children experiencing viral-bacterial co-infections, RV and *S. aureus* were co-cultured more frequently than any other viral-bacterial pair (69). Together, these studies present strong clinical evidence for association of respiratory viral co-infection with presence of bacterial pathogens in the airways and suggest associations between specific species of viruses and bacteria co-cultured that may vary with age.

Toward Mechanisms Underlying Viral-Bacterial Interactions

Mechanistic studies evaluating the outcomes of viral-bacterial co-infections have been made possible through the availability of isogenic immortalized CF airway epithelial cell lines (72) and access to well-differentiated primary airway epithelial cells cultured from CF lung tissue following lung transplant (73). In cell culture experiments by our group and others, simultaneous inoculation of CF and non-CF airway cells with RSV and *P. aeruginosa* increased adherence of both mucoid and non-mucoid *P. aeruginosa* strains (74, 75). This effect could be blocked by pre-treating non-polarized airway cells with heparin prior to inoculation with RSV and *P. aeruginosa* (75). A recent study from our group found no significant difference in *P. aeruginosa* adherence to polarized CF airway cells with a preceding RSV infection (24–72 h) compared to control CF cells; however, co-infection with RSV, RV, and adenovirus each promoted the growth of *P. aeruginosa* biofilms (74). Treatment of CF airway cells with exogenous type I or type III interferon prior to *P. aeruginosa* inoculation also stimulated biofilm growth,

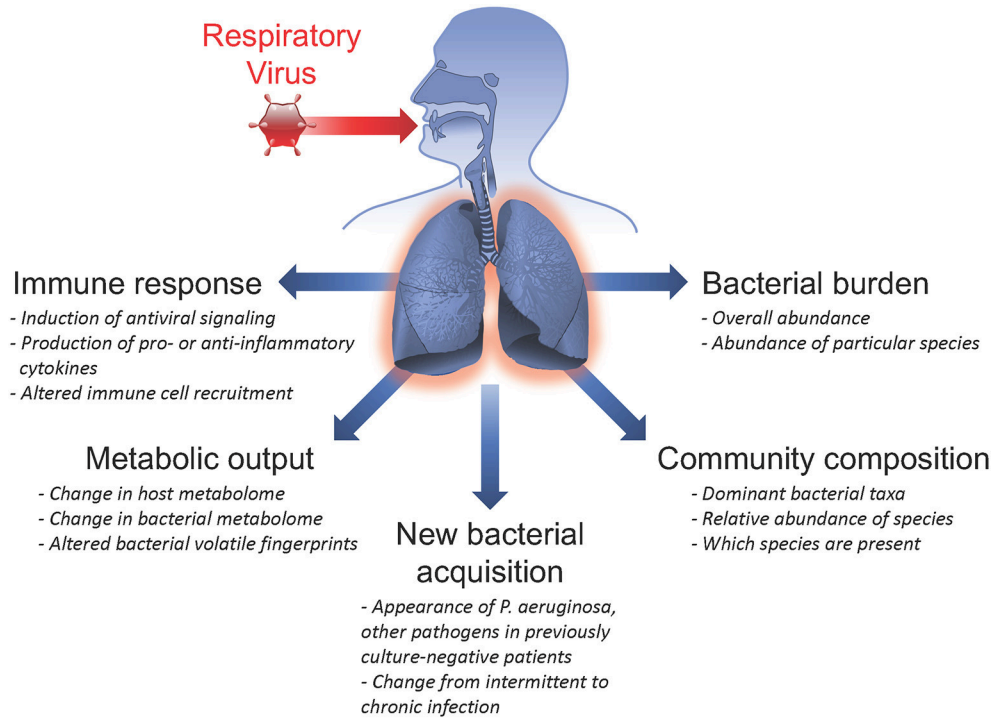

FIGURE 1 | Summary of respiratory virus infection outcomes impacting viral-bacterial interactions in Cystic Fibrosis. Acute respiratory viral infections are known to affect subsequent infection with bacterial pathogens and influence pre-existing chronic bacterial infections in individuals with Cystic Fibrosis. Some ways in which respiratory viruses have been shown to impact bacterial infections include: inducing the host immune response; altering metabolic output of both host and infecting bacteria; causing new bacterial acquisition in patients who were previously culture-negative; altering bacterial community composition by shifting relative abundance of specific bacterial species; and inducing fluctuations in total bacterial burden.

suggesting *P. aeruginosa* benefits from the innate antiviral response in CF airway cells. Together, these studies suggest physical binding of RSV to *P. aeruginosa* or the CF airway cell surface may facilitate initial adherence of *P. aeruginosa* to the epithelium, whereas progression of viral infections and activation of interferon-stimulated innate antiviral signaling pathways may play a role in promoting chronic *P. aeruginosa* growth in biofilms. New work from our group finds co-infection with RSV and RV also promotes *S. aureus* biofilm growth on CF airway cells through as yet unidentified mechanisms (76).

Bacteria depend largely on nutrients and metabolites supplied by the host during infection, and virus co-infection has been shown to alter nutritional availability, thereby influencing bacterial responses. Iron is known to be a key nutrient required for growth and pathogenesis of many pathogens (see the Frontiers Research Topic, "Role of Iron in Bacterial Pathogenesis"). The host normally sequesters iron and other essential metals from invading microbes through a process termed nutritional immunity (77), but these mechanisms have been found to be dysregulated during virus infection. In CF airway cell studies, it was discovered that RSV infection promoted increased secretion of iron-bound host transferrin protein, which stimulated *P. aeruginosa* biofilm growth (74). Lipocalin-2, a host antimicrobial protein that sequesters iron (78), was found to be reduced during influenza A infection through virus-mediated suppression of NF-kB activation and IL-1B expression, exacerbating *S. aureus* acute pneumonia in mice (58). Other potential nutrient sources in the airways, such as surfactant proteins (79–81), and mucins (82–84), are known to change during virus co-infections, and there is evidence that CF bacterial pathogens can utilize these nutrient sources (85–88), but specific links between these nutrient shifts and viral-bacterial co-infections CF have not yet been confirmed.

Conversely, bacterial interactions with the CF airway epithelium can also alter subsequent virus infection. In one study, pre-infection of CF human bronchial cells with *P. aeruginosa* was found to diminish the interferon response to RV infection and resulted in a higher RV load than RV infection alone (89). As CF cells showed increased generation of reactive oxygen species (ROS) at baseline compared to non-CF cells, treatment of CF cells with antioxidants prior to *P. aeruginosa* infection helped to restore the IFN response, and it was observed that while RV infection alone acted through PI-3 kinase to induce Akt phosphorylation, this was prevented by pre-infection of CF airway cells with *P. aeruginosa*. A later study evaluating the effects of *P. aeruginosa* secreted factors on primary CF and non-CF airway cells observed no effect of pre-treatment on RV load or antiviral gene expression; however, pre-exposure of cells to *P. aeruginosa* secreted factors did potentiate IL-8 production upon subsequent RV infection (90). Another secreted

P. aeruginosa protein, Cif, also potentiated virus infections in CF airway epithelial cells by preventing MHC class I antigen presentation and CD8 T cell-mediated clearance of influenza A-infected cells (91). Taken together, these findings suggest a complex interplay between bacterial pathogens, respiratory viruses, and the innate immune response in the CF airway epithelium, where an appropriate immune response to one pathogen may alter secondary infection by another pathogen. The effects of virus co-infection on many prominent CF bacterial pathogens has yet to be evaluated, and relationships between respiratory viruses and bacteria in multi-species polymicrobial infections representative of the complex communities existing in the CF airways is also an underexplored area.

IMPACT OF THERAPEUTICS ON CO-INFECTIONS

Antimicrobial Treatments for Viral and Bacterial Infections

As we've observed with the host immune response, attempts to clear one type of pathogen may have unintended effects on other microbes in the CF airways. The same may be true for cases of antiviral or antibacterial treatments administered to CF patients. It is appreciated that despite intense antibiotic therapy and even with alternating antibiotic courses, chronic infections with bacterial pathogens established as biofilms in the CF airways resist clearance through multiple mechanisms reviewed in Høiby et al. (92) and Lambert (93). Several therapies are now shown to impact both viral and bacterial pathogens, potentially leading to new therapeutic options for polymicrobial infections. We recently reported that an engineered antimicrobial peptide therapy, WLBU2, reduced both bacterial biofilm, and RSV titers in a mixed infection model *in vitro* (94). In addition, members of the macrolide class of antibiotics, including erythromycin, azithromycin, and bafilomycin, which are known to effect antibacterial activity by binding to bacterial ribosomal subunits to inhibit protein synthesis (95) were also found to have anti-inflammatory effects. By blocking production of the pro-inflammatory cytokines IL-6 and IL-8, macrolide antibiotics reduced neutrophil recruitment to sites of injury, and infection (96). In non-CF bronchial epithelial cells, azithromycin (97), bafilomycin (98), and clarithromycin (99) treatments were found to reduce RV replication by increasing induction of interferon-stimulated antiviral genes, demonstrating that in addition to its anti-inflammatory, and anti-bacterial properties, azithromycin has anti-viral activity. In CF airway cells, azithromycin also reduced RV replication and increased RV-induced expression of interferon and interferon-stimulated antiviral genes; however, azithromycin did not prevent induction of IL-6 or IL-8 during RV infection, suggesting that the anti-inflammatory effects of azithromycin are diminished during a virus infection (51). Administration of azithromycin as an antiviral or anti-inflammatory agent could provide a potential therapeutic option for CF patients, yet as macrolide resistance is known to be widespread in clinical isolates from chronic airway infections (100), it is important to keep in mind the broader effects antibiotic administration may have on the CF microbiome.

As viral infections can lead to severe respiratory morbidity and are linked to exacerbations in CF populations, there is a demand for effective anti-viral therapies, especially for major CF pathogens like RV and RSV for which no successful vaccine exists. RSV immunotherapy was shown to be effective at preventing lower respiratory tract infections and reducing symptom severity in high-risk infants, and young children (101). A humanized monocolonal antibody treatment for RSV, palivizumab, was developed (102) and prophylactic treatment with palivizumab significantly reduced hospitalizations (103), and incidences of respiratory-related illness (104) in CF children compared to untreated control groups. While potentially effective at preventing RSV infection, palivizumab prophylaxis is costly and has shown limited benefits for populations that do not regularly have high incidence of RSV-related hospitalizations (105), leading some to propose that anti-RSV therapy would be best-reserved for treatment during infections, not as prophylaxis, or for fall, and winter seasons when probability of virus-related illnesses and hospitalizations typically increases (106). As it has been observed that virus co-infection promotes *P. aeruginosa* colonization, a secondary benefit of antiviral therapies could be a delay in acquisition of bacterial pathogens in CF children. However, a recent study found that prophylactic treatment of CF infants with palivizumab to prevent RSV infection did not delay acquisition of either *P. aeruginosa* or *S. aureus* (107). A separate retrospective study found that although palivizumab reduced RSV-related hospitalizations and overall *P. aeruginosa* chronic colonization rates did not differ between treatment and control groups, the time to first *P. aeruginosa* isolate was significantly earlier in palivizumab-treated CF children (108). Many factors could have affected these outcomes, including patients' genetics, environmental exposures, and differences in clinical care quality and access. Broader studies evaluating the impact of palivizumab on the CF microbiome, including changes in abundance of commensal and pathogenic bacterial species, could shed light on how anti-viral therapies affect viral-bacterial-host interactions.

