CROSS-TALK BETWEEN INFLAMMATION AND BARRIER FRAMEWORK AT MUCOSAL SURFACES IN THE LUNG: IMPLICATIONS FOR INFECTIONS AND PATHOLOGY

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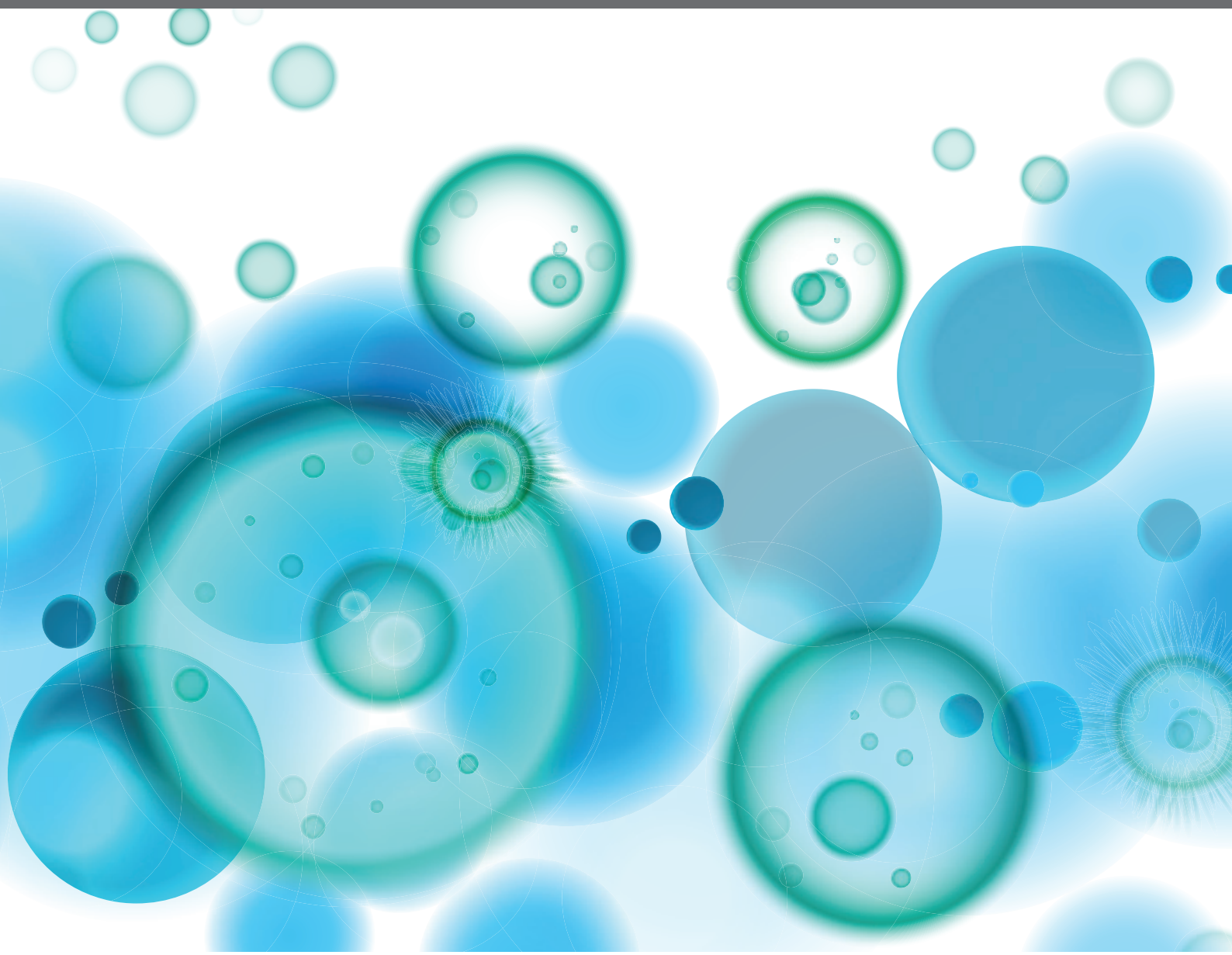

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CROSS-TALK BETWEEN INFLAMMATION AND BARRIER FRAMEWORK AT MUCOSAL SURFACES IN THE LUNG: IMPLICATIONS FOR INFECTIONS AND PATHOLOGY

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Table of Contents

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EDITORIAL
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Editorial: Cross-Talk Between Inflammation and Barrier Framework at Mucosal Surfaces in the Lung: Implications for Infections and Pathology

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Keywords: lung infection and pathology, mucosal inflammation, epithelial barrier function, mucosal immune response, therapeutic interventions

Editorial on the Research Topic

Cross-Talk Between Inflammation and Barrier Framework at Mucosal Surfaces in the Lung: Implications for Infections and Pathology

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Inflammation at lung mucosal surfaces is required to resolve infections and preserve barrier integrity. A carefully orchestrated optimal immune response is a desirable outcome that leads to the resolution of infection while mounting a simultaneous reparative response to maintain the lung homeostasis and barrier integrity during acute and chronic inflammation. However, defects in immune regulation result in developing an aberrant immune response that causes immune pathology and exacerbates the disease. Therefore, the targets for immune therapies against acute and chronic inflammatory diseases in the lung require a comprehensive understanding of immune mechanisms associated with immune dysregulation and tissue damage. In this special issue, several original, review, and opinion articles highlighted the role of inflammation in acute infections and chronic conditions in the lung.

The perspective article from Alcorn encapsulates the significant role of interleukin−22 (IL-22) in promoting epithelial integrity and repair following the lung's infectious pathogen challenge. The pre-clinical animal models suggest that IL-22 has significant therapeutic potential in the context of infectious diseases. While the reparative role of IL-22 has been shown broadly in the context of epithelial repair and lung barrier integrity, the article highlights the need to further assess the effects of IL-22 on epithelial cells in inflammatory settings, perhaps in combination with pathogen-associated molecular patterns (PAMPs) or toxins.

The review article by LeMessurier et al. elaborates on the role of the respiratory barrier as a safeguard and regulator of defense against influenza and *Streptococcus pneumoniae*. The article highlights the role of leukocyte-epithelial as well as inter-epithelial crosstalk in the regulation of barrier integrity during influenza infection. Furthermore, the article elaborates the role of several host factors, such as TRAIL, interferons, and other inflammatory cytokines, in the altering epithelial junctions and permeability, which is associated with dysregulated inflammation, leading to the permissiveness of influenza-infected airway cells for *Streptococcus pneumoniae* co-infection. Finally, the article proposes that the crosstalk at the interface of microbial pathogens and human

host epithelium presents multiple opportunities for the development of clinically relevant therapies during respiratory infections.

In a mini review article by Samarasinghe and Rosch, they described the convergence of inflammatory pathways in allergic asthma and sickle cell diseases (SCD). Asthma and SCD share a number of similarities in terms of the immunological factors associated with their respective disease states. The immunologic sequelae associated with SCD and asthma are complex but have some overlap. The review provided a concise overview of inflammatory pathways impacted during SCD and asthma, and how pulmonary physiology and inflammation are impacted during SCD and asthma comorbidity.

The original article by Allard et al. describes the role of asthmatic bronchial smooth muscle (BSM) derived CCL5 and its role in monocyte migration in response to the rhinovirus-infected epithelium. Asthma exacerbations, a significant concern in therapeutic strategies, are most commonly triggered by viral respiratory infections, particularly with human rhinovirus (HRV). The study assessed whether or not BSM could increase monocyte migration induced by HRV-infected bronchial epithelial cells. An *in vitro* model of co-culture of human bronchial epithelial cells in air-liquid interface with human BSM cells from control and asthmatic patients was developed to address that. HRV-induced monocyte migration was substantially increased in the co-culture model with asthmatic BSM, compared with control BSM. However, the well-known monocyte migration chemokine, CCL2, was not involved in this increased migration. Instead, the recruitment was CCL5 dependent. Therefore, the findings highlighted a new role of BSM cells in HRV-induced inflammation *via* CCL5.

Gao et al.'s original research article describes bacterial porin, OprC mediated impairment of host defense by increased quorum sensing mediated virulence of *Pseudomonas aeruginosa*. *P. aeruginosa*, found widely in the wild, causes infections in the lungs and several other organs in healthy people but more often in immunocompromised individuals. The authors reported that oprC deletion severely impaired bacterial motility and quorum-sensing systems, as well as lowered levels of lipopolysaccharide and pyocyanin in *P. aeruginosa*. In addition, oprC deficiency impeded the stimulation of TLR2 and TLR4 and inflammasome activation, resulting in decreased proinflammatory cytokines and improved disease phenotypes, such as attenuated bacterial loads, lowered lung barrier damage, and prolonged mouse survival. The findings summarize OprC as a critical virulence regulator, providing the groundwork for further dissection of the pathogenic mechanism of OprC as a potential therapeutic target of *P. aeruginosa*.

The original research article by Zhang et al. describes the Mycobacterium abscessus components and their crosstalk with human bronchial epithelial cells (HBECs). Mycobacterium avium complex (MAC) and Mycobacterium abscessus (MAB) are two of the most common causative pulmonary infection agents. The reaction between bronchial epithelia and components in the envelope of the mycobacterial cell wall is poorly understood. The results importantly demonstrate the role of Type I IFN in cross-talk between NHBE cells and MAB, suggesting an immune response by HBECs cells may play a central role in the imitation of innate immunity. Furthermore, the study underscores the importance of mycobacterial cell wall in initiating an innate immune response.

Finally, the review article by Aguilera and Lenz discusses the role of inflammation as a modulator of host susceptibility to influenza, pneumococcus, and co-infections. The article summarized the role of different leukocyte subsets and immune sensing to pulmonary infections. Specifically, the article elegantly summarizes the findings on alveolar macrophages, monocytes, NK cells, and cytokine mediators IFN-γ, TNF-α, IL-10 in influenza and influenza pneumococcal infections. The article concludes that regulation of lung innate immune responses in susceptible populations and in the context of complex environmental elements (such as the microbiota) are needed to provide avenues for the development of new treatments.

The contributions in the form of original and review articles to this Research Topic highlight the complex interplay between pathogen and inflammation in the lung and chronic conditions that dysregulate inflammation in a complex manner. The articles broadly underline the significance of safeguarding the mucosal barrier in the lung during infection or chronic inflammation by therapeutic interventions or tailoring immune response to allow more effective resolution of infection/inflammation and mitigate tissue damage and immune pathology.

AUTHOR CONTRIBUTIONS

RM, GM, and MK conceived, designed, and wrote the manuscript. All authors have read and approved the finalized version of the manuscript for publication.

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ORIGINAL RESEARCH
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Mycobacterium abscessus—Bronchial Epithelial Cells Cross-Talk Through Type I Interferon Signaling

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Introduction: *Mycobacteria* are aerobic non-motile organisms with lipid rich, hydrophobic cell walls that render them resistant to antibiotics. While there are over 150 different species of NTM, *Mycobacterium avium complex* (MAC) and *Mycobacterium abscessus* (MAB) are two of the most common culprits of pulmonary infection. MAB has been found to be most common in southeastern United States (Florida to Texas) and the third most rapidly growing NTM infection. It is responsible for chronic lung infections. Mycobacterial cell wall components initiate the interaction between bacteria and host. The reaction between bronchial epithelia and components in the envelope of mycobacterial cell wall is poorly understood.

Methods: A lung-on-membrane model was developed with normal human bronchial epithelial (NHBE) cells re-differentiated at the air-liquid interface (ALI) and human endothelial cells on a transwell® polyester membrane. Microparticles from MAB cell walls were developed by an inhouse protocol and added to the ALI side of lung model. NHBE cells were harvested at day 3. RNA was isolated and analyzed with RNASeq. NHBE cells were lysed and protein assay was performed with western blot. We tested whether lung INF-alpha expression would increase in mice treated with intratracheal MAB cell wall particles. A paired *t*-test is used to compare two population means using GraphPad Prism 7 software.

Results: RNAseq analysis identified 1759 differentially expressed genes between NHBE cells challenged with and without MAB microparticles with FDR < 0.5. 410 genes had a 2.5-fold change (FC) or greater. NHBE cells exposure to MAB microparticles significantly enriched the IFN I signaling pathway. Protein overexpression of IFN I family (2′-5′-Oligoadenylate Synthetase 1, Interferon-induced GTP-binding protein Mx1, Interferon-stimulated gene 15) was found in bronchial epithelial cells following exposure to MAB cell wall microparticles. IFN-α protein and gene expressions were significantly increased in mice lung challenged with microparticles in comparison with controls.

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Conclusion: These data strongly support the role of Type I IFN in cross-talk between NHBE cells and MAB. They also suggest that initiating immune response by NHBE cells may play a central role in innate immunity. Furthermore, this study underscores the importance of mycobacterial cell wall in initiating innate immune response.

Keywords: *Mycobacterium abscessus*, bronchial epithelial cells, IFN, Interferon, mycobacteria

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are ubiquitous organisms responsible for clinically significant lung infections that have increased 5–10% annually over the past two decades with an annual burden of ∼84,000 cases (1). In the United States, *Mycobacterium Avium Complex* (MAC) is the most frequently isolated species followed by *Mycobacterium kansasii* and *Mycobacterium abscessus* (MAB) (2, 3). MAB is the most challenging NTM to treat due to high antibiotic resistance rates (4).

Mycobacterial cell walls contain multiple peptidoglycans including D-glucosamine and a mycolic acid layer (5) that initiate the interaction between bacteria and host upon inhalation (6). Macrophages are a critical immune cell in combatting mycobacterial infections with a significant proportion of their response dependent on type I IFN signaling (7, 8). However, the response of bronchial epithelial cells to mycobacterial infection is not well-described. Normal human bronchial epithelial (NHBE) cells express type I IFN that suppress viral replication, induce apoptosis and enhance Th1 immunity (9). NHBE cells exposed to MAB are known to upregulate expression of cytokine transcripts (10). We hypothesize that NHBE cells play a vital role in initiating the host response to MAB through production of pro-inflammatory type I IFN cytokines. To determine the effects of MAB exposure on NHBE production of type I IFN signaling, we investigated the gene expression profile, and protein expression changes in NHBE cell cultures. The immunologic effects of MAB-cell wall microparticles in lung bronchial and immune cells were tested in a mouse model.

METHODS

Lung-on-Membrane Model (LOMM)

Our dual chamber lung model contains normal human bronchial epithelial (NHBE) cells re-differentiated at the air-liquid interface (ALI) on one side and human endothelial cells (Human Lung Microvascular Endothelial Cells, Lonza, Walkersville, MD) on the other side of a transwell® polyester membrane cell culture inserts (12 mm diameter, 0.4 μm pore size; Corning Life Sciences, Amsterdam, The Netherlands). NHBE cells were collected from lungs rejected for transplant at University of Miami where epithelial cells were isolated from upper bronchi and cultured as previously reported (11–13). Both sides of the membrane were coated with collagen IV from human placenta (Millipore Sigma, St. Louis, MO, USA). 5×10^5 NHBE cells were cultured on top of the membrane in bronchial epithelial cell growth medium (BEGM) until cells were confluent. The cells were placed on air and fed with ALI Media from bottom chamber thereafter. When NHBE cells were fully differentiated and became ciliated, 2×10^5 endothelial cells were plated on the opposite side of the transwell membrane when membrane was upside down. The upside-down membrane was placed into humidified incubator at 37°C, 5% CO_2 for 8 h to let endothelial cells to adhere. The transwell was flipped to the original position and both cells lines were feed with a 50:50 mixture of endothelial and epithelia cell media in the bottom chamber and were incubated for 24 h. NHBE cells were washed and the media was changed every 2 days. Two days after adding the endothelial cells, the lung model was used for experiment and the media was changed every 2 days. This lung model has been previously published (14). For the current study, primary NHBE cells from five individuals were used to develop LOMM. **Table 1** shows demographic data and smoking history of lung donors.

MAB Microparticle Production

MAB cell wall microparticles were isolated from a strain of MAB with a rough colony isolated from the sputum of an 11-year old boy with cystic fibrosis (isolate # CCUG 47942, gift from Dr. Malin Ridell, University of Gothenburg, Sweden). MAB is grown in Middlebrook 7H9 broth with ADC enrichment medium (Millipore Sigma, St. Louis, MO, USA) at 37°C. When the culture OD600 reached 1.0–1.2, cells were collected by centrifugation at 4,000 g for 10 min, washed once in PBS, centrifuged, resuspended using a 15:1 (volume to volume) ratio of lysis buffer, sonicated and incubated on ice for 30 min. The lysis buffer contains 137 mM sodium chloride, 10 mM sodium phosphate, 2.7 mM potassium chloride, and detergents and protease inhibitors. Lysed cell samples were then centrifuged at 3,000 g for 5 min to remove intact MAB cells. The supernatant was transferred to a new tube and centrifuged for 20 min. Twenty milliliters of fresh lysis buffer was and the pellet was resuspended by brief sonication and centrifuged at 12,000 g for another 20 min. The pellet was resuspended in 20 ml volume of PBS and kept at 95°C for 15 min. After cooling to room temperature, the lysate was centrifuged at

TABLE 1 | Shows age, race, and smoking history of lung donors.

Subjects	Age	Race	Smoking
1	60–65	European American	NS
2	65–70	Latino	NS
3	75–80	European American	NS
4	20–25	Latino	NS
5	35–40	European American	NS

12,000 g for 20 min and the pellet was washed with PBS buffer 3 times at 12,000 g for 10 min. Finally, the pellet is suspended in Dulbecco's Modified Eagle Medium (DMEM) and stored at −80°C. The concentration of the microparticles is calculated by the following equation: Final concentration = Volume of original culture × OD600 × (2.2×10^8 bacteria/ml)/final volume. High quality images of MAB particles were obtained by scanning electron microscope (SEM) and proven to be non-infectious by absence of growth of MAB in culture.

Exposure of Epithelial Cells to MAB Microparticles

LOMM (bronchial epithelial cells side) were exposed to 100 μL of MAB microparticles diluted to a concentration equal to a multiplicity of infection (whole bacterium) of 10:1. Bronchial epithelial cells were harvested 72 h after exposure.

Mouse Model Exposure to MAB Microparticles

We used 6-week-old age C57Bl/6 male mice purchased from the Jackson Laboratory (Bar Harbor, ME) in experiments approved by the Animal Studies Subcommittee (IACUC) at the Miami VA Health system. Individual mice were challenged intratracheally every 3 days with MAB microparticles for 4 doses using a 20 G angiocatheter inserted into the trachea. After tube placement, microparticles were injected with the first dose injecting 50 μL ($\sim 5 \times 10^8$ CFU) and next three doses receiving 20 μL ($\sim 2 \times 10^8$ of CFU). The control group received equivalent volumes of PBS intratracheally. **Figure S1** shows the procedure and **Figure S2** shows proof of correct instillation within the lung with use of methylene blue instillation.

Mice were sacrificed on day 14 and the left lungs were harvested for pathology after perfusion to remove blood. Lungs were filled with 10% buffered formalin and fixed in formalin for at least 72 h before immunohistochemistry (IHC) staining. H&E staining was used to determine inflammatory pathology. Lungs were stained with antibodies against CD4 (rabbit, Abcam, catalogue# ab133616), CD8 (rabbit, Abcam, #ab12512), CD68 (rabbit, Abcam, #ab12512), PD-L1(rabbit, Proteintech, #17952-1-AP), and IFN-α (rabbit, Abcam, #ab193055) antibodies to identify infiltrating immune cells. Lung inflammation was scored using the three fields with the highest infiltrate's intensity at 100X power magnification. The area of inflammation was measured and averaged for the three examined high power fields. The right lungs were removed and frozen at −80°C for later protein extraction and western blot analysis. Protein extracted from lung tissue was performed as previously described (15).

RNAseq and Pathway Analysis

Total RNA from NHBE cells was extracted by using a Direct-zol™ RNA MicroPrep kit (R2060, Zymo Research Zymo Research, Irvine, CA), following the manufacturer's protocol. Briefly, cells were washed with PBS, lysed in TRIreagent and RNA was purified using a Direct-zol RNA column. DNase I treatment was performed on the column and RNA was eluted in DNase/Rnase Free water.

RNA from mouse lungs were extracted using RNA Miniprep Plus Kit (Zymo Research). Briefly, whole lung was homogenized in TRI reagent and total RNA extraction was performed following the instructions provided by the manufacturer with additional DNase treatment. Quantity and quality of the samples was determined by NanoDrop spectrophotometer and Agilent Bioanalyzer 2100, respectively. Samples with RNA integrity number > 8 were used for the analysis.

Preparation and sequencing of RNA libraries was performed at the John P. Hussman Institute for Human Genomics Center for Genome Technology. Briefly, total RNA quantity and quality were determined using the Agilent Bioanalyzer. At least 300 ng of total RNA was used as input for the KAPA RNA HyperPrep Kit with RiboErase (HMR) according to manufacturer's protocol to create ribosomal RNA-depleted sequencing libraries. Sequencing was performed on the Illumina NextSeq 500 generating ∼40 million single-end 75 base reads per sample. Sequencing data were processed with a bioinformatics pipeline including quality control, alignment to the hg19 human reference genome, and gene quantification. Count data was inputted into edgeR software (16) for differential expression analysis. Counts were normalized using the trimmed mean of M-values (TMM) method (17) to account for compositional difference between the libraries and paired differential expression analysis using a generalized linear model with sample as a blocking factor. Genes were considered statistically different with a false discovery rate *p*-value (FDR) ≤0.05.

Pathway enrichment analyses was performed using Enrichr online (18) and DAVID bioinformatics resource (19) to obtain the enriched biological processes (BPs) and pathways with genes with a linear fold change (FC) >2.5.

Western Blotting

NHBE cells and lung tissue cells were lysed in lysis buffer (Cell Signaling Technology, Beverly, MA) with protease inhibitor cocktail (Cell Signaling Technology, Beverly, MA and sonicated

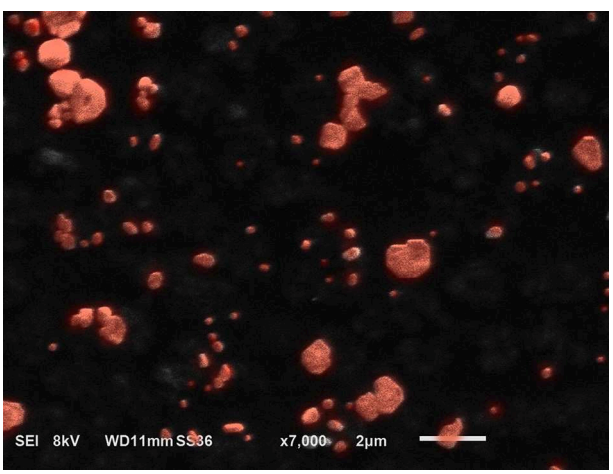

FIGURE 1 | SEM image from microparticles developed from MAB. The particles are submicron to 2 microns in size.