CFTR Modulators and Impact on Infections

In the field of CF research and patient care, there is great excitement surrounding the promise of CFTR modulating drugs that improve mutated CFTR rescue to the cell surface (correctors) or modulate activity of dysfunctional CFTR protein channels (potentiators). Three drugs have undergone clinical trials and are now options for CF patients with specific CFTR mutations: the potentiator ivacaftor and correctors lumacaftor and tezacaftor (109, 110). Trials showed CF patients treated with CFTR modulators had improved lung function and decreased rates of pulmonary exacerbations, hospitalization, and IV antibiotic use, and the first studies on how these treatments impact respiratory microbiology in CF patients are now becoming available. During clinical trials, CF patients receiving ivacaftor, and lumacaftor were still found to experience adverse events, including upper respiratory infections (usually attributed to acute respiratory viral infection), at rates similar to placebo groups (111–113), and CF patients experiencing acute upper or lower respiratory

infections have also been excluded from trials on the basis that this could confound results, although this practice may limit knowledge of the impact of these drugs during infection (113).

In vitro studies of ivacaftor, whose structure resembles that of quinolone antibiotics, found that ivacaftor has dose-dependent antibacterial activity against *S. aureus* and *S. pneumoniae* clinical isolates, and synergy of ivacaftor and the anti-Gram positive antibiotic vancomycin was observed (114). Ivacaftor was also observed to have a milder antimicrobial effect toward *P. aeruginosa* that was improved in combination with the anti-pseudomonal antibiotic ciprofloxacin. These results suggest that in addition to the intended ability of CFTR modulators to improve CFTR production and function, these treatments could have the added benefit of helping to reduce certain bacterial populations in the CF airways.

Studies evaluating changes in the airway microbiome of CF patients undergoing CFTR modulator therapies can begin to inform us if the above observed properties of CFTR modulators translate to the clinic. A small study using quantitative PCR and 16S sequencing to evaluate the microbiome of three CF children undergoing ivacaftor treatment found no significance in overall bacterial burden or bacterial genera represented prior to and following treatment, and individual patients' microbiomes pre- and post-treatment were found to be more similar than treated vs. non-treated microbiomes across all patients (115). Recent work evaluating CF adults showed that ivacaftor treatment reduced *P. aeruginosa* and overall bacterial density in sputum samples, yet despite immediate effects, the same clonal isolates of *P. aeruginosa* sampled prior to treatment persisted in the airways of CF patients after ivacaftor (116). Inflammatory markers in patient sputum were found to be decreased in patients through mass spectrometry analysis of sputum proteins, and a separate study unexpectedly found that ivacaftor treatment dampened the interferon-gamma response and impaired monocyte recruitment, effects that modulate immune responses in the respiratory tract and could potentially influence disease outcome (117). Trials conducted in adult populations, many of whom are already chronically colonized with *P. aeruginosa*, do not allow for evaluation of the potential impact of CFTR modulators in preventing *P. aeruginosa* acquisition. Initiating new studies in younger patient populations following modulator treatment and following patients that begin treatment culturing *P. aeruginosa*-negative for longer periods of time post-treatment will be important to address the question of whether CFTR modulators impact *P. aeruginosa* acquisition. No specific effects of CFTR modulators on viral-bacterial co-infections have been reported to date, and valuable information could be gained from testing for the presence of specific respiratory viruses in patients undergoing modulator therapy, along with measuring changes in bacterial microbiome constituents. The above described effects of CFTR modulators on specific bacterial pathogens and the host immune response suggest co-infections will likely be impacted by such therapies and warrant further study.

NEW TECHNIQUES FOR EVALUATION OF VIRAL-BACTERIAL CO-INFECTIONS

Traditional means of diagnosing viral and bacterial infections through specific PCR panels or culture-based techniques, respectively, have provided the majority of our current knowledge regarding which microbes comprise the CF respiratory microbiome and can be considered pathogens in the CF airways. However, as previously mentioned, these methods of identification are limited, as each requires prior knowledge of which viruses to screen for or which bacteria to culture, and therefore we may be underestimating the number of species that exist in the airway environment and the impact they have on CF respiratory disease. The recent trend toward 16S studies has identified additional members of the bacterial microbial community, and the application of new techniques such as metagenomics, metatranscriptomics, and metabolomics to the study of the CF microbiome may begin to reveal previously unknown roles for new microbial species and offer unprecedented insight into their functions in the CF respiratory tract.

Metagenomics and Metatranscriptomics

Metagenomic studies evaluating the total genomic content of samples have great potential to identify a broader range of viruses and other microbes in CF, in addition to bacterial species. Such evaluations are technically challenging from a computational standpoint, and to date, few metagenomic studies have been published applying this technique to the study of viromes in CF populations. While RNA viruses have been the most appreciated viruses in causing acute respiratory infections in CF, a metagenomic study evaluating DNA viruses identified genomes of herpesviruses, and retroviruses in CF sputum and found overall eukaryotic viral diversity was low in both CF and non-CF individuals (118). The majority of viral diversity in airway microbial communities was found to be derived from populations of bacteriophage, with CF phage communities being more similar to one another than non-CF phage, and indicative of the dominant bacterial species residing in the CF airways that comprise the host range of the phage (118, 119). Although phage are not traditionally thought of as viruses that impact human health, recent work has shown that phage affect *P. aeruginosa* biofilm assembly and promote survival of bacteria in biofilms by enhancing adhesion and tolerance to antibiotics (120), and the role of phage in promoting a healthy microbiome in other organ systems, namely the gastrointestinal tract, has been a rapidly growing area of research outside of CF (121, 122). Going further, functional genomic analyses that consider the predicted functions of all genes present in the total DNA from a sample predicted the viromes of CF patients had a separate set of core metabolic functions compared to healthy subjects (118), with enrichment of genes for metabolizing aromatic amino acids. This suggests the host organisms of these phage have a specialized metabolism specific to CF disease, and the genes carried by phage represent factors necessary for survival in the CF airways.

Similar to metagenomics, metatranscriptomics is the analysis of the total RNA content of a sample and can thus account

for changes in expression of host and microbial genes. To date, limited metatranscriptomics studies have been performed evaluating both host and pathogen gene expression in the same sample. In a mouse model of acute *P. aeruginosa* infection, metatranscriptomics revealed genes related to *P. aeruginosa* outer membrane vesicle production, and iron uptake and utilization were significantly upregulated, indicating the importance of iron-mediated regulation and scavenging *in vivo* (123). In infected mice, expression of pro-inflammatory cytokines and chemokines associated with toll-like receptor signaling were induced. Recent dual-RNA sequencing experiments from our group show polarized CF airway cells and *S. aureus* exhibit altered transcriptional profiles during co-culture in the presence of RSV co-infection (76). In CF cells, differences in innate immune and inflammatory signaling were observed in the presence of RSV, *S. aureus* or viral-bacterial co-infection, and *S. aureus* exhibited an altered metabolic transcriptional profile during virus co-infection, with upregulation of genes for cofactor biosynthesis and amino acid utilization, perhaps reflecting altered availability of protein substrates in the airway surface liquid of virus-infected CF cells. The use of next-generation sequencing approaches is expanding, and these techniques as applied to CF in future studies will no doubt expand our view of viruses and other underappreciated microbes that exist in the CF airways.

Metabolomics

Supplementing metagenomic studies that identify which microbial species are present in the airways, metabolomics studies can account for microbial and host-derived metabolites and proteins. For studies of biological samples, liquid chromatography-mass spectrometry (LC-MS) is often used to identify small molecules, and liquid chromatography-tandem mass spectrometry (LC-MS/MS) can be applied to identify sequences of individual peptides for proteomic analysis in complex samples (124).

A novel application of MS techniques is analysis of volatile compounds found in breath to diagnose infections. Volatile compounds produced during microbial metabolism can provide species-specific signatures based on knowledge of individual species' metabolic capabilities from genome sequences, and variation in volatile compound production can be an indicator of compounds available for use in the environment. Early studies in CF utilized the knowledge that *P. aeruginosa* produces cyanide, and using cyanide as a biomarker, selected ion flow mass spectrometry (SIFT-MS) was able to identify the presence of *P. aeruginosa* in the airways by sampling the breath of infected CF patients (125, 126). Later studies showed that beyond identifying presence of a specific molecule, groups of *P. aeruginosa*-colonized CF patients could be differentiated from non-colonized CF patients based on overall volatile breath profiles (127). Using genomic data available for other CF-related bacterial species to predict organisms capable of producing specific volatile compounds, high levels of acetaldehyde, ethanol, and methanol in CF subjects' breath were linked to *Lactococcus*, *Escherichia* and *Rothia* species, respectively (128), confirming that metabolic predictions based on genetic sequence could translate to positive identification in patient samples for species other than *P. aeruginosa*.

Translating breath detection to bench studies, a volatile fingerprint could be identified for CF airway cells co-cultured with *P. aeruginosa* (129). Evaluating RSV-*P. aeruginosa* co-infections in CF airway cells, different levels of volatile compounds were found to be produced during co-infection rather than infection with either *P. aeruginosa* or RSV alone, and predictive models were able to discriminate *P. aeruginosa*-infected cells, but not cells undergoing only RSV infection (129). Breath diagnosis could prove to be a quick, non-invasive, and culture-independent means for diagnosing the presence of CF pathogens, and with additional knowledge of the links between overall metabolic state and respiratory function, breath testing could serve as an indicator of a stable or exacerbating CF airway environment. The prospect of using breath analysis to identify a virus co-infection is intriguing, and *in vitro* studies require further investigation and translation into CF patients undergoing respiratory viral infections to confirm specific volatile signatures for co-infections.

In summary, the individual roles of bacterial and viral infections in CF respiratory disease have long been appreciated, and independently, viral infections, and chronic bacterial infections are known to influence pulmonary exacerbations and progression of respiratory function decline. Now, advances in sequencing technology have facilitated our understanding of the complexity of the microbial communities in the CF airways, bringing to light new interactions between bacteria and viruses in the airways that suggest microbial population dynamics and interplay between microbes and the host, and not just the presence of known pathogens, could be the true drivers of CF respiratory disease. While metabolomics has not yet been used to evaluate differences in metabolite profiles in CF patients with respiratory viral infections or viral-bacterial co-infections, the technology used in the above described studies provide an exciting window into potential host- and bacterial-associated changes in metabolism that likely accompany co-infections. Expanding the use of such new technologies in clinical and basic CF research will undoubtedly allow us to better understand these microbe-microbe and microbe-host interactions, improving our ability to more accurately diagnose and treat respiratory infections in CF patients and informing us of underlying mechanisms of microbial pathogenesis in the CF respiratory tract.