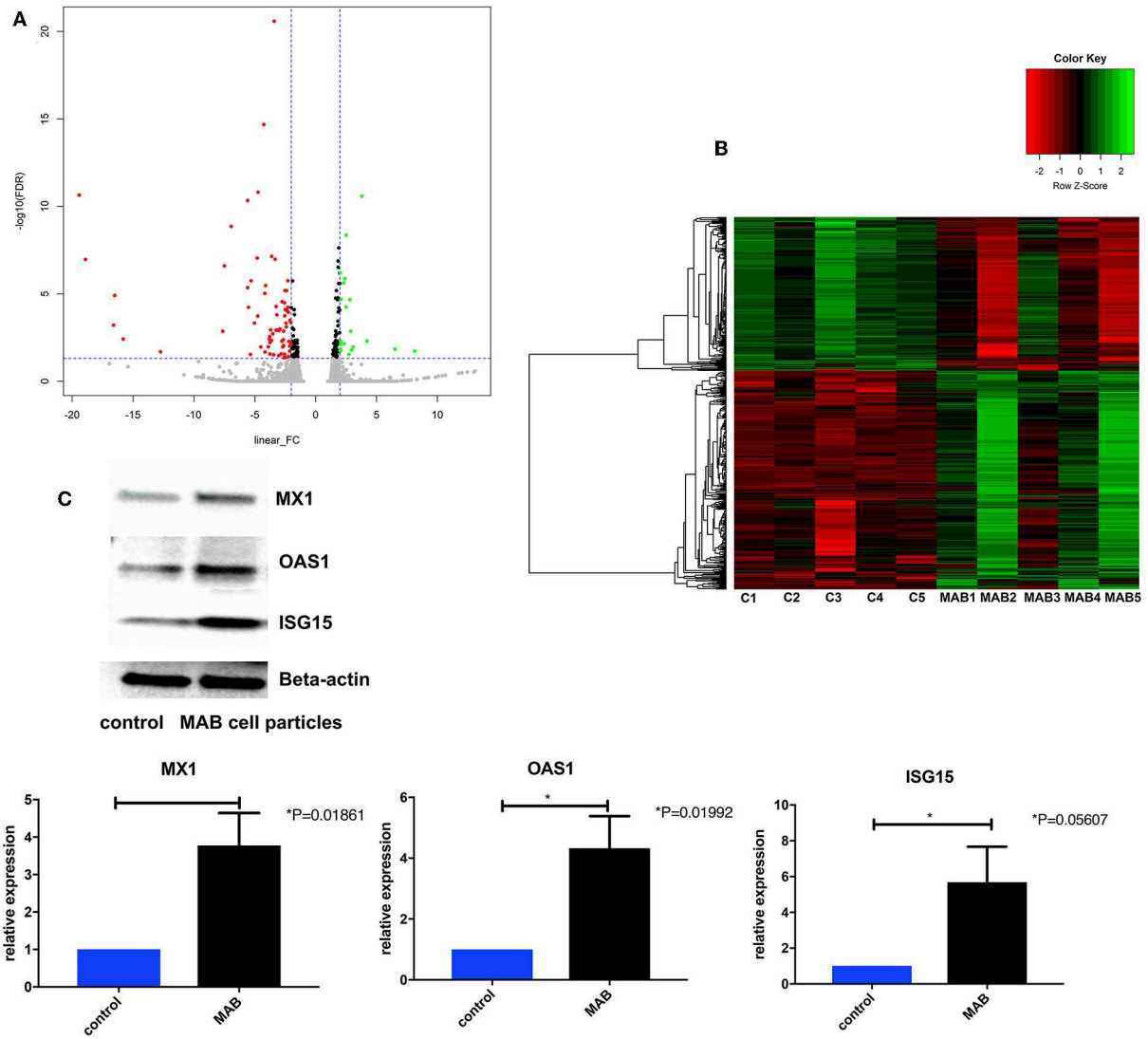

FIGURE 2 | (A) Volcano plots differentiating genes of NHBE cells with and without exposure to MAB microparticles. **(B)** Heat map of RNA-Seq transcriptome analysis of bronchial epithelial cells with and without exposure to MAB. Genes that were identified as significantly different between two groups with a >2.5-fold increase (green) or decrease (red) in expression level (sample size was 5 for each group). **(C,D)** Representative western blot analysis of MX1, OAS1, and ISG15 expression in bronchial epithelial cells exposed to MAB cell wall particles.

three times for 2 s each with at least 1-min rest on ice between each 2-s pulse. Samples were centrifuged at 10,000 × g for 5 min at 4°C and the supernatant was collected. Protein concentration was determined by BCA protein assay kit from Cell Signaling Technology.

Thirty micrograms of total protein were mixed in a reducing sample buffer, and then electrophoresed on a 10–15% Tris gel with Tris running buffer, blotted to PVDF membrane, and sequentially probed with primary antibodies against 2′-5′-Oligoadenylate Synthetase 1 (OAS1), Interferon-induced GTP-binding protein Mx1 (MX1), Interferon-stimulated gene 15 (ISG15) (Proteintech Group, Inc. Rosemont, IL). A horseradish peroxidase-conjugated goat anti-rabbit antibody was then added, and secondary antibodies were detected using enhanced chemiluminescence (ECL Plus, General Electric Healthcare, and Milwaukee, WI).

Statistical Analysis

A paired *t*-test is used to compare two population means using GraphPad Prism 7 software. Results with $p < 0.05$ were defined as statistically significant.

RESULTS

IFN I Signaling Pathway Genes Are Overexpressed in NHBE Cells Following MAB Exposure

The MAB cell wall particles (**Figure 1**) with a size that ranged from less than a sub-micron to 2 μm were exposed to NHBE cells

and RNA and protein expression was analyzed. RNAseq analysis identified 1759 differentially expressed genes between NHBE cells challenged with and without MAB microparticles (FDR < 0.5) and found 410 genes had at least a 2.5-fold change (FC). Volcano plots show marked differences in gene expression between NHBE cells with and without exposure to MAB microparticles (**Figure 2A**). **Figure 2B** shows the heatmap for unsupervised clustering of the RNAseq transcriptomes according to pearson correlation. Individual gene expression was normalized across samples to percentages ranging from marked downregulation (deep red) to marked upregulation (deep green).

The pathway enrichment analysis for gene differentially expressed 2.5 fold between NHBE cells with and without exposure to MAB microparticles. NHBE cells exposure to MAB microparticles significantly enriched the IFN I signaling pathway (GO:0060337) and cellular response to type I IFN (GO:0071357) (adjusted $p = 0.00001047$, and $p = 0.00001047$ respectively) in pathway analysis (**Table 2**). The top upregulated genes from the IFN I family (with FC>2.5 and FDR<0.5) were Radical S-Adenosyl Methionine Domain Containing (RSAD2) (FC 6.67), Myxovirus resistance 2 (MX2) (FC 5.66), Interferon induced protein 44 like (IFI44L) (FC 4.34), Interferon stimulated gene (ISG)15 (FC 4.34), Interferon Induced Protein With Tetratricopeptide Repeats 1 (IFIT1) (FC 4.20), Interferon Alpha Inducible Protein 6 (IFI) (FC 3.66), MX1 (FC 3.1), 2′-5′-Oligoadenylate Synthetase (OAS)1 (FC 2.79), and OAS3 (FC 2.69). **Figures 2C,D** show confirmation of increased protein expression of MX1, OSA1, and ISG15 using western blot in cultures exposed to MAB microparticles.

TABLE 2 | The pathway enrichment analysis for gene differentially expressed 2.5 fold between NHBE cells with and without exposure to MAB microparticles.

Index	Name	*P*-value	Adjusted *p*-value
1	Epidermal cell differentiation (GO:0009913)	8.304e-13	9.309e-10
2	Peptide cross-linking (GO:0018149)	5.967e-12	2.230e-9
3	Type I interferon signaling pathway (GO:0060337)	6.541e-8	0.00001047
4	Keratinocyte differentiation (GO:0030216)	3.633e-12	2.037e-9
5	Epidermis development (GO:0008544)	2.621e-9	5.876e-7
6	Regulation of nuclease activity (GO:0032069)	0.00005144	0.004436
7	Skin development (GO:0043588)	9.947e-10	2.788e-7
8	Negative regulation of viral genome replication (GO:0045071)	1.165e-7	0.00001632
9	Cellular response to type I interferon (GO:0071357)	6.541e-8	0.00001047
10	Negative regulation of viral life cycle (GO:1903901)	5.545e-7	0.00006907

Overexpression of Cytokine Genes in NHBE Cells Following MAB Exposure

Cytokine genes expression profile of NHBE cells following exposure to MAB cell wall microparticles also showed significant upregulation of IL36β (FC 41.3), IL36α (FC 18.4), IL36γ (FC 3.2), IL 23A (FC 3.2), IL1RL1 (FC 3.1), IL1RN (FC 3.1), and IL1RN (FC 2.6). Chemokine profiles showed significant expressions of CCL5 (FC 8.8), CXCL11 (FC 3.1), CCL22 (FC 2.8), and CXCL10 (FC 2.5). We also found Matrix Metallopeptidase (MMP) 9 (FC 4) was differentially expressed between two groups.

Granulomatous Reaction in the Lungs Following Exposure to MAB

Figure 3 shows mouse lungs developed non-caseating granuloma after MAB microparticles challenge. The inflammatory lesions were scored and showed significant increase in inflammation by H&E staining with marked increase in cells staining for macrophage marker, CD68, and PD-L1. IHC staining for IFN-α also showed significant increasing in bronchial cells in comparison with controls ($P < 0.00001$).

IFN I Signaling Pathway Genes Are Overexpressed in Mouse Lungs Following Exposure to MAB

RNAseq analysis identified 1759 differentially expressed genes between NHBE cells challenged with and without MAB microparticles with FDR < 0.5, 1155 genes had a 2.5-fold change (FC) or greater. Volcano plots reveal differential expression of genes between NHBE cells with and without exposure to MAB microparticles (**Figure 4A**). **Figure 4B** shows the heatmap for unsupervised clustering of the RNAseq transcriptomes according to pearson correlation. Gene expression for each gene was normalized across samples to percentages ranging from marked upregulation (deep red) to marked downregulation (deep blue). Many immunogens were significantly upregulated in challenged mice by MAB microparticles (**Figure 4C**). **Figure 4D** shows significant upregulation of *IL-17a* and *IL-17f* genes in mice lung after exposure to microparticles.