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MK and JB conceptualized and wrote the manuscript.

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OPINION
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Using Lung Organoids to Investigate Epithelial Barrier Complexity and IL-17 Signaling During Respiratory Infection

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MICROBIAL CAUSES OF RESPIRATORY TRACT INFECTIONS

The respiratory system is the first point of contact with airborne microbial compounds. Consequently, lung mucosal immunity has been extensively studied to understand the mechanisms of host resistance to respiratory infections. The lungs exhibit highly active innate and adaptive mucosal immune mechanisms: they are infiltrated with a wide spectrum of immune cells in steady state and possess the capacity to recruit vast numbers of infiltrating cells upon infection or encounter with inflammatory stimuli. Despite the existence of such protective mechanisms, respiratory tract infections (RTIs) with epidemic and pandemic potential are one of the most common causes of morbidity and mortality worldwide. In recent years, studies using new lung culture systems, such as air liquid interface (ALI), spheroids, tissue explants and advances in DNA sequencing technology have helped identify that the upper and lower respiratory tracts represent distinct biomes in terms of their commensal microorganism colonization, immune barriers and host defense mechanisms (1–3). Most lower respiratory tract infections (LRTIs) cause bronchitis, bronchiolitis and pneumonia as a result of *Streptococcus pneumonia* or *Haemophilus Influenzae* infection. In children, respiratory viruses are responsible for an enormous amount of serious LRTIs (4, 5). In addition, most upper respiratory tract infections are of viral etiology (6). Fungal infections of the lower respiratory tract are also typically caused by pathogenic dimorphic fungi (7). In addition, opportunistic fungi as *Aspergillus fumigatus* commonly cause pneumonia. There is an extraordinary need to better understand human respiratory tract infections, as LRTI represent one of the ten most common causes of death in the world (8).

Technical limitations are inherent with pneumonia animal models and *in vitro* lung infections modeled using immortalized cell lines. In particular, for *in vivo* models, lung anatomy, namely the distribution of the bronchial glands, differs between rodents and humans, and complex processes such as mucus production, or organization of the epithelial barrier are not accurately reproduced experimentally. For *in vitro* lung infections, it is not possible to reproduce *in vivo*-like architecture, the microenvironment, the pulmonary cell complexity in composition. Moreover, bronchial epithelial cells lack cilia and tight junctions. Although lung epithelial barrier cell signaling is today more deeply understood, it has still not been fully evaluated in reproducible lung infection models.

Recent advances in the stem-cell field, including the generation of protocols allowing tissue differentiation from induced pluripotent stem cells (iPSCs), have provided new opportunities to study host–pathogen interactions in a human experimental system that maintains controlled tissue complexity. For this reason, recently developed techniques now allow for innovative and more

meaningful investigations of 3D human lung tissue. Here we outline the complexity of the epithelial barrier to opportunistic microbes and the new 2D and 3D lung models of infection, and explain how these models may be used to improve our knowledge on epithelial cell signaling events upon infection.

COMPLEXITY OF THE LUNG EPITHELIAL BARRIER

Epithelial cells represent the first point of contact for opportunistic microbes or pathogens in the respiratory tract (9). The lung mucosa senses infection through pattern recognition receptors expressed by the airway epithelia (10–12), alveolar cells (13, 14), and mesenchymal stem cells (15, 16). Several cell types then orchestrate mucosal barrier immunity: club cells, ciliated cells, basal cells, goblet cells and neuroendocrine cells as tuft cells decorate the proximal airways, while type-1 and type-2 alveolar cells populate the distal epithelium. The lungs can also be divided into a conducting zone and a respiratory zone, which are populated by different progenitor cell types. The conducting zone is abundant in basal cells (17, 18), airway secretory club cells and lineage-negative epithelial cells (18). The respiratory zone is mainly populated by alveolar type II cells (AEC II) that can proliferate and act as progenitor cells, replacing AECII and AECI cells (19).

The complex barrier functions executed by the lung epithelial layer, including mucociliary clearance and antimicrobial production, cooperate to clear inhaled pathogens. Unsurprisingly, gaining a clear understanding of the lung epithelial barrier has been restrained by this described complexity of the lung organization and its underlying cell types. Early research strategies based on immortalized airway epithelial cells or lung primary cells thus may not replicate the conditions where inhaled microorganisms become pathogens that trigger infections.

DEVELOPING 2D AND 3D TOOLS TO MIMIC LUNG STRUCTURE

In vivo lung epithelial barrier experiments are challenging: dissecting the roles of individual cell types is complex due to the heterogeneity of the lung and the lack of specific cell markers. Much research has thus utilized the 2D ALI system and immortalized, lung cancer-derived cell culture approaches to study airway epithelial barrier–pathogen interactions *in vitro* (20, 21). The ALI system has been successfully used to differentiate progenitor cells, such as primary bronchial epithelial cells, into the corresponding airway tract upon exposure to the appropriate culture conditions (22). For example, basal cells cultured in an ALI system, can differentiate into a pseudo-stratified epithelium containing ciliated, goblet and basal cells (23). So far, this method has helped elucidate the transcriptomic profile of basal cells (24) and the impact of virus-infected basal cells on epithelium development (25). The limits of these "conventional" approaches, however, are namely the lack of tissue architecture; indeed, they are not able to faithfully recapitulate the phenotypic and morphological characteristics of the native epithelium (**Table 1**).

To overcome such limitations of the ALI 2D system, an ALI 3D culture system has been developed, in which human bronchial epithelial cells are cultured on a permeable membrane submerged in media supplemented with stromal cells or growth factors (26).

A precursor to lung organoid culture was the development of a "Lung-on-a-Chip": a microphysciological device that replicates on a chip the functional unit of the breathing lung. This system is based on communication between alveolar and endothelial cells through a microporous elastomeric membrane. The alveolar cells located in the upper chamber, are exposed to air and fed by endothelial cells grown in the bottom layer. Although this Lung-on-a-Chip system has been used for drug discovery and toxicology studies (27), it is not possible to recreate the lung architecture, which has a central role in various physiological functions (**Table 1**) (28).

Over the past 15 years, new *in vitro* strategies have facilitated the production of miniature 3D structures, known as "mini organs" or organoids. Organoids are multi-cellular, stem-cell-derived systems in which cells spontaneously self-organize into properly differentiated, functional cell types that resemble *in vivo* counterparts and recapitulate the key features of the entire organ (**Table 1**). Organoids can help dissecting the role of individual cell types because are deprived of immune cells and endothelial cells. The overall approach is based on using hydrogels containing a gelatinous mixture, such as laminin and collagen, to mimic the extracellular matrix and self-organizing iPSCs. The human adult lung stem cells that are essential for epithelial renewal and tissue repair have proven capable of generating such 3D human lung organoids when cultured in the appropriate differentiating conditions. Adult stem cells, once isolated from the peripheral tissue, are usually cultured in enriched medium in the presence of a hydrogel scaffold that provides structural support and mediates instructive signaling for cell polarization, retention and mobilization. This organoid medium needs to be changed every 4 days, and the organoids must be passaged every 2 weeks. After 3 weeks of culture, starting from adult stem cells, the first small organoids reproduce the faithful microanatomy of the lung and recapitulate some specific lung functions (**Figure 1**) (29). The protocols using human tissue stem cells and iPSCs have been developed further to study organ-related pathologies and ontogeny (30–32). Over the past decade, although lung organoids have been used for much translational research, such as lung engraftment (33), the main application has been *in vitro* disease modeling.

USING ORGANOIDS TO MODEL RTIs

Access to the organoid lumen for experimental perturbation is challenging; thus, many researchers add bacteria to the supernatants of organoid-derived 2D cultures to monitor host–pathogen interactions. Cutting-edge technologies, however, now permit microinjection of microbes into the organoid lumen (34), allowing host–microbiota interplay within the 3D structure. More recently, a high-throughtput organoid

microinjector system has been developed that can deliver microbial communities into the organoid lumen (35).

The first tissue 3D organoid models used to study host–pathogen interactions were intestinal organoids (36). To date, lung organoids have been used in microbial infection studies to understand the molecular mechanisms of epithelial renewal upon viral infection (37) and to study the cytokine profile released in response to pattern recognition receptor activation by *Pseudomonas aeruginosa* (38). Here, wild-type and transgenic lung organoids were treated with bacterial flagellar hook proteins eliciting IL-1β and IL-6 release (38). Another study using organoids infected with *Cryptosporidium* oocytes provided deep understanding of the microbial life cycle and showed that the parasite is able to infect secretory and non-secretory cells, triggering the Type I interferon release from epithelial cells (39). Of note, even though organoids have reproduced the 3D lung architecture during infection, a role for immune cells has not yet been evaluated.

While the above-mentioned studies have been based on the use of 3D organoids differentiated from murine adult stem cells, several efforts are ongoing to generate organoids from immature lung epithelial and iPSCs. iPSCs are obtained by transfecting and reprogramming adult somatic cells with pluripotency transcription factors (40).

As well as studying host–pathogen interactions, lung organoids have proven valuable in understanding cystic fibrosis pathology (41, 42). Here, major breakthroughs have been achieved through using iPSCs derived from patients carrying genetic mutations to generate organoids. These organoids modeling cystic fibrosis have permitted drug testing directly on patient cells with affected organ properties (43). Further studies that aim to elucidate the molecular nature of the protective immune barriers in the lower and upper respiratory tracts will rapidly advance the rational design of novel therapeutics targeting such important diseases. Future studies using patient-specific organoids may permit bio-banking and the development of personalized medicines and targeted therapies for opportunistic pulmonary infections.

USING LUNG ORGANOIDS TO DELINEATE IL-17R SIGNALING IN EPITHELIAL LUNG CELLS

Recent studies have identified the importance and complexity of interleukin-17 receptor (IL-17R) signaling by lung epithelial cells and highlighted the need for deeper investigations into the regulatory network activated by IL-17 cytokines in acute or chronic inflammation. Thus far, studies have shown that high IL-17R expression on lung epithelial cells has a prominent role in the innate immune defense against pulmonary fungal pathogens, including *Blastomyces dermatitidis* (44) and *Aspergillus fumigatus* (45). These epithelial cells may orchestrate innate antifungal immunity by first up-regulating the number of lymphocytes that secrete interleukin-17A (IL-17A) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (44). They then respond to secreted IL-17 *via* the IL-17R, which regulates the secretion of antimicrobial peptides and chemokines that recruit neutrophils. IL-17R expressed on lung club cells orchestrates neutrophil recruitment and *Klebsiella pneumonia* resistance (46).

IL-17A and IL-17F homodimers both bind the IL-17R subunits IL-17RA and IL-17RC (47–49).

Also, the human IL-17A/F heterodimer may bind the complex IL-17R (50) since it may mimic the IL-17A as well as IL-17F behaving as a two-face cytokine. During *Aspergillus* fungal infection, the fungus increases IL-17F expression, which subsequently induces IL-33 and IL-17RC expression on lung epithelial cells, especially in the context of IL-17RA deficiency (45).

A polarized lung epithelium is required for IL-17R expression and innate immune functions, such as mucus production (51). Because organoid cultures recapitulate tissue polarity, they thus provide an exciting possibility of using lung organoids to comprehensively investigate IL-17R signaling in the lung. Improving our understanding of IL-17R signaling by lung epithelial cells is likely to offer new opportunities to develop and test therapeutics for inflammatory diseases and identify new molecular targets to improve resistance to infections. Such

TABLE 1 | Comparison between advanced cell culture techniques for lung infection studies.

Tecnique	Advantage	Disadvantage
Air Liquid Interface	Easy to use protocol	Not *in vivo*-like architecture
	High reproducibility	Lack of morphological characteristics of the native epithelium
	Compliant with high-troughtput screening (HTS)/ High Content Screening (HCS)	
	Patient specific	
	Low cost system	
Lung-on-a-Chip	Physiological environment (perfusion, stretch)	Not adaptable to HTS
	Alveolar–capillary interface	High cost
	Surfactant production and electrical resistance	Difficult to manufacture
Lung organoids	*in vivo*-like complexity and architecture	Lack of vascolature and immune cells
	Histological structures and function of native tissue	Require meticulous maintenance
	Scalable to different plate format	
	High reproducibility	
	Compliant with HTS/HCS	
	Patient specific	

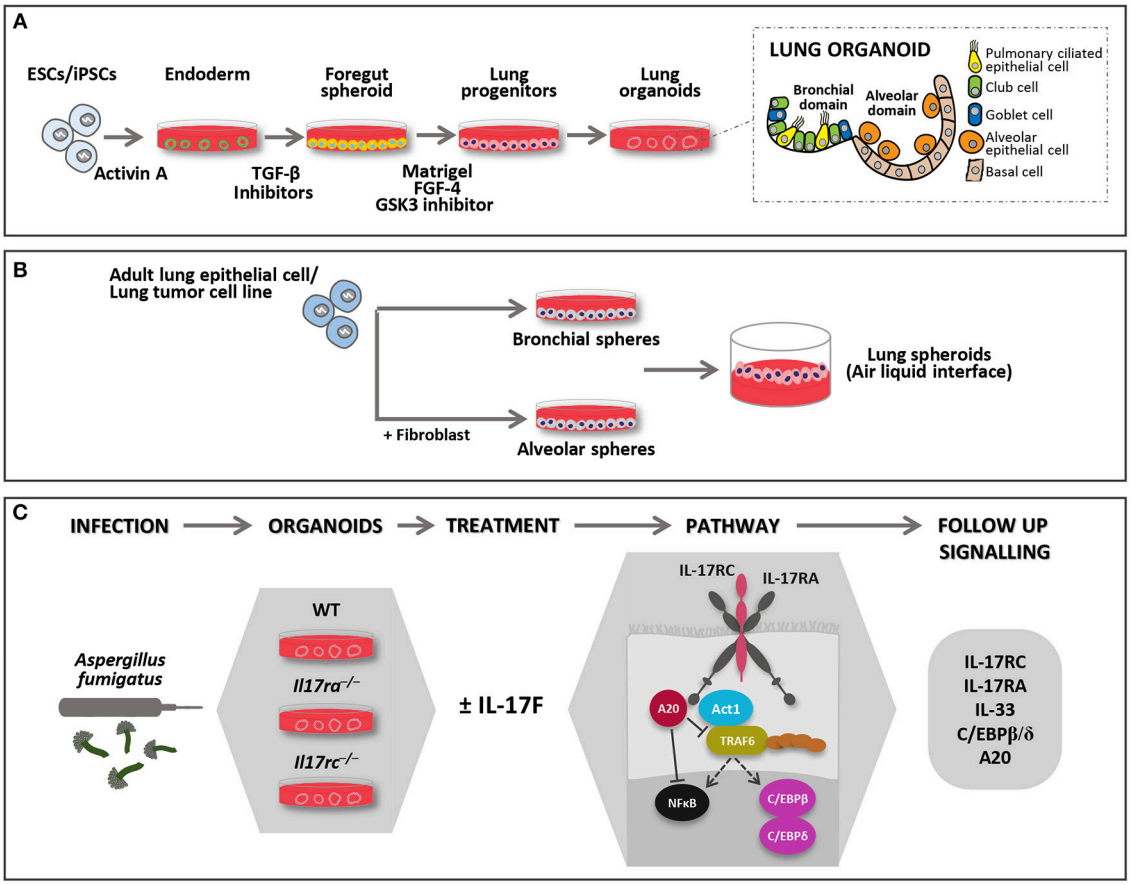

FIGURE 1 | Models of 3D lung organoid infections. Lung organoids can be developed from iPSCs or adult stem cells (upper panel). **(A)** iPSCs derived lung organoids: cells are differentiated into endoderm by Activin A and further to anterior foregut followed by lung progenitor spheroids through the activation and inhibition of several signaling pathways. The progenitor spheroids are further embedded in matrigel to develop 3D lung organoids when supplemented with appropriate growth factors, which resemble lung tissue in morphology and function. They form the bronchial and alveolar like domains of the lungs and has both functional (epithelial cells) and supportive (basal mesenchymal cells) pulmonary tissue. **(B)** Adult lung progenitor cells can form spheroids, which can be further cultured on ALI to mimic lung environment. Spheroids do not form the exact morphology and lack some of the functional cell types. They can either form alveolar or bronchial branch based on various protocols, some of them require co-culture with support cells. **(C)** In the lower panel, the description of an experimental model of RTIs where lung organoids derived from different genotypes may be injected with Aspergillus fumigatus. The model may be used to study IL-17R signaling pathways in 3D system where the complex role of IL-17F may be studied. FGF-4 (Fibroblast growth factor 4); GSK3 (Glycogen synthase kinase 3); TGF-β (Transforming growth factor beta 1).

work is important given that human studies have demonstrated the importance of IL-17-driven immunity in LRTI infections, with mutations in IL-17RA or IL-17RC conferring increased susceptibility to RTIs (52, 53). In addition, the immune-free organoid microenvironment favors IL-17RC signaling studies, as epithelial cells express high levels of IL-17RC compared to immune cells; furthermore, the best characterized IL-17A-targeted cells are non-immune cells, including epithelial cells and mesenchymal cells of the lung (51).

Another important area of research in which lung organoids are anticipated to be of value is in deciphering IL-17F function in asthma. It is produced by multiple cell types including bronchial epithelial cells. IL-17F mediates asthma *via* IL-17R binding on bronchial epithelial cells, eosinophils, fibroblasts and airway smooth muscle cells (54). A certain extent of pulmonary IL-17F, however, is also released by immune cells, which are not differentiated in 3D-organoids. This limitation may be compensated by using exogenous IL-17F or by co-culturing organoids with IL-17F-producing cells. It would be interesting to study the independent role of epithelial IL-17F in modulating airway remodeling, asthma and steroid resistance in 3D cultures. In addition, transgenic lung organoids for IL-17R subunits expression may be useful to better disentangle IL-17F receptor signaling (**Figure 1**). The need to elucidate the function of the receptor subunits comes from the evidence that IL-17RA or IL-17RC mutations have been also described in human fungal infections (52, 53). Clearly, there is a need to recapitulate the 3D structure of lung organoids with the appropriate cell mixture in order to properly investigate pulmonary intercellular networks and immune receptor signaling pathways as IL-17R.

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GP, AL, MA, IT, and SSJ critically read, analyzed, and discussed the literature and conceived the outline of the manuscript. JF and TZ wrote the manuscript. All the authors edited the manuscript and provided valuable discussions and criticisms.

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REVIEW
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Mechanisms of Bacterial Superinfection Post-influenza: A Role for Unconventional T Cells

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Despite the widespread application of vaccination programs and antiviral drug treatments, influenza viruses are still among the most harmful human pathogens. Indeed, influenza results in significant seasonal and pandemic morbidity and mortality. Furthermore, severe bacterial infections can occur in the aftermath of influenza virus infection, and contribute substantially to the excess morbidity and mortality associated with influenza. Here, we review the main features of influenza viruses and current knowledge about the mechanical and immune mechanisms that underlie post-influenza secondary bacterial infections. We present the emerging literature describing the role of "innate-like" unconventional T cells in post-influenza bacterial superinfection. Unconventional T cell populations span the border between the innate and adaptive arms of the immune system, and are prevalent in mucosal tissues (including the airways). They mainly comprise Natural Killer T cells, mucosal-associated invariant T cells and γδ T cells. We provide an overview of the principal functions that these cells play in pulmonary barrier functions and immunity, highlighting their unique ability to sense environmental factors and promote protection against respiratory bacterial infections. We focus on two major opportunistic pathogens involved in superinfections, namely *Streptococcus pneumoniae* and *Staphylococcus aureus*. We discuss mechanisms through which influenza viruses alter the antibacterial activity of unconventional T cells. Lastly, we discuss recent fundamental advances and possible therapeutic approaches in which unconventional T cells would be targeted to prevent post-influenza bacterial superinfections.

Keywords: unconventional T cells, influenza A virus, secondary bacterial infection, *Streptococcus pneumoniae*, *Staphylococcus aureus*, immune suppression, barrier function, immunotherapy

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INFLUENZA VIRUS INFECTION AND BACTERIAL SUPERINFECTION

Respiratory infections are one the biggest health concerns worldwide. They account for a substantial rate of morbidity and mortality in Western and developing countries (1). Amongst respiratory pathogens, influenza viruses, commonly known as "the flu," represent one the most important concern despite ongoing vaccine campaigns and anti-viral drugs. Each year, seasonal influenza infection affects 5 to 15% of the population and is a major contributor of

pneumonia-related death worldwide (500,000 deaths per year) (2). Seasonal influenza is due to two main subtypes in humans, H1N1, and H3N2. Antigenic variations due to mutation in the hemagglutinin (HA) and neuraminidase (NA) genes, a phenomenon known as the antigenic drift, occur every year, and result in the circulation of new strains with sometime enhanced virulence and lethality potential. In parallel, in general every 10 to 20 years, new influenza subtypes distinct from circulating seasonal strains can emerge (due to antigenic shift) and provoke pandemic waves with sometime devastating consequences (3). Relative to seasonal influenza, pandemics exhibit a higher transmissibility and a higher rate of mortality, particularly among younger people who lack specific immunity against these new strains. Mortality attributed to influenza infection can be of high incidence during pandemics. During the Spanish flu (1918–1919), more than 40 million people died from influenza infection (4, 5). During the 2009 pandemic, influenza infection had a substantial impact on human mortality (3, 6). As discussed below, viral-bacteria pneumonia contribute significantly to morbidity and mortality during influenza epidemics and pandemics (5). Due to medical and economical burdens, and considering the threat of new pandemics and the emergence of antibiotic resistance, it is urgent to find novel options to fight against influenza infections and their complications, including secondary bacterial infections.