Pathway enrichment analysis for selected genes with a FDR<0.05 differentially expressed between mice lung cells treated with saline (control) vs. MAB microparticles. MAB microparticle challenged lungs significantly showed gene pathway enrichment in the type I interferon pathway (GO:0071357) and type I IFN signaling pathway (GO:0060337) (adjusted $p = 1.757e\text{-}19$, and $p = 8.783e\text{-}20$, respectively). The top upregulated genes from the IFN I family were *IRF1* (FC:2.54), *IFIT3* (FC:2.55), *ISG15* (FC:2.56), *MXD3* (FC:3), *IRF8* (FC:3.05), and *MX1* (FC:3.10).

IFN-α Proteins Overexpression in the Lungs Following Exposure to MAB

Expression of IFN-α in bronchial and granulomatous inflammatory cells were significantly increased following exposure to MAB cell wall microparticles ($P < 0.00001$). Western blot analysis of IFN-α protein expression found significant increase in microparticle challenged lung tissue ($P = 0.0002$) compared to negative controls (**Figure 5**).

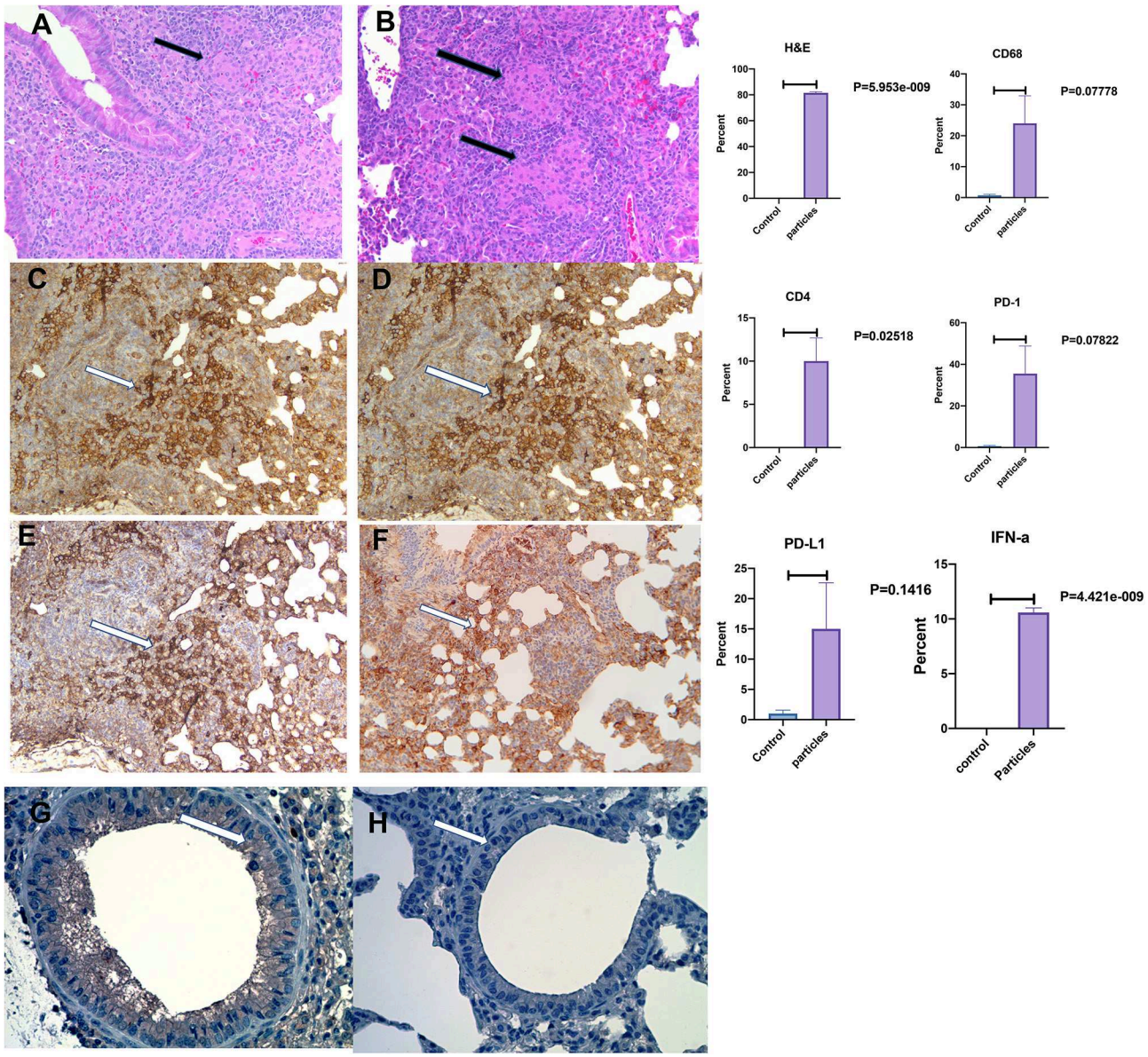

FIGURE 3 | Development of non-caseating granuloma in the mouse lung after MAB microparticle challenge. **(A,B)** Black arrows show granulomas in the lung (HandE staining), **(C)** CD68, **(D)** CD4, **(E)** PD-L1 staining, **(F)** PD-1 staining, **(G)** IFN-α in challenged lung, **(H)** IFN-α in control. White arrow shows a group of positive cells for each staining. Magnification are x20 for all representative images. *P*-value shows percentage differences of lung stained cells between challenged mice and controls (sample size 3 controls and 4 challenged mice).

DISCUSSION

This study found upregulation of 11 genes of the IFN 1 signaling pathway, upregulation of all 3 species of IL-36 (α, β, and γ) and upregulation of leukocyte chemokines in NHBE cells after exposure to microparticles of MAB. Mouse lungs challenged with MAB cell wall microparticles showed a granulomatous reaction with significant upregulation of IFN 1 genes. We also demonstrated that protein expression of MX1, OSA1, ISG15, and IFN-α were upregulated after MAB-host interaction in *in vitro* and *in vivo* models. *IL-17a* and *IL-17f* were upregulated in mice lung after exposure to microparticles. These data show MAB cell walls elicit a proinflammatory reaction from NHBE cells that likely initiates the host response to MAB infection. Finding similar gene expression changes in mice exposed to MAB particles confirms the bronchial epithelia response in an intact organism.

IFN I genes play an important role in controlling viral infections in bronchial epithelia and our study implicates their role in the host response to mycobacterial disease. IFIT1, ISG15, ISG20, and OAS 1,2, and 3 inhibit protein synthesis and cell proliferation in viral infected host cells. MX1 protein inhibits viral nucleoprotein synthesis and endocytosis (20). Given that mycobacteria are also intracellular pathogens,

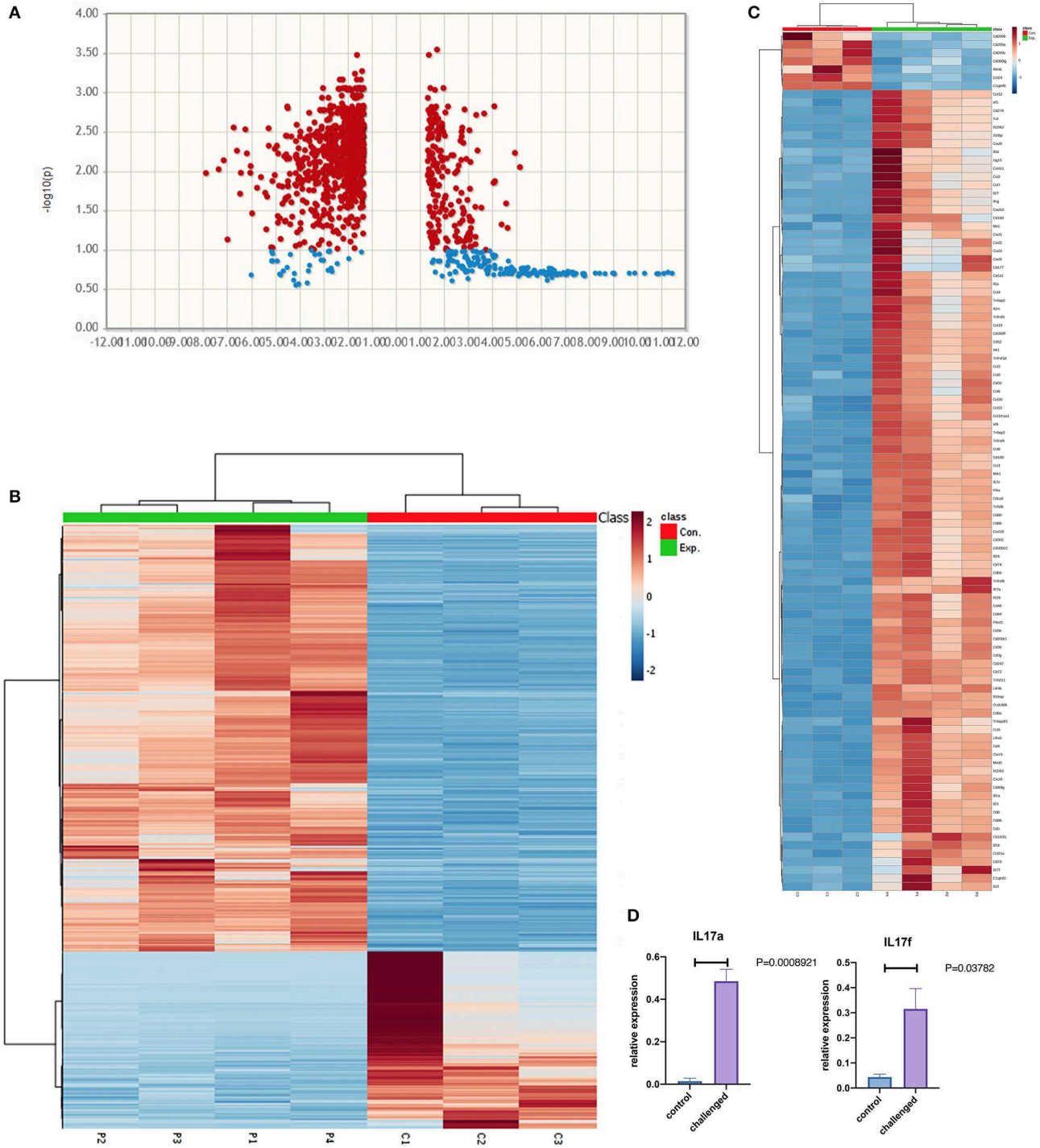

FIGURE 4 | (A) Volcano and **(B)** heatmap for unsupervised clustering of the RNAseq transcriptomes according to pearson correlation. **(C)** Heatmap of immunogens, and **(D)** Lung *IL17a* and *IL-17f* gene expression in the lung of MAB microparticle challenged mice (sample size=controls and 4 challenged mice).

IFN response genes may also form the first layer of innate defense in upregulating macrophage and T cell specific genes including IL-17.

NHBE cells treated with MAB microparticles significantly upregulated all three subtypes of IL36 (α, β, and γ). IL36 belongs to the IL1 superfamily and is expressed by bronchial epithelial cells. IL-36 activates the pro-inflammatory transcriptional factor nuclear factor kappa B (NFκB), induces T Helper cell type 1 (Th1) responses by enhancing cell proliferation and IL2 secretion (21, 22) and is implicated in the inflammatory response from skin epithelial cells in psoriasis (23). IL36 is known to control IFN I related gene expression in a time dependent manner (24) and

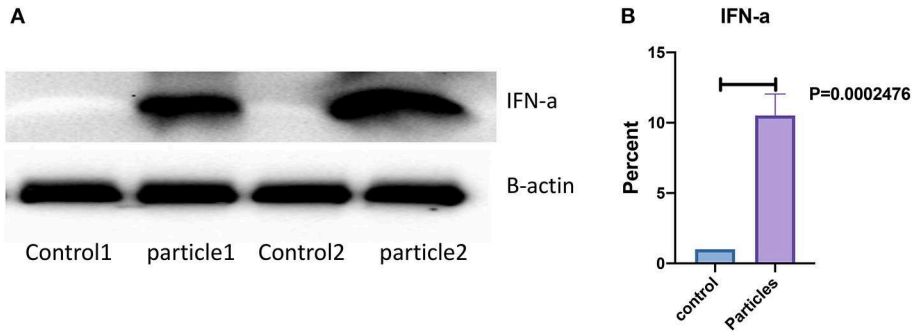

FIGURE 5 | IFN-α protein expression was significantly increased in lung after MAB microparticle challenge. **(A)** Shows a representative image of western blot bands, **(B)** shows significant difference in IFN-α protein expression in the lung of challenged mice (sample size 4 for each group).

may play a role in NHBE response to MAB cell wall components. Our *in vitro* study also showed a significant upregulation in IL-36 genes and members of the *IL1* superfamily genes suggesting a possible link between IL-36 expression from NHBE cells leading to type I IFN gene expression via autocrine loop.

MAB exposed NHBE also produced chemokines *CCL5* and *CCL22* that are strong leukocyte chemoattractants. The gene of both chemokines were upregulated in the mice lung after challenging with MAB cell wall microparticle. CCL5 is a potent monocyte and macrophage attractant recruiting important immune cells to combat mycobacterial infections. The immune response to MAB is T cell dependent and that macrophages develop pathologic features of mycobacterial disease known as granulomas. We found significant granulomatous reaction in the lung of challenged mice that could suggest functional activity of upregulated *CCL5* and *CCL22*. Interestingly, MMP9 is a critical protein required to recruit macrophages and develop well organized granulomas in *M.TB* infections (25). Thus, NHBE expression of MMP may also initiate the granuloma formation commonly seen in mycobacterial infections.

These data strongly support the role of NHBE cells in the host defense against MAB infections. They suggest that bronchial epithelial cells play a central role in initiating an innate immune response producing the initial signal alerting resident macrophages to the site of infection and producing IL36 and type I IFN genes to add to the host defense. Furthermore, this study underscores the importance of mycobacterial cell wall antigens in initiating the innate immune response. Understanding the direct impact of the IFN I genes and IL36 production by NHBE cells during MAB infection will provide data to develop strategies to treat or prevent NTM infections.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Miami Ethic Committee. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Miami VA Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

CZ and NF performed experiments and helped in manuscript preparation. HA conducted literature review, helped to develop first draft of manuscript. GH and MC assisted with analyzing results and manuscript preparation. AG performed bioinformatic analysis and assisted in manuscript preparation. PB reviewed lung pathology slides. MM conducted literature review, conducted exploratory analysis, performed data analysis and pathway analysis, and manuscript preparation.

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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.2019.02888/full#supplementary-material

Figure S1 | Development of granulomas in mouse lung. Anesthetized 8 week old male, C57Bl/6 mice **(A)** are injected with an 18-G IV catheter **(B)** to install *Mycobacterium abscessus* cell wall microparticles **(C)** into the lung **(D)**.

Figure S2 | Shows injection of methylene blue in trachea using the method described in **Figure S1**. Lung shows blue color that confirms the correct technique.

REFERENCES

1. Prevots DR, Marras TK. Epidemiology of human pulmonary infection with nontuberculous mycobacteria: a review. *Clin Chest Med.* (2015) 36:13–34. doi: 10.1016/j.ccm.2014.10.002
2. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. *Am J Respir Crit Care Med.* (2007) 175:367–416. doi: 10.1164/rccm.200604-571ST
3. Mirsaeidi M, Vu A, Leitman P, Sharifi A, Wisliceny S, Leitman A, et al. A patient-based analysis of the geographic distribution of mycobacterium avium complex, mycobacterium abscessus, and mycobacterium kansasii infections in the United States. *Chest.* (2017) 151:947–50. doi: 10.1016/j.chest.2017.02.013
4. Luthra S, Rominski A, Sander P. The role of antibiotic-target-modifying and antibiotic-modifying enzymes in mycobacterium abscessus drug resistance. *Front Microbiol.* (2018) 9:2179. doi: 10.3389/fmicb.2018.02179
5. Mirsaeidi M, Banoei MM, Winston BW, Schraufnagel DE. Metabolomics: applications and promise in mycobacterial Disease. *Ann Am Thorac Soc.* (2015) 12:1278–87. doi: 10.1513/AnnalsATS.201505-279PS
6. Reuschl AK, Edwards MR, Parker R, Connell DW, Hoang L, Halliday A, et al. Innate activation of human primary epithelial cells broadens the host response to Mycobacterium tuberculosis in the airways. *PLoS Pathog.* (2017) 13:e1006577. doi: 10.1371/journal.ppat.1006577
7. Monack DM, Mueller A, Falkow S. Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat Rev Microbiol.* (2004) 2:747–65. doi: 10.1038/nrmicro955
8. Novikov A, Cardone M, Thompson R, Shenderov K, Kirschman KD, Mayer-Barber KD, et al. Mycobacterium tuberculosis triggers host type I IFN signaling to regulate IL-1beta production in human macrophages. *J Immunol.* (2011) 187:2540–7. doi: 10.4049/jimmunol.1100926
9. Kalliolias GD, Ivashkiv LB. Overview of the biology of type I interferons. *Arthritis Res Ther.* (2010) 12 (Suppl. 1):S1. doi: 10.1186/ar2881
10. Matsuyama M, Martins AJ, Shallom S, Kamenyeva O, Kashyap A, Sampaio EP, et al. Transcriptional response of respiratory epithelium to nontuberculous mycobacteria. *Am J Respir Cell. Mol Biol.* (2018) 58:241–52. doi: 10.1165/rcmb.2017-0218OC
11. Randell SH, Walstad L, Schwab UE, Grubb BR, Yankaskas JR. Isolation and culture of airway epithelial cells from chronically infected human lungs. *In vitro Cell Dev Biol Anim.* (2001) 37:480–9. doi: 10.1290/1071-2690(2001)037<0480:iacoae>2.0.co;2
12. Valencia-Gattas M, Conner GE, Fregien NL. Gefitinib, an EGFR tyrosine kinase inhibitor, prevents smoke-mediated ciliated airway epithelial cell loss and promotes their recovery. *PLoS ONE.* (2016) 11:e0160216. doi: 10.1371/journal.pone.0160216
13. Kunzi L, Holt GE. Cigarette smoke activates the parthanatos pathway of cell death in human bronchial epithelial cells. *Cell Death Discov.* (2019) 5:127. doi: 10.1038/s41420-019-0205-3
14. Korukonda AZC, Rodriguez L, Guerrero A, Campos M, Holt G, Fregien Nl, Conner G, et al. Electronic cigarettes enhance replication of *Mycobacterium abscessus* in airway epithelial cells. *Am J Respir Cell Mol Biol.* (2019) 60:717–9. doi: 10.1165/rcmb.2018-0339LE
15. Decaris ML, Gatmaitan M, Florcruz S, Luo F, Li K, Holmes WE, et al. Proteomic analysis of altered extracellular matrix turnover in bleomycin-induced pulmonary fibrosis. *Mol Cell Proteomics.* (2014) 13:1741–52. doi: 10.1074/mcp.M113.037267
16. Robinson MD, Mccarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616
17. Anders S, Mccarthy DJ, Chen Y, Okoniewski M, Smyth GK, Huber W, et al. Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. *Nat Protoc.* (2013) 8:1765–86. doi: 10.1038/nprot.2013.099
18. Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics.* (2013) 14:128. doi: 10.1186/1471-2105-14-128
19. Huang Da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* (2009) 4:44–57. doi: 10.1038/nprot.2008.211
20. Kochs G, Janzen C, Hohenberg H, Haller O. Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes. *Proc Natl Acad Sci USA.* (2002) 99:3153–8. doi: 10.1073/pnas.052430399
21. Vigne S, Palmer G, Martin P, Lamacchia C, Strebel D, Rodriguez E, et al. IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. *Blood.* (2012) 120:3478–87. doi: 10.1182/blood-2012-06-439026
22. Gabay C, Towne JE. Regulation and function of interleukin-36 cytokines in homeostasis and pathological conditions. *J Leukoc Biol.* (2015) 97:645–52. doi: 10.1189/jlb.3RI1014-495R
23. Ding L, Wang X, Hong X, Lu L, Liu D. IL-36 cytokines in autoimmunity and inflammatory disease. *Oncotarget.* (2018) 9:2895–901. doi: 10.18632/oncotarget.22814
24. Swindell WR, Beamer MA, Sarkar MK, Loftus S, Fullmer J, Xing X, et al. RNA-Seq Analysis of IL-1B and IL-36 responses in epidermal keratinocytes identifies a shared MyD88-dependent gene signature. *Front Immunol.* (2018) 9:80. doi: 10.3389/fimmu.2018.00080
25. Taylor JL, Hattle JM, Dreitz SA, Troudt JM, Izzo LS, Basaraba RJ, et al. Role for matrix metalloproteinase 9 in granuloma formation during pulmonary *Mycobacterium tuberculosis* infection. *Infect Immun.* (2006) 74:6135–44. doi: 10.1128/IAI.02048-05

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Asthmatic Bronchial Smooth Muscle Increases CCL5-Dependent Monocyte Migration in Response to Rhinovirus-Infected Epithelium

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Asthma exacerbations, a major concern in therapeutic strategies, are most commonly triggered by viral respiratory infections, particularly with human rhinovirus (HRV). Infection of bronchial epithelial (BE) cells by HRV triggers inflammation, notably monocyte recruitment. The increase of bronchial smooth muscle (BSM) mass in asthma, a hallmark of bronchial remodeling, is associated with the annual rate of exacerbations. The aim of the present study was to assess whether or not BSM could increase monocyte migration induced by HRV-infected BE. We used an advanced *in vitro* model of co-culture of human BE cells in air-liquid interface with human BSM cells from control and asthmatic patients. Inflammation triggered by HRV infection (HRV-16, MOI 0.1, 1 h) was assessed at 24 h with transcriptomic analysis and multiplex ELISA. *In vitro* $CD14^+$ monocyte migration was evaluated with modified Boyden chamber. Results showed that HRV-induced monocyte migration was substantially increased in the co-culture model with asthmatic BSM, compared with control BSM. Furthermore, the well-known monocyte migration chemokine, CCL2, was not involved in this increased migration. However, we demonstrated that CCL5 was further increased in the asthmatic BSM co-culture and that anti-CCL5 blocking antibody significantly decreased monocyte migration induced by HRV-infected BE. Taken together, our findings highlight a new role of BSM cells in HRV-induced inflammation and provide new insights in mucosal immunology which may open new opportunities for prevention and/or treatment of asthma exacerbation.