Influenza a Virus: Main Characteristics

The influenza viruses (types A, B, and C) are negative sense single-stranded RNA viruses (7). These enveloped viruses belong to the family of *Orthomyxoviridae*. In humans, the most common cause of respiratory illness is influenza A virus (IAV) (8). To reach successful replication, sense messenger RNAs must be generated from the viral genome, a process due to the viral RNA polymerase. Sense messengers comprise eight RNA segments encoding eleven proteins (9, 10). The mature virion contains eight of these proteins surrounded by a protein envelope, which mainly includes two viral antigenic determinants: HA, which binds to terminal sialic acids expressed by airway and alveolar epithelial cells and NA, a critical enzyme necessary for releasing the viral progeny from infected cells. Eighteen HA subtypes and 11 NA subtypes have been identified to date. Two IAV subtypes (H1N1 and H3N2) along with one or two influenza B viruses co-circulate annually causing influenza epidemics. The primary targets (and site of replication) of IAV are airway and alveolar epithelial cells. Shortly after infection, the viral machinery, with the unintentionally help of host factors, is at work and generate the release of virions (11). Productive replication in epithelial cells results in cell death and to epithelial/endothelial damages leading to barrier rupture and exudation of fluids and proteins into the airways and alveolar spaces, greatly impairing gas exchanges (12). Meanwhile, an intense infiltration of immune cells (neutrophils, monocytes) occurs. Clinically, severe IAV infection can lead to acute respiratory distress syndrome, a severe form of respiratory failure associated with 40 % of mortality (5).

IMMUNE RESPONSE TO IAV

Innate immune response is rapidly triggered after IAV infection (13–18). This relies on the presence of viral RNA in the cytosol of infected cells and on different and complementary innate sensors including Toll-like receptors (TLRs; primarily TLR3 and TLR7), retinoic acid inducible gene-1 and inflammasomes. Activation of these innate sensors results on the production of massive amounts of type I and type III interferons (IFNs) as well as interleukin (IL)-1β and IL-18. Type I and type III IFNs, though autocrine and paracrine (myeloid cells) effects, elicit the production of a myriad of IFN-stimulated genes that strongly participate in virus clearance. Meanwhile, activation of inflammasomes and NF- κB promotes the release of pro-inflammatory cytokines and chemokines and the subsequent recruitment and activation of numerous immune cells such as monocytes/macrophages and neutrophils. These events limit and/or prevent viral entry and replication and attenuate the severity of the disease. All of these responses however contribute to tissue injury. For instance, inflammatory monocytes greatly participate in epithelial cell damage and death (19, 20). On the other hand, although they participate in virus clearance, neutrophils are also strong contributors of lung damage and lethality (21). While epithelial cells are critical to initiate innate immunity, other resident sentinel cells also play a role in virus clearance. Alveolar macrophages promote the elimination of viruses through the phagocytosis of collectin-opsonised viral particles or infected apoptotic cells (efferocytosis) and production of inflammatory cytokines and chemokines (22, 23). In parallel, other resident and/or recruited innate immune cells, including natural killer cells, unconventional T cells, and innate lymphoid cells play a part in disease outcomes (mouse model of influenza), an effect associated—or not—with effector functions (24–33). On the other hand, depending on the infectious dose, some of them (e.g., NK cells) may also participate in immunopathology (34, 35). Several days after IAV entry and elicitation of innate immunity, a strong antigen (Ag)-specific $CD8^+$ T cell response develops in the lungs. In this phenomenon, the migration of antigen-loaded $CD103^+$ dendritic cells to the draining lymph nodes is critical. Even though Ag-specific $CD8^+$ T cells are sufficient to contain viruses (e.g., through lysis of infected cells), they also contribute to alveolar epithelium and endothelium damage (36, 37).

RESOLUTION OF INFLAMMATION AND SECONDARY BACTERIAL INFECTION

After the inflammatory burst and pulmonary tissue damage, a resolving/repair phase takes place, in general 7 to 14 days after the primary IAV infection. It leads to resolution of infiltrates and regeneration of damaged lung tissue thus restoring gas exchange. In this setting, murine studies of influenza infection suggested that $CD8^+$ T cells, by producing the anti-inflammatory cytokine IL-10, are important to resolve inflammation (38). Activated macrophages can also promote the

expansion of Foxp3-expressing regulatory T cells to suppress the deleterious production of inflammatory cytokines by neutrophils (39). By suppressing IL-17, a cytokine involved in neutrophil recruitment, type I IFNs also contribute to resolution of inflammation post-influenza. Recent data also indicate a role for M2 macrophages in this process (40). Unconventional T cells may also play a critical role in recovery from influenza infection (27, 33, 41, 42). Finally, through amphiregulin production, innate lymphoid cells restore airway epithelial integrity and tissue homeostasis during IAV infection (25). Resolution of inflammation during influenza infection is critical for lung resiliency and restoration of physiological functions (which can take several weeks). This regenerating response corresponds to a period of enhanced susceptibility to respiratory bacterial (particularly Gram-positive) infections. Indeed, this process creates a favorable environment to the emergence of opportunistic pathogens that can eventually result in bacterial superinfection, bacterial pneumonia and bacterial dissemination from the lungs. The two later are major contributors to lethality. Post-mortem examination of autopsy specimens collected during the last pandemic (as well as the 1918 pandemic) suggests that a substantial proportion of patients died from bacterial infections once the virus was cleared (5, 43). The most common bacteria found in autopsied individuals were *Streptococcus pneumoniae* (the pneumococcus) and *Staphylococcus aureus*, two major ubiquitous upper respiratory opportunistic pathogens. Two main mechanisms (mechanical and immunological) explain bacterial superinfection post-influenza: loss of the epithelial barrier function and altered innate immune defense. Before reviewing the role of "innate-like" unconventional T cells in this setting, we summarize the main mechanisms by which IAV favors secondary bacterial infection.

MECHANICAL AND IMMUNOLOGICAL MECHANISMS LEADING TO SUPERINFECTION

Several excellent reviews have described the current mechanistic understanding of how IAV enhances susceptibility to secondary bacterial infections (44–49). Current data, mostly derived from experimental (mouse) models, point toward a multifactorial mechanism. Briefly, IAV disrupts the functions of the respiratory barrier by inducing, in a direct or indirect (through inflammatory monocytes) fashion, epithelial cell death, and by degrading mucins (20, 50). This alteration leads to exposure of new attachment sites for bacteria and allows bacterial translocation (51–53). Influenza A virus can also alter respiratory ciliary function, thereby impairing the clearance of aspirated bacteria from the lungs (54). As stated above, alteration of the innate immune response is critical in post-influenza bacterial superinfections. In particular, poor bacterial control in the context of prior IAV infection is due to the loss and/or dysfunction of macrophages and neutrophils (55–59). For these later, their ability to sense and clear (phagocytosis and killing activity) bacteria is profoundly altered (60–62). Along with these effector cells, dysfunction of natural killer cells also depresses host's antibacterial capabilities (63). Some cytokines are critical in bacterial superinfection. The immune-suppressive cytokine IL-10 inhibits the functions of macrophages and neutrophils (64, 65). IL-27, another immunosuppressive cytokine downstream of type I interferon receptor (IFNAR) signaling pathway also impairs innate immune response against secondary bacterial challenge (66, 67). Type I interferons, which are massively produced during IAV infection to limit viral replication, are also detrimental in bacterial superinfection. Mechanistically, they inhibit the production of chemokines (CXCL1 and CXCL2) important for the recruitment of macrophages and neutrophils to the lung and impair their phagocytic responses (57, 68, 69). Of note, type III IFNs (which share similarities with type I IFNs) favor bacterial superinfection post-influenza by disrupting the nasal microbiome, which often includes potential pathogens (70). The underlying mechanisms are still elusive but may depend on altered barrier functions of the nasal epithelium and dysfunctional innate defense. Although IFN-γ is critical in host defense against respiratory bacterial infections, it might favor secondary bacterial infection, for instance by decreasing the expression of the scavenger receptor MARCO on macrophages (56). In fact, the role of IFN-γ in bacterial superinfection is controversial since a protective role has also been suggested (71). Finally, IAV infection reduces, through signal transducer of activation and transcription-1 (STAT-1), the production of Th17-related cytokines, a critical family of cytokines involved in the control of respiratory bacterial infections (66, 72–75). Hence, the accumulating literature (experimental models) provides a clearer understanding of mechanisms leading to bacterial superinfection and suggests several targets to prevent it. In humans, impairment of innate immunity by pre-existing viral (IAV) infections has also been shown to hamper the control of carriage load and clearance of upper respiratory bacteria such as *S. pneumoniae* (76). This, along with mechanical defects (respiratory ciliary and barrier functions), may favor bacterial superinfection and secondary bacterial pneumonia. While some progresses have been made recently, much remains to be learned about the way that the virus alters pulmonary barrier functions and undermines protective antibacterial immunity during IAV-bacterial (co)infection. As outlined below, recent evidences suggest that unconventional T cell functions are targeted during IAV infection, a process that may be important in secondary bacterial infections.

UNCONVENTIONAL T LYMPHOCYTES

Natural Killer T Cells

Natural killer T (NKT) cells represent a subset of lipid-reactive αβ T cells. In response to lipid Ags presented by the monomorphic Ag presenting molecule CD1d, NKT cells swiftly produce a large amount of cytokines, thus promoting and orientating immune responses (77). Lipid recognition by NKT cells is mediated by a conserved T cell receptor (TCR) repertoire. Natural killer T cells can be divided into two major populations: type I NKT cells and type II NKT cells. Type I NKT cells express a semi-invariant TCR α-chain (Vα14-Jα18 in mice and Vα24-Jα18 in humans) paired with a limited set of TCR β-chains (77, 78). These cells respond strongly to alpha-galactosylceramide (α-GalCer),

a glycolipid under clinical development, particularly in cancer settings (79). Type I NKT cells also recognize endogenous lipids which are necessary for their selection in the thymus and for their activation at peripheral sites. Type I NKT cells can also react to microbial-derived lipids (80). Of importance, type I NKT cells also activate in response to a wide array of cytokines, including IL-12 and IL-23. Despite a relatively conserved TCR, type I NKT cells are heterogeneous and can be further divided into distinct subsets (81, 82). NKT cells produce a wide range of cytokines, with sometime opposite functions, a property that depends on the cell subset activated and on the nature of the stimulation (e.g., lipids and/or activating cytokines). Through this unique property, type I NKT cells can influence different types of immune responses ranging from T helper (Th)1-like, Th2-like, Th17-like, or T regulatory-like responses (83). This property is critical in pathological situations during which type I NKT cells can either exert positive or negative functions. Of note, type I NKT cells not only produce cytokines and display cytotoxic functions toward transformed cells and virally-infected cells (84). Type II NKT cells represent a much broader family of CD1d-restricted αβ T cells that react to lipids, but not to α-GalCer. They express a more diverse TCR repertoire that recognizes lipid Ags of various nature and origin (mammalian and microbial) (85). Due to the lack of specific tools, the functions of type II NKT cells have mainly been proposed indirectly by comparing the phenotypes observed in Jα18-deficient (which lack type I NKT cells) vs. CD1d-deficient (which lack both type I and type II NKT cells) mice in various settings. Type II NKT cells appear to share conserved phenotypic and functional features with type I NKT cells including an effector memory phenotype, cytotoxic potential and secretion of numerous cytokines/chemokines (85). Akin to type I NKT cells, type II NKT cells play important functions during (bacterial) infections. NKT cells, which are more abundant in mice relative to humans, populate both lymphoid tissues and mucosal sites, including the lungs (86, 87).