Keywords: mucosal immunology, bronchial remodeling, asthma, monocyte, rhinovirus, epithelium, smooth muscle, co-culture

INTRODUCTION

Asthma is a chronic respiratory disease characterized by chronic inflammation, bronchial hyperresponsiveness and bronchial remodeling. Asthma exacerbations still represent a major concern in therapeutic strategies since they are characterized by an increase in symptoms and a decrease in lung function that is sufficient to require a change in treatment (1). Asthma

exacerbations have been often associated with viral respiratory infections, with an estimated rate of 65–85% of all viral exacerbations in children and 50% in adults being caused by human rhinovirus (HRV) (2). HRV infection was largely restricted to the bronchial epithelium (BE) (3). HRV infection of BE triggered the release of a various range of mediators, such as antiviral interferons and pro-inflammatory cytokines (4). HRV-infected BE also produced chemokines, such as IL-25, IL-33, and thymic stromal lymphopoietin, therefore inducing immune cell migration toward lung tissue and subsequent inflammation (5).

HRV also presented the ability to enter and replicate in monocytes when they are co-cultured with BE cells (6). Monocytes are myeloid cells that give rise to macrophages, dendritic cells (7), and fibrocytes (8). While macrophage and dendritic cell involvement in asthma has been well-documented, the role of monocytes themselves has not been studied extensively, whereas several publications showed that they are recruited after viral- or bacterial-infection (9). Indeed, several chemokines have been shown to attract monocytes from blood circulation. CCL2 was considered as the main monocyte chemoattractant and has been shown to be associated with monocyte migration in many diseases. During viral- or bacterial-infection, increased amount of both CCL2 and CCL5 has been observed, in serum from asthmatic patients and these data correlate with those found in mouse models of asthma, in whom expression level of CCL2 in lung tissue and bronchoalveolar lavage fluids were also enhanced compared to control mice (10). In addition to CCL2, many other chemokines presented chemotactic properties on monocytes such as CCL3, CCL5, or CCL7 (9, 11). Importantly, monocyte may have an important role in HRV-induced exacerbation since sCD86, a pro-inflammatory mediator secreted by the intermediate monocyte subset, was highly expressed in serum of asthmatic patients under exacerbation (12).

An important feature of bronchial remodeling was the increase of bronchial smooth muscle (BSM) mass, which has been correlated with decreased lung function (13, 14). Interestingly, this enhanced BSM mass was also associated with an increased annual rate of exacerbations (15). Moreover, the reduction of BSM area by bronchial thermoplasty drastically decreased the rate of exacerbations (16, 17), but its mechanisms remain unknown. Surprisingly, the role of BSM on monocyte migration during exacerbation has never been explored.

Therefore, the goal of the present study was to assess whether or not BSM could increase monocyte migration induced by HRV-infected BE. The present study showed that HRV-induced monocyte migration was substantially increased in the co-culture of BE with asthmatic BSM, compared to that with control BSM. Furthermore, we determined that the major monocyte chemoattractant CCL2 was not involved but instead this increased migration was CCL5-dependent. Taken together, our findings highlight a new role of BSM cells in HRV-induced inflammation and provide new insight in mucosal immunology which may open new opportunities for the prevention and/or treatment of asthma exacerbation.

MATERIALS AND METHODS

Study Populations

Patients with asthma were recruited from the "COBRA" cohort ("Cohorte Obstruction Bronchique et Asthme"; i.e., Bronchial Obstruction and Asthma Cohort) in the Clinical Investigation Center of Bordeaux (CIC, Hôpital Haut-Lévêque, Pessac, France) according to GINA (1). Non-asthmatic control subjects were recruited after surgical resection if they had normal lung function. Bronchial specimens from all subjects were obtained by either fiberoptic bronchoscopy or lobectomy, as previously described (18). All subjects gave their written informed consent to participate to the study after the nature of the procedure has been fully explained. The COBRA study received approval from the National Ethics Committee. Patients' characteristics are presented in **Table 1**.

Cell Culture and Co-culture Model

Primary BSM cell culture was established, as described previously (19, 20). BSM cells (see subjects' characteristics **Table S1**) were only used from passages 2 to 5 to avoid BSM cell dedifferentiation into myofibroblasts or fibroblasts. Cell culture purity was assessed by immunocytochemistry using BSM-specific markers, with a requirement of αSMA- and calponin-positive cells ≥90%.

BE cells were obtained from surgical specimen of control subjects (see subjects' characteristics **Table S2**), as described previously (21). Briefly, BE cells were cultured in Pneumacult-Ex medium (Stemcell Technologies, Vancouver, Canada). After reaching 70% confluence, BE cells were grown on 0.4 μm pore-diameter insert with Pneumacult-ALI (complemented with hydrocortisone and heparin according to the manufacturer, Stemcell) and cultured in air-liquid interface (ALI) for 21 days, in order to obtain a fully differentiated BE. ALI-BE was either infected or not infected with HRV-16 (Gift from Dr. Brian Oliver, Woolcock Institute of Medical Research, Sydney, Australia) at a MOI of 0.1 for 1 h (100 μl of DMEM containing HRV-16, was dropped on the top of the insert and removed after 1 h). For HRV-16 infection, hydrocortisone was removed from Pneumacult-ALI medium. Supernatant were collected 24 h after the infection. The absence of HRV

TABLE 1 | Patients' characteristics.

Characteristics	Controls	Asthmatics	*p*-value
No. of patients	32	25	
Age, yr	64.48 ± 9.44	54.31 ± 18.19	0.08
Body mass index, kg/m^2	25.34 ± 4.98	26.37 ± 5.98	0.72
Treatments			
LABA, No. of patients	0	24	
ICS, No. of patients	0	24	
OCS, No. of patients	0	5	
FEV_1			
Liters	2.21 ± 0.49	2.11 ± 0.79	0.37
Percentage of predicted value	82.4 ± 24.23	78.93 ± 22.18	0.79
Percentage of FVC	72.45 ± 10.14	82.24 ± 15.77	0.04

particles in the co-culture supernatant was confirmed by digital PCR (data not shown).

The co-culture was established by adding the ALI-BE insert to the BSM well for 1 week. Medium used for co-culture was a mix of that used for BSM and BE (50% of DMEM supplemented with 10% FBS and 50% of Pneumacult-ALI without hydrocortisone). The BE co-cultured with BSM were infected, as mentioned above for BE alone. The absence of HRV particles within BSM cells was confirmed by digital PCR (data not shown). Supernatant were collected from three independent experiments and stored at −80°C for further analysis. In total, 18 controls and 12 asthmatic BSM cells were co-cultured with at least three different BE cells.

Rhinovirus Production and Infection

HRV-16 was used in this study since it represents the "major group" (which utilize the cell surface receptor intercellular adhesion molecule 1) and is used in a large number of experimental studies on human primary cells. The HRV-16 was propagated in HeLa cells with 2% serum, as previously described (22). HeLa cells were maintained in DMEM supplemented with 10% FBS. HRV-16 titration assay was established in Hela cells and digital PCR (Functional Genomic Centre of Bordeaux). The absence of any mycoplasma, bacterial and fungal contamination was confirmed by the use of PCR and bacterial/fungal cultures (Eurofins Genomics, Germany and Parasitology-Mycology department of Bordeaux University Health Centre, respectively).

Monocyte Isolation

Primary monocytes were obtained from blood of asthmatic patients (see patients' characteristics **Table S3**). Briefly, peripheral blood mononuclear cells were separated from buffy coat using a Ficoll-gradient centrifugation method followed by a positive depletion with CD14 microbeads. Cells were then suspended at the adequate concentration in RPMI + Glutamax (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 8% FBS (Eurobio, Evry, France).

Migration Assay

Primary monocyte migration was performed using transwell chambers (Thermo Fisher Scientific) with monocytes added to the upper chamber and culture supernatant to the lower chamber of 3.0 μm pore-diameter inserts, pre-coated with PLL(20)-g[3.5]-PEG(2) (SuSoS, Dübendorf, Switzerland) to prevent monocyte adhesion. The number of migrated cells was assessed after 4 h of migration by cell counting. The effect of chemokines on monocyte migration was assessed using neutralizing antibodies anti-CCL2 at 5 μg/ml (Biolegend, San Diego, CA) or anti-CCL5 at 1 μg/ml (R&D) antibodies, Armenian hamster IgG isotype control at 5 μg/ml (Biolegend) or mouse IgG1 isotype control at 1 μg/ml (Abcam, Cambridge, UK). For each experiment, results were first normalized with control medium (DMEM/ALI medium). Then, all the data were further normalized by the mean of the control condition (co-culture without HRV) and multiplied by 100 to display the results as percentage of migration.

Monocyte migration was also assessed using micro-optical coherence tomography (μ-OCT) (23). Briefly, it is a bio-imaging technic producing trans-sectional images based on the sample reflectance, with an axial and lateral resolution of 1 micron. μ-OCT measured variation of electric field amplitude of light scattered by the structure of the tissue. Primary BE cells seeded on 3 μm inverted transwell were placed in a customized holder specifically designed for transwells. The reconstituted BE was stimulated with TNF-α (100 ng/ml) 24 h before the migration assay. Cells were imaged using the μ-OCT imaging device in its inverted configuration and rotated around 10° to minimize direct reflection of the beam on flat surfaces. A 75 W lamp was circled with aluminum foil to transfer the heat and obtained a temperature around 37°C near the sample. 2D images were acquired by scanning the beam in a linear path (B-scan) over 1 mm length and 3D images were obtained by multiplying B-scan to scan 1 × 1 mm area. Time-lapsed acquisition was made by taking 512 two-dimensional sections every 10 min for 4 h. Typical Fourier-domain OCT reconstruction was applied to convert raw interferometric data to depth-resolved images that could be processed an analyzed in ImageJ to obtain 3D images.

Multiplex Gene Expression Analysis

BE cells were lysed by adding 200 μl of lysis buffer RLT according to manufacturer's instruction (AllPrep® DNA/RNA/Protein Mini Kit, Qiagen, Hilden, Germany), 24 h after HRV infection. mRNA extracts were stored at −80°C and send to PARS-I (Plateforme Analytique de Recherche en Santé-Immunologie, Dr. Isabelle Pellegrin, Bordeaux, France) to be assessed with nCounter® FLEX using the inflammation panel (nanoString, Seattle, WA). Analysis was performed with nSolver™ Analysis software.

Protein Expression

Protein expression of CCL2 and IL-6 was assessed by ELISA in supernatants (100 μl undiluted for controls and diluted 1/10 for HRV-16) following the manufacturer's instruction (Qiagen, Hilden, Germany). A custom Bio-Plex Assay (BioRad, Hercules, CA) was performed to assess chemokine expression in co-culture supernatants (50 μl undiluted) according to the manufacturer's instruction. Special plate reader (Bio-Plex MAGPIX™, BioRad) and software (Bio-Plex manager) were used. The assay running was based on the same principle as a classic ELISA, except that all the washing steps were made with a wash station (Bio-Rad), equipped with a magnetic field that kept the microbeads to the bottom of the well while performing washing steps.

Statistical Analysis

All statistical tests were performed on Graphpad Prism 6 software (Graphpad Software, San Diego, CA). Results were displayed as mean ± SEM values of repeated independent experiments. Statistical tests used were ordinary one-way ANOVA with Newman-Keuls or Bonferonni's multiple comparisons test and Wilcoxon tests. Results were considered as statistically significant when $p < 0.05$.

RESULTS

Enhanced Monocyte Migration Mediated by Rhinovirus-Infected BE

Since BE is the first line of defense against respiratory viruses, we first sought to assess monocyte migration in response to supernatant of HRV-infected BE cells alone, cultured in ALI. As anticipated, a significant increase of monocyte migration (62%) was observed with HRV-infected BE supernatant (**Figure 1A**). Since HRV infection of BE cells induce CCL2 production (24), the major monocyte chemoattractant protein, we assessed CCL2 mRNA and protein levels in BE cell lysates. Although there was no difference in mRNA level at 24 h (data not shown) a significant increase of CCL2 protein was observed in HRV-infected BE (**Figure 1B**). We then used an anti-CCL2 neutralizing antibody to confirm that HRV-mediated monocyte migration was dependent on CCL2. As expected, this antibody virtually abrogated monocyte migration (**Figure 1A**).

We further assessed whether monocyte may cross the ALI-BE barrier. To that extent, we designed an inverted model with BE cells seeded on the inverted side of the insert of the transwell and we used an advanced system of OCT-imaging (μOCT), to perform live-imaging of inflammatory cell migration for 4 h (**Figure S1**). While neutrophil trans-epithelial migration could be demonstrated (**Figure S1B**), monocytes migration was not observed within the time of the experiment (**Figure S1C**). Thus, all subsequent migration assays were then performed using the modified Boyden Chamber.

Asthmatic Bronchial Smooth Muscle Co-culture Increased Rhinovirus-Mediated Monocyte Migration

Surprisingly, HRV infection of BE co-cultured with control BSM did not increase monocyte migration (**Figure 1C**). By contrast, a significant 2-fold increased migration was observed in HRV-infected BE when co-cultured with BSM from asthmatic

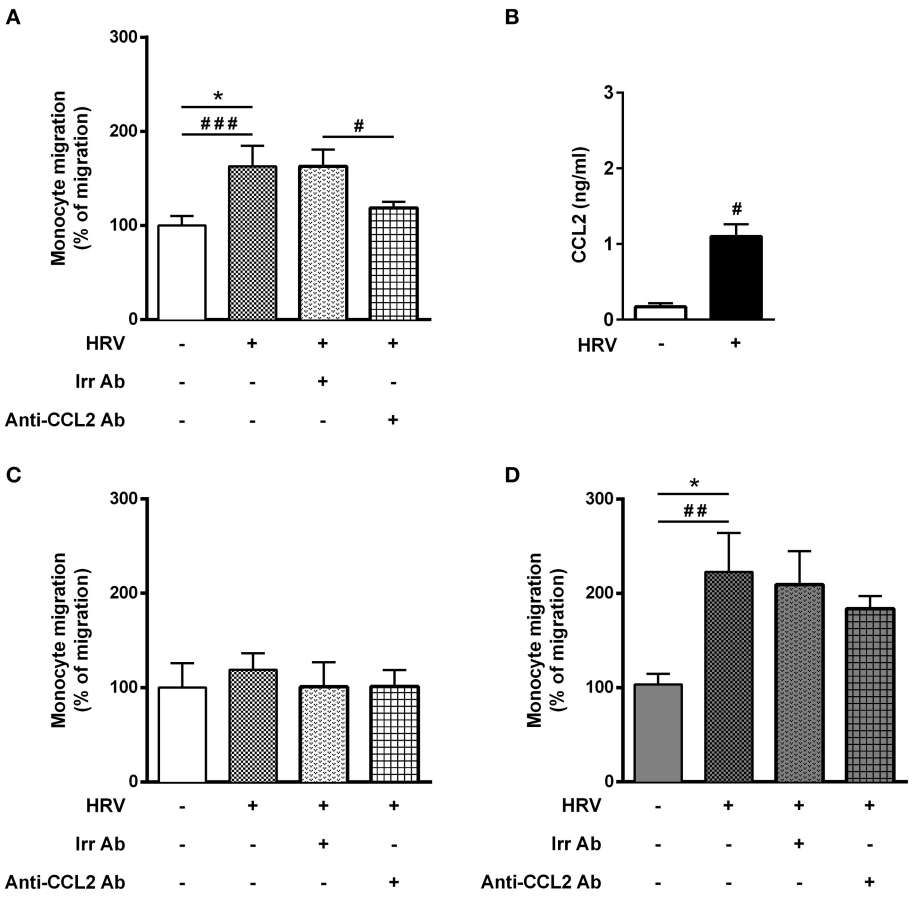

FIGURE 1 | Asthmatic bronchial smooth muscle cell co-culture increases rhinovirus-mediated monocyte migration. **(A)** Monocyte migration was assessed in response to supernatants of reconstituted bronchial epithelial cells in air liquid interface infected or not with human rhinovirus (HRV-16; at MOI 0.1 for 1 h). The effect of CCL2 on rhinovirus-induced monocyte migration was evaluated by addition of blocking antibody ($n = 7$–11 per group). **(B)** CCL2 proteins were assessed from epithelial cell supernatant ($n = 5$ per group). **(C)** Monocyte migration was assessed in response to supernatants of reconstituted bronchial epithelial cells in air liquid interface co-cultured for 1 week with bronchial smooth muscle cells from control ($n = 5$–9 per group) or **(D)** asthmatic patients ($n = 5$–8 per group). Data are presented as mean ± SEM values of three independent experiments (Wilcoxon test, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ and ordinary one way anova, Bonferroni's multiple comparisons test, $^{*}P < 0.05$ compare the mean of HRV+ alone with the mean of every other columns).

patients (**Figure 1D**). Moreover, this migration was not related to CCL2, since the use of an anti-CCL2 neutralizing antibody did not alter monocyte migration, suggesting the involvement of other chemokines (**Figure 1D**). Importantly, no HRV-16 particle has been detected in both co-culture supernatant and BSM cells (data not shown) suggesting minor modification of epithelium integrity. In order to identify potential monocyte chemoattractants in this co-culture model, we performed a transcriptomic analysis of BE cell lysates from the different co-culture conditions. Several chemokines emerged as potential actors for monocyte migration since they presented mRNA levels significantly increased in HRV-infected BE co-cultured with asthmatic BSM compared to that co-cultured with control BSM: CCL2, CCL5, CCL17, CXCL1, CXCL2, CXCL5, CXCL6, and CXCL9 (**Figure 2**). Whereas both CCL4 and CCL23 mRNA levels were also increased in HRV-infected BE co-cultured with asthmatic BSM compared to that co-cultured with control BSM, the absolute counts were too low to be considered as pertinent (**Figure S2**). Additional chemokine transcriptomic levels were also measured but did not present any significant difference between HRV-infected BE co-cultured with control vs. asthmatic BSM (**Figure S2**). Transcriptomic analyses also demonstrated an increased expression of genes involved in the pro-inflammatory response, such as IL-1A, IL-6, or TNF-α (**Figure S3A**), as well as HRV-induced genes, like IFNA1, IFNB1, IFIT1, IFIT3, which all belonged to the interferon pathway (**Figure S3B**).

The protein level of the chemokines of interest was then measured using multiplex ELISA assay (**Figures 3A–H**). Since anti-CCL2 blocking antibody was unable to alter monocyte migration induced by HRV-infected BE co-cultured with asthmatic BSM (**Figure 1D**), it was not surprising to identify no significant difference in CCL2 protein expression (**Figure 3A**). From the eight targets pre-selected from the transcriptomic analysis, only CCL5 presented a differential protein expression between HRV-infected BE co-cultured with asthmatic BSM compared to that co-cultured with control BSM (**Figure 3B**).

Enhanced Monocyte Migration Was CCL5-Dependent

To finally assess the role of CCL5 on monocyte migration, we performed additional migration experiments. First, adding recombinant CCL5 to non-infected-BE cell medium increased monocyte migration, which was abolished using an anti-CCL5 neutralizing antibody (**Figure 4A**). Second, anti-CCL5 neutralizing antibody did not decrease monocyte migration in HRV-infected-BE co-cultured with control BSM cells (**Figure 4B**), whereas it abolished the increased monocyte migration in HRV-infected-BE co-cultured with asthmatic BSM cells (**Figure 4C**).

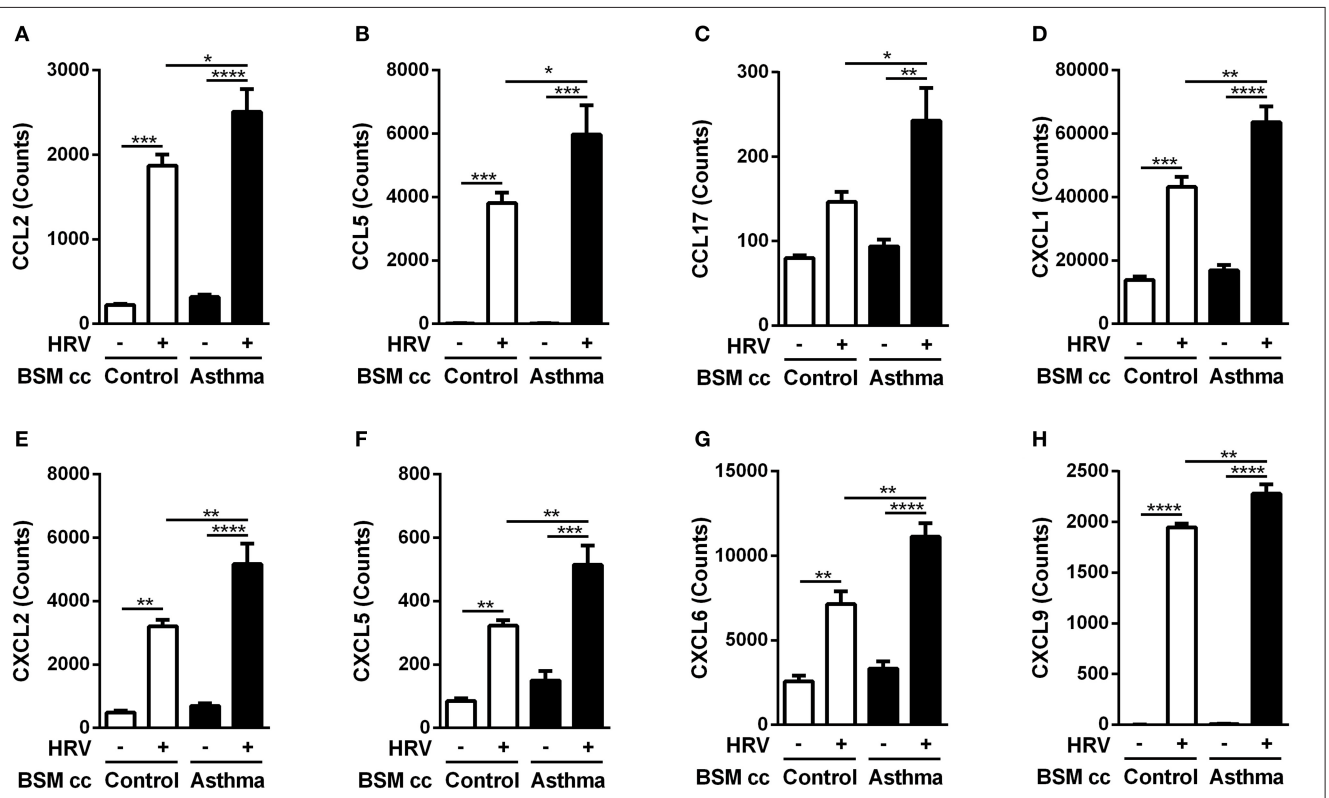

FIGURE 2 | Asthmatic bronchial smooth muscle cell co-culture increases chemokines gene expression in bronchial epithelial cells after rhinovirus infection. **(A)** CCL2, **(B)** CCL5, **(C)** CCL17, **(D)** CXCL1, **(E)** CXCL2, **(F)** CXCL5, **(G)** CXCL6, **(H)** CXCL9 mRNA were quantified in epithelial cells by multiplex gene expression analysis. Data are presented as mean ± SEM values ($n = 3$ per group, one-way ANOVA, Newman-Keuls multiple comparisons test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