Mucosal-Associated Invariant T cells

Mucosal-associated invariant T (MAIT) cells present many common features with NKT cells and γδ T cells including the capacity to rapidly react to non-peptide Ags. MAIT cells are defined by their restriction to the major histocompatibility complex class I-related molecule 1 (MR1) (88, 89). The majority of MAIT cells (referred to as classical MAIT cells) (90) express a semi-invariant TCR composed of a canonical TCRα-chain (Vα19-Jα33 in mice and Vα7.2-Jα33 in humans) associated with a restricted set of Vβ segments (88, 89, 91, 92). Through their TCR, MAIT cells recognize small intermediate metabolites from the riboflavin (vitamin B2) pathway of bacteria, mycobacteria and yeast (93–95). They can react to products derived from the non-enzymatic reaction between a riboflavin precursor and small aldehydes of both microbial and host origin. The high instability of these ligands has so far limited their use in the clinics. Reminiscent with NKT cells, MAIT cells can respond to TCR signals and/or to various activating cytokines, including IL-12 and IL-18 (96–98). Upon activation, MAIT cells produce large amounts of Th1- and Th17-related cytokines (99). Additionally, MAIT cells can kill bacteria-infected cells (100). Unlike NKT cells, MAIT cells are abundant in the blood (up to 10% of the T cell compartment) in humans. They are also present at mucosal sites, including the lungs (10% of respiratory mucosal T cells) (101), where they sense the environment and exert a role of sentinels of the immune system. Due to their scarce representation in common laboratory mouse strains (unlike NKT cells), understanding MAIT cell biology is challenging even using *Mr1*$^{-/-}$ mice. To better assess their role in preclinical models, transgenic mice (*Vα19i*Tg x Cα$^{-/-}$) displaying high content of MAIT cells have been developed (102). Given their cytokine profile and cytotoxic potential, MAIT cells intuitively emerged as a specialized cell population in host defense against bacteria.

γδ T Cells

γδ T cells represent approximately 1–10% of peripheral blood T cells in humans and are important components of both innate and adaptive immunity. They display vast effector and immune regulatory functions (103). Akin to other members of the unconventional T cell family, γδ T cells display a pre-activated status that allows rapid induction of effector functions following the detection of tissue stress (104–106). Another important feature of γδ T cells is their tropism for epithelial surfaces including lungs, to where they migrate shortly after development and persist as resident cells. They frequently express invariant or closely related γδ TCRs in a given tissue site (e.g., Vγ1, Vγ4, and Vγ6 in the mouse lung tissue), which confer them specific Ag recognition capabilities from one tissue to another (103). Reminiscent to other unconventional T cells, γδ T cells can kill infected cells and initiate adaptive immune responses through the release of substantial amounts of Th1- and Th17-related cytokines (103). Thus, γδ T cells have emerged as essential constituents of the antimicrobial immunity in both preclinical and clinical settings (107). While the Ags for mouse γδ TCRs have not been reported yet, human γδ T cell subsets (e.g., Vγ9Vδ2^{+} cells) can recognize both natural (of microbial and mammalian origins) and synthetic phosphoantigens (108). However, it is now clear that the phosphoantigens are not directly sensed by the γδ TCRs but rather require the involvement of butyrophilin BTN3A as an intermediate. The precise molecular mechanisms involved in this TCR-dependent γδ T cell activation are still a matter of debate (109). Whatever the mechanisms involved, phosphoantigens have been shown to strongly activate (*in vivo* and *ex vivo*) human Vγ9Vδ2 γδT cells to induce their proliferation and to increase their cytotoxic capacities as well as their cytokine secretion including IFN-γ and TNF-α. Given this, harnessing γδ T cell functions in therapeutic protocols is currently highly considered by clinicians especially in the context of cancer (110).

ROLE OF UNCONVENTIONAL T CELLS IN RESPIRATORY PNEUMOCOCCAL AND STAPHYLOCOCCAL INFECTIONS

Evidence in both preclinical and clinical settings have suggested a key role for unconventional T cells in host response against lung bacterial pathogens. Here, we compared their mode of activation

and functions during respiratory bacterial infections with a focus on the two major opportunistic pathogen bacteria implicated in bacterial superinfection post-influenza, namely *S. pneumoniae,* and *S. aureus*.

Streptococcus pneumoniae

Streptococcus pneumoniae (also referred to as the pneumococcus) is the leading cause of community-acquired bacterial pneumonia worldwide (2 million deaths per year), with infants and the elderly exhibiting higher susceptibility. This Gram-positive bacterium, which comprises a group of more than 90 serotypes, colonizes asymptomatically nasopharynx of healthy individuals. However, when the immune equilibrium is broken, pneumococcus carriage can lead to mild disease such as otitis media or sinusitis and more occasionally turns into severe complications such as pneumonia, sepsis, and meningitis (111). *Streptococcus pneumoniae* is often found in biological fluids of hospitalized patients diagnosed for influenza infection as well as patients with exacerbated chronic lung inflammation (112, 113). Despite vaccination prevents pneumococcus spread and controls infections, the available vaccines have however some issues (114). In addition, the emergence of antibiotic-resistant strains represents an important threat for the management of pneumococcal infections in clinics (115).

In the mouse system, both type I NKT cells and γδT cells activate in response to *S. pneumoniae* (**Figure 1**). While type I NKT cells produce IFN-γ early after pneumococcal challenge, γδT cells produce IL-17A (66, 74, 116–118). Activation of type I NKT cells during *S. pneumoniae* infection depends on pneumococcal-derived lipid(s) (α-glucosyldiacylglycerol), cytokines (IL-12) or both according to the strain studied (86, 116, 119). Of note, we and others have highlighted the role of $CD103^+$ dendritic cells in the activation of type I NKT cells during pneumococcal infection (119, 120). The mechanisms through which murine γδT cells activate (IL-17) mainly depends on IL-1β and IL-23 (117, 121). The lack of type I NKT cells ($J\alpha18^{-/-}$ mice) (119, 122, 123) or γδT cells ($Tcrd^{-/-}$ mice) (66, 117) results in higher bacterial loads and mortality. The underlying mechanisms of this protective activity rely on IFN-γ and IL-17 secretion and on the early recruitment of neutrophils. Hence, both type I NKT cells and γδT cells play a natural positive role in host defense against experimental pneumococcal infection (**Figure 1**). The potential role of type II NKT cells and MAIT cells during pneumococcal infection is still elusive. Of interest, *S. pneumoniae* expresses enzymes involved in the synthesis of riboflavin metabolites (124, 125) and human MAIT cells produce, in an MR1-dependent manner, IFN-γ in response to dendritic cells and airway epithelial cells exposed to *S. pneumoniae* (126, 127). Using Vα19iTg x $C\alpha^{-/-}$ mice, a small proportion of lung MAIT cells were shown to produce IFN-γ and IL-17A during pneumococcal infection (127). Although a more detailed kinetic analysis is required, these levels were relatively low compared to those produced by NKT cells and γδT cells. The use of $Mr1^{-/-}$ or Vα19iTg x $C\alpha^{-/-}Mr1^{-/-}$ mice will be instrumental to address the role of MAIT cells during experimental pneumococcal infection.

The potential effects of exogenous activation of unconventional T lymphocytes on pneumococcal infection have been examined. Inoculation of the type I NKT cell superagonist α-GalCer protects against lethal pneumococcal infection in the mouse system (123, 128). Mechanistically, this protective activity relies on respiratory $CD103^+$ dendritic cells and on both IFN-γ and IL-17A production and neutrophils (128). The potential effect of exogenous γδ T cell and MAIT cell activation on host defense against pneumococcal infection is presently unknown. Despite emerging evidence for a critical role in host response to pneumococcus in experimental models, information regarding the phenotype and dynamics of unconventional T cells in patients with severe *S. pneumoniae*-driven pneumonia are rather limited. Of note, the level of circulating MAIT cells in critically ill patients with severe bacterial infection is markedly decreased compared to age-matched healthy controls (129). Although this decrease is less striking in patients with streptococcal infections, these data suggest that MAIT cells may migrate into the lungs, and thus may exert a potential role during pneumococcal infection.

Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium with a potent pathogenic potential to cause a variety of community and hospital-acquired infections. In normal conditions, it commonly colonizes the upper airways. Under certain circumstances, including influenza infection, it can cause localized and serious invasive infections, as well as a severe septic shock syndrome (130). The frequency of these infections is increasing. The ability of *S. aureus* to form biofilms and the emergence of multidrug-resistant strains (e.g., methicillin-resistant *Staphylococcus aureus*) are the main reasons why their treatment is becoming more difficult. The capacity of *S. aureus* to become pathogenic is related to the expression of virulence factors, among which the production of a wide variety of toxins. Staphylococcal superantigens (SAgs) constitute a family of potent exotoxins secreted by *S. aureus* (131). They can cross-link MHC class II molecules with TCRs to stimulate an uncontrolled polyclonal activation of T lymphocytes (cytokine storm), potentially leading to severe illnesses including toxic shock syndrome.