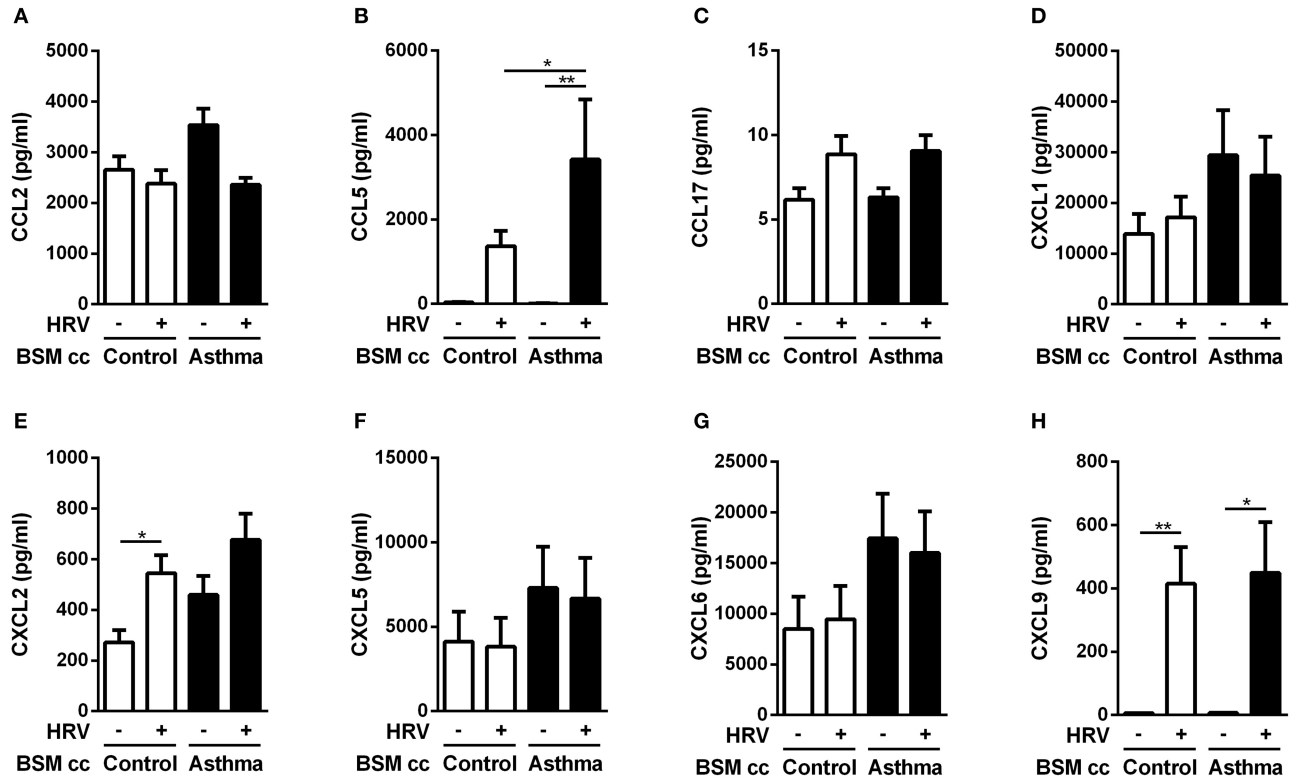

FIGURE 3 | Asthmatic bronchial smooth muscle cell co-culture increases CCL5 expression in epithelial cells after rhinovirus infection. **(A)** CCL2, **(B)** CCL5, **(C)** CCL17, **(D)** CXCL1, **(E)** CXCL2, **(F)** CXCL5, **(G)** CXCL6, **(H)** CXCL9 proteins were quantified in co-culture supernatants by multiplex ELISA. Data are presented as mean ± SEM values ($n = 8$–11 per group, one-way ANOVA, Newman-Keuls multiple comparisons test, $^{*}P < 0.05$; $^{**}P < 0.01$).

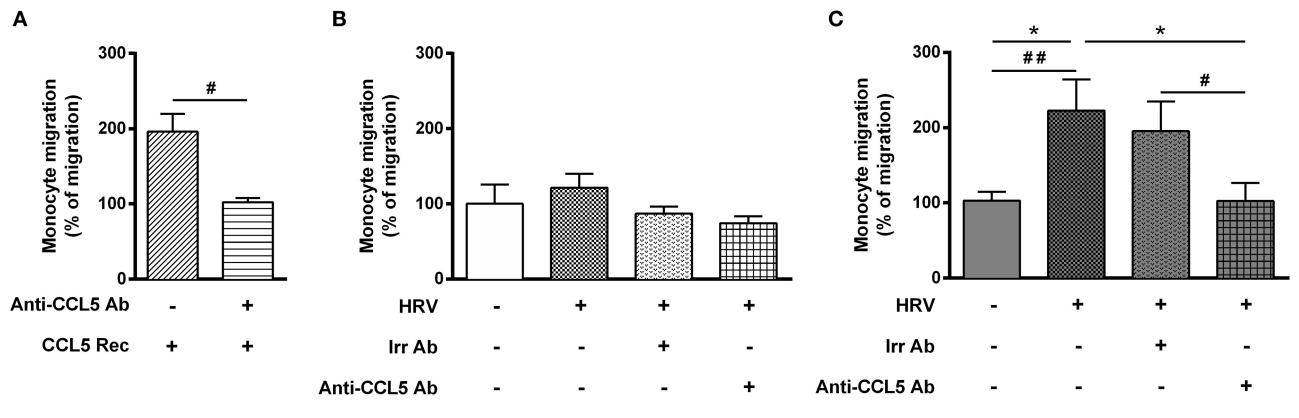

FIGURE 4 | Increase rhinovirus-mediated monocyte migration induced by asthmatic bronchial smooth muscle is CCL5 dependent. Monocyte migration was assessed in response to recombinant CCL5 (CCL5 Rec) **(A)** as a positive control, or to supernatants of reconstituted bronchial epithelial cells in air liquid interface co-cultured with bronchial smooth muscle cells from **(B)** control ($n = 6$–9 per group) or **(C)** asthmatic patients ($n = 6$–8 per group). The effect of CCL5 on rhinovirus-induced monocyte migration was evaluated by addition of blocking antibody (Anti-CCL5 Ab). Data are presented as mean ± SEM values of three independent experiments (Wilcoxon test, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ and ordinary one way anova, Bonferroni's multiple comparisons test, $^{*}P < 0.05$ compare the mean of HRV+ alone with the mean of every other columns).

DISCUSSION

Taken together, these results showed that the BSM from asthmatic patients increased the effects of HRV infection of the BE in terms of both pro-inflammatory response and monocyte migration in a CCL5-dependent manner.

In the present study, we focused our attention on the effects of HRV infected-BE on monocytes migration. Indeed, monocytes

are recruited in the bronchial wall of asthmatic patients after infection (25, 26). Moreover, Shi et al. found that sCD86, a pro-inflammatory mediator secreted by the intermediate monocyte subset, was highly expressed in serum of asthmatic patients under exacerbation (12). In addition, monocyte-derived dendritic cells are sufficient and necessary to induce airway inflammation in mouse models of asthma (27, 28). In a mouse model of allergic airway inflammation using cockroach antigen, the increased monocytes/macrophage infiltration was related to subepithelial accumulation of versican and hyaluronan, two important proteins from the extracellular matrix involved in airway remodeling (29).

These results demonstrated the interest of using an advanced model of co-culture, since the crosstalk between BSM and BE cells altered the "classical" pattern of monocyte migration. Indeed, BSM can produce chemotactic proteins and therefore attract many inflammatory cells. In the context of asthma, BSM may attract mast cells (30), as well as T cells (14). However, to the best of our knowledge, there is no data regarding the attraction of monocytes by BSM cells. Using the co-culture model with asthmatic BSM cells, blocking CCL2 did not inhibit monocyte migration, suggesting a role of other chemokines in monocyte migration. Instead, transcriptomic and protein analyses showed an increased expression of CCL5, and functional migration assay demonstrated that the migration was CCL5-dependent. CCL5 can bind the chemokine receptors CCR1, CCR3, and CCR5 and monocytes express CCR1 and CCR5 on their surface (31). Whether CCL5/CCR1 axis was more important than CCL5/CCR5 axis in monocytes recruitment was not investigated in the present study. CCL5 is a potent chemoattractant for monocytes, T helper cells and eosinophils (32–34). Using a similar co-culture model, Malavia et al. showed that secreted mediators, such as IL-8, IL-6, or CCL2, were enhanced, especially when BE was mechanically injured with a pipette tip to mimic epithelial disruption (35). Moreover, HRV-induced CCL5 production by BE can also induce BSM cell chemotaxis (36). However, all these experiments were performed only with non-asthmatic BSM cells. Therefore, our own co-culture model with asthmatic BSM on BE chemokine expression and monocyte migration provided new insights regarding the role of asthmatic BSM in HRV-induced inflammation. Surprisingly, the co-culture system with control BSM abrogated the monocyte migration induced by HRV infection. These results suggest that a crosstalk between BE and BSM in healthy subjects could moderate inflammatory signaling in response to HRV infection whereas asthmatic BSM altered this crosstalk toward a pro-inflammatory response. Indeed, HRV-infection of BE co-cultured with asthmatic BSM increased the production of pro-inflammatory mediators (IL-1A, IL-6, TNF-alpha) at the transcriptional level compared to that co-cultured with control BSM cells. Moreover, HRV-infected BE-co-cultured with asthmatic BSM also increased IL-8 and IL-15 levels. By contrast, such an experimental condition did not alter type 2 inflammation (i.e., IL-4, IL-5).

In this study, we showed that CCL2 remained the main monocyte chemoattractant upon HRV infection when BE was cultured alone in ALI. CCL2 was considered as the main monocyte chemoattractant and was produced by many cell types, including BSM (37). Constitutive or stimulated CCL2 secretion by human asthmatic BSM has already been shown *in vitro* (38). BE cells were also able to produce CCL2 in response to other respiratory virus infection (39). However, *in vitro,* no effect of HRV-16 infection of BE on CCL2 production could be found in the literature. For instance, Keininger et al. did not observe any difference in CCL2 expression after HRV-16 infection, unlike infection with HRV-1B (40). These contradictory findings might be related to the infection protocol and the multiplicity of infection used. In addition, some of these studies used a monolayer of BE cells grown in liquid phase and not a pseudostratified BE grown in ALI (41). Similarly, it has been previously shown that HRV infection of such a monolayer of primary BE cells induced CCL5 expression