Despite a relatively poor literature in the field, unconventional T cells might play role during *S. aureus* infection. They also recently emerged as potential targets of Staphylococcal SAgs. Indirect evidence suggest that IL-17 production by γδ T cells might be important in the control of *S. aureus* lung infection (72, 73) (**Figure 1**). Mice lacking γδ T cells have a reduced ability to clear bacteria and to control pulmonary inflammation (132). The role of NKT cells and MAIT cells in the control of *S. aureus* is still unknown. On the other hand, emerging evidence suggest that unconventional T cells (at least NKT cells and MAIT cells) are involved in toxic shock syndrome induced by Staphylococcal SAgs. Intranasal inoculation of Staphylococcal enterotoxin B promotes the activation of type I NKT cells and lung injury (133). Staphylococcal enterotoxin B activates mouse and human type I NKT cells via a MHC class II (but not CD1d) Vβ8-dependent pathway (134). More recently, Szabo et al., using SAg-sensitive HLA-DR4-transgenic mouse demonstrated that type I NKT cells are pathogenic (toxic shock syndrome) in response to Staphylococcal enterotoxin B (135). Of interest, administration of a Th2-polarizing glycolipid agonist

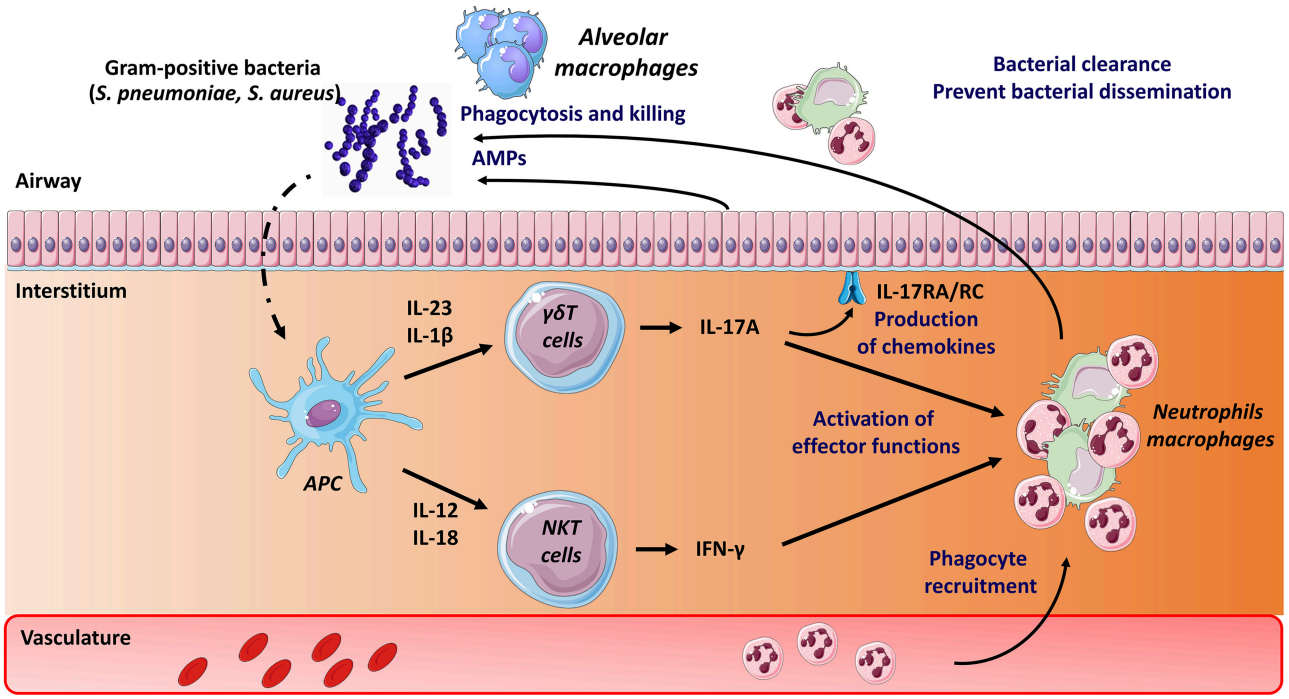

FIGURE 1 | Mode of activation and role of unconventional T cells during pneumococcal and staphylococcal infections. Early after *S. pneumoniae* and *S. Aureus* infection, Ag presenting cells, including dendritic cells, produce a wide array of cytokines that activate pulmonary γδ T cells (IL-17A) and type I NKT cells (IFN-γ). Lipids from *S. pneumoniae* can also directly activate type I NKT cells through their TCR. The protective role mediated by γδ T cells and type I NKT cells comprises activation of innate effector cells, such as macrophages and neutrophils (IFN-γ, IL-17). The latter are rapidly recruited in response to chemokines produced, amongst other cell types, by epithelial cells (i.e., IL-17 receptor signaling pathways). Mechanisms leading to bacterial clearance include killing (bactericidal) activity of macrophages and neutrophils and release of antimicrobial peptides (AMPs). The potential role of MAIT cells in pneumococcal and staphylococcal infections is still unknown.

for type I NKT cells reduced morbidity and mortality. Type I NKT cells may therefore constitute an attractive therapeutic target in SAg-mediated illnesses. Mouse and human MAIT cells can also activate in response to Sags in a largely TCR-independent, cytokine-driven manner (136). They produce a huge amount of pro-inflammatory cytokines and thereafter become unresponsive to stimulation with bacterial Ags. Through this mechanism, they might participate in cytokine storm and subsequent immunosuppression. Akin to type I NKT cells, MAIT cells may therefore provide an attractive therapeutic target for the management of both early and late phases of severe SAg-mediated illnesses.

ROLE OF UNCONVENTIONAL T CELLS IN BACTERIAL SUPERINFECTION POST -INFLUENZA

As outlined below, mouse models of viral-bacterial infection have been used to assess the role of unconventional T cells in bacterial superinfection post-influenza. These cells are activated during influenza infection (24, 27–33, 137) and, through their ability to control barrier function, they may limit bacterial superinfection. On the other hand, although activation during influenza infection may preset their antibacterial effector functions, immune suppression arising from influenza counteracts their antibacterial potentials.

Role of Unconventional T Cells in Pulmonary Barrier Functions

Disruption of the pulmonary barrier functions strongly contributes to enhanced bacterial colonization, bacterial superinfection and bacterial pneumonia in the context of prior influenza. Emerging evidences suggest that unconventional T cells play a natural role in the maintenance of tissue integrity and/or in tissue repair processes (138). Recent studies have addressed the role of unconventional T cells in tissue homeostasis and barrier functions during experimental influenza. Type I NKT cells and γδ T cells produce the tissue protective cytokine IL-22 (through IL-1β- and IL-23) during the early course of IAV infection (24, 139). Although IL-22 does not affect viral loads during influenza, several independent groups have demonstrated the protective effect of IL-22 against epithelial damages caused by viral replication (24, 139–143). The mechanisms through which IL-22 prevents epithelial barrier dysfunction during influenza infection might include an inhibitory effect on the recruitment of inflammatory monocytes and a direct effect on the expression of genes involved in barrier functions (143). Interleukin-22 might also participate in airway epithelial regeneration and barrier repair (141, 142). Interestingly, through

its protective effect on barrier functions, IL-22 reduces secondary bacterial infection (139, 143). Of note, MAIT cells have recently been reported to accumulate in the lungs and to activate (through IL-18) during experimental IAV infection, a process associated with protection against a lethal viral challenge (33). Although not firmly established, MAIT cell activation during IAV infection may reduce pulmonary epithelial damage and reinforce barrier functions. Hence, by rapidly producing tissue protective factors, unconventional T cells, including NKT cells, γδ T cells, and MAIT cells, may limit the extent of secondary bacterial infection post-influenza. It is noteworthy that NKT cells can also indirectly activate the synthesis of protective barrier factors by other cells (e.g., amphiregulin by group 2 innate lymphoid cells) (25, 41). These functions might be exploited for therapeutic purposes.

Role of Unconventional T Cells in Pulmonary Innate Responses

Alteration of innate immune defense also strongly contributes to bacterial superinfection post-influenza. As stated above, unconventional T cells play a part in host defense against *S. pneumoniae* and *S. aureus*. Here, we summarize host factors that (may) compromise their protective functions in the context of double viral and bacterial infection (mouse system). In this setting, IL-10 and type I IFNs appear to play a relevant role. During IAV infection, IL-10 is massively produced by innate and adaptive immune cells. This includes $CD4^+$ (including regulatory T cells) and $CD8^+$ T cells as well as NK cells and myeloid cells, mostly inflammatory monocytes (38, 119). Our data indicate that in the context of prior influenza, type I NKT cells fail to produce the protective cytokine IFN-γ (**Figure 2**), an effect associated with worse secondary pneumococcal infection (119). Blockade of IL-10 rescues activation of type I NKT cells (through restoration of IL-12 production by Ag-presenting cells), reduces bacterial outgrowth and dissemination and improves disease outcomes. Hence, the lack of type I NKT cell activation participates, at least in part, to bacterial (pneumococcal) superinfection post-influenza. Along with IL-10, type I IFNs favor bacterial superinfection post-influenza (57, 66, 68, 72–74, 144). γδ T cells appear to be the main target of type I IFNs. In the context of double IAV-bacterial (both pneumococcal and staphylococcal) infection, γδ T cells fail to secrete IL-17 in a type I IFN-dependent manner (66, 72, 74) (**Figure 2**). This ultimately leads to altered neutrophil recruitment and activity and to inhibition of the IL-17 antimicrobial pathway, including production of antimicrobial peptides. In this setting, the mode of action of type I IFNs is multiple. Type I IFNs can block the secretion of Th17-promoting cytokines IL-1β and IL-23 by Ag-presenting cells (72, 73). On the other hand, type I IFNs can directly target γδ T cells, via IFNAR, to inhibit IL-17 production (74) (**Figure 2**). Finally, type I IFNs indirectly inhibit IL-17 release by γδ T cells by promoting IL-27 production (66, 67). IL-27 targets γδ T cells to decrease expression of the IL-17-promoting factors RORγt and IL-23 receptor (66). The later mechanism is probably dominant as exogenous administration of IL-27 reverses the resistance phenotype of IFNAR-deficient mice upon post-influenza bacterial infection via down-regulating IL-17 production by γδ T cells and neutrophil response. Whether MAIT cell functions are affected by influenza, for instance through IL-10 or type I IFNs, is still ignored. Type III IFNs have also been shown to favor bacterial superinfection post-influenza (70). Regarding the role of unconventional T cells in barrier functions and innate antibacterial immunity, one can speculate that type III IFNs (like type I IFNs) also alter the functions of these cells to favor bacterial superinfection.

Human Studies

Whilst the use of experimental models suggests a role for NKT cells and γδ T cells in bacterial superinfection post-influenza (the potential role of MAIT cells has not yet been appreciated), few studies have so far investigated unconventional T cells during human influenza and secondary infections. MAIT cells are more abundant in the blood relative to NKT cells and, to a lesser extent, γδ T cells. Compared to healthy donors, the frequency of circulating MAIT cells decreased in patients hospitalized for severe pneumonia due to infection with the Asian lineage avian IAV (H7N9) (96). Of interest, individuals who recovered from pneumonia had a higher level of circulating MAIT cells compared with patients who succumbed (96). This study suggested a protective role of MAIT cells in human influenza. Another clinical study confirmed the reduced peripheral blood MAIT cell frequencies (and enhanced granzyme B expression) in patients with acute IAV infection (2009 H1N1 pandemic) (145). This decrease was even more pronounced in critically ill patients admitted in intensive care unit compared to patients with mild symptoms. Reduction of MAIT cell numbers during acute human influenza infection (critically ill patients) could impair protective anti-bacterial immunity increasing the risk of secondary bacterial infections, which would enhance disease severity and mortality. The frequency, number and functional state of NKT cells and γδ T cells during human influenza have not yet been examined. In influenza vaccinated individuals, γδ T cells proliferate and activate although this intensity weakens with age (146).

THERAPEUTIC OPPORTUNITIES

The unique biologic features of unconventional T cells are now being harnessed in the fight against cancer (79, 110, 147). Although this field is still in its infancy, exploitation of these cells in the management of lung infections appears to have therapeutic promise (148). Targeting unconventional T cells has several advantages. Firstly, the cells' restriction to non-polymorphic Ag-presenting molecules renders most patients eligible for unconventional T cell-based therapy using universal ligands. The question of whether unconventional T cells are potential immune targets in post-influenza bacterial superinfections has recently been addressed in preclinical models. The results suggest that these cells can indeed be exploited therapeutically. One major obstacle is the difficulty in balancing the induction of effective bacterial clearance and the avoidance of excessive inflammation. Cytokine-based strategies, neutralizing antibodies, and treatment with agonists that are specific for unconventional

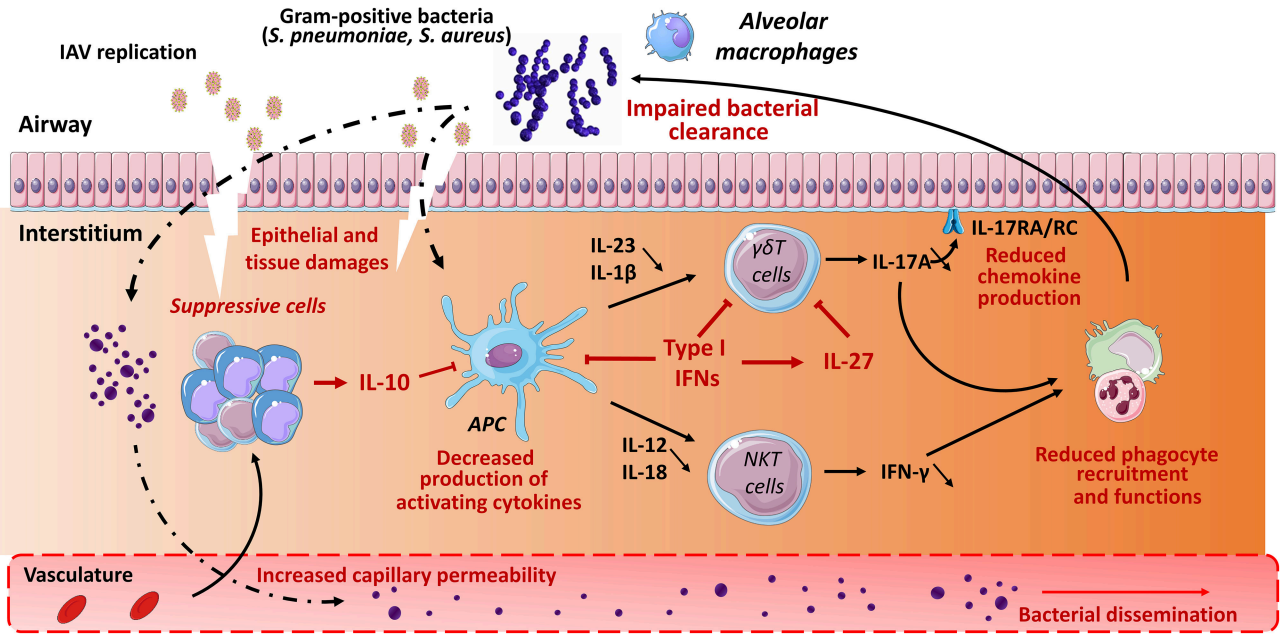

FIGURE 2 | Role of unconventional T cells in bacterial superinfection post-influenza. Influenza A virus replicates in epithelial cells, thus leading to cellular damage and pulmonary inflammation. Along with typical inflammatory cells (neutrophils, inflammatory monocytes), the lungs is infiltrated with various populations of immune suppressive cells expressing for instance IL-10. The later inhibits the production of IFN-γ by type I NKT cells. Meanwhile, due to reduced IL-1β and IL-23 production, γδ T cells display a defective ability to release IL-17A. Multiple mechanisms are involved including a direct role for type I IFNs on Ag presenting cells (reduced IL-1β and IL-23) and γδ T cells (reduced IL-17A) and a promoting effect of type I IFNs on IL-27 synthesis. Interleukin-27 in turn targets γδ T cells to reduce IL-17A production. During IAV infection, there is also a numeric and/or functional defect of alveolar macrophages and neutrophils. As a result, the development of respiratory bacteria in the lung compartment is not controlled, leading to severe bacterial pneumonia and bacterial dissemination from the lungs. The potential positive role of γδ T cells and type I NKT cells (IL-22) in the maintenance of the epithelial barrier is not mentioned. The potential role of MAIT cells in bacterial superinfection is still unknown.

T cells have generated promising results over the last few years (mouse system).

The overexpression and/or inoculation of IL-23 or IL-1β restores the defective production of IL-17 by γδ T cells and T helper cells, and improves the clearance of pneumococci and staphylococci (72, 73). Furthermore, neutralization of IL-10 and IL-27 by blocking antibodies during the course of influenza restores the respective abilities of NKT cells and γδ T cells to combat secondary bacterial infections (66, 119). It is important to note that this approach is associated with better disease outcomes, including higher survival rates. In the mouse system, it has been suggested that treatment with the superagonist α-GalCer can enhance the beneficial activity of type I NKT cells. Inoculation of α-GalCer during IAV infection markedly reduces the bacterial (pneumococcal) burden in the lungs and bacterial dissemination from the lungs (149). However, the efficacy of this type of treatment is limited by its narrow therapeutic window; on day 7 (when susceptibility to superinfection peaks), α-GalCer has no effect. This is due to the disappearance of $CD103^+$ dendritic cells (150)–a critical population involved in activation of type I NKT cells in the lungs—at this time point (128). In contrast, α-GalCer treatment early in the IAV infection (on day 4) or during the resolution phase (day 14) is associated with lower pneumococcal outgrowth and dissemination. Less intense viral-bacterial pneumonia and a lower morbidity rate were observed in superinfected mice treated with both α-GalCer and the anti-inflammatory corticosteroid dexamethasone (149). However, this combination therapy was not associated with a lower mortality rate during secondary bacterial superinfection. In contrast to type I NKT cells, the potential effects of agonists on γδ T cells and MAIT cells in the context of post-influenza secondary bacterial infections have yet to be investigated.

Although the above-mentioned findings (the restoration of NKT cell and/or γδ T cell functions) have revealed a novel aspect of immunotherapy against superinfection in animal models, their clinical relevance remains to be proven. In the search for an effective balance between effective bacterial clearance and the avoidance of excessive inflammation, it is likely that additional therapeutic approaches (e.g., anti-inflammatory drugs) will have to be implemented. One can also speculate that combination treatment with antibiotics might enhance the efficacy of immunotherapy. It was recently shown that the application of a combination of antibiotics and immune stimulators (e.g., Toll-like receptor agonists) improved the outcome of post-influenza bacterial superinfection in a murine system (151). On the same lines, it would be useful to study the effects of a combination of an agonist (e.g., α-GalCer) and an antibiotic. Another key challenge relates to cell targeting. As discussed above, unconventional T cells are heterogeneous, and comprise subpopulations with sometime opposite functions. It will be necessary to target

subpopulations of interest (e.g., IL-17 producers) more accurately by engineering T-helper-polarizing agonists (which have only been developed for NKT cells so far) and/or co-factors polarizing their functions. This would open the way to immunotherapies tailored to match a patient's immune profile. Importantly, it seems that this type of treatment must also take account of the nature of secondary bacterial infection. As discussed above, Staphylococcal SAgs have a critical role in the pathogenesis of *S. aureus* infections. In this setting, antagonists or T-helper-polarizing agonists could be used to manipulate type I NKT cells and MAIT cells—both of which are hyper-responsive to SAgs.

CONCLUSIONS AND PERSPECTIVES

Unconventional T cells have attracted growing interest from researchers and clinicians. The literature on the cells' roles in immune and inflammatory responses has grown tremendously over the last 10 years. In view of their immunoregulatory potential, unconventional T cells are well poised to help fight lung infections and the latter's complications. However, there is a paucity of preclinical and clinical research on the cells' potential roles in the context of influenza and secondary bacterial infections. Further research into (i) the role of unconventional T cells in bacterial (super) infections of the respiratory tract and (ii) how influenza modulates the cells' functions is now needed. Furthermore, the use of novel mouse models will be essential for defining the respective roles of unconventional T cells and their subsets in influenza and secondary bacterial infections. Given that the mechanisms of post-influenza bacterial superinfections are multifactorial (with the exploitation of mechanical and/or immune alterations in the host), future therapeutics will probably have to include several components that target several host factors in addition to the viruses and bacteria themselves. Although this approach is in its infancy, the manipulation of unconventional T cells during influenza (cytokines, α-GalCer) has shown its potential in the fight against secondary infections. As mentioned above, this strategy is not problem-free, and must be considered with caution. Research on the effects of combining immunostimulatory factors with antimicrobial drugs (e.g., antibiotics) should be encouraged, and might help to lessen the development of drug resistance. Given the physiological role of unconventional T cells in tissue repair and barrier functions, strategies for promoting these functions might also be of value. Lastly, given the role of type I NKT cells and MAIT cells in the cytokine storm that follows exposure to Staphylococcal SAgs, the manipulation of these cells might help to control the outcomes of secondary staphylococcal infection—including necrotizing pneumonia. As discussed in this review, there is also a critical knowledge gap between preclinical and clinical studies; hence, analyses of the frequency/number and functional states of patients' unconventional T cells should be encouraged. Counts of circulating unconventional T cells are not negligible; considering the critical role they exert in many diseases, one can expect to see some major breakthroughs in the near future. Promising research initiatives might include a complete analysis of the whole family of unconventional T cells, i.e., NKT cells, group 1 CD1-restricted T cells, MAIT cells and γδ T cells. High-throughput RNA sequencing (at the bulk population and single-cell levels) and the computer modeling of cytokine signatures in patients should also be encouraged. Although the work will be time-consuming and arduous, it might translate into improved clinical outcomes.

In conclusion, we critically analyzed the available evidence on the potential role of unconventional T cells in post-influenza bacterial superinfections. In view of these cells' extraordinary immunostimulatory and immunoregulatory properties and the proven safety of unconventional T cell agonists, further research in this field should be encouraged.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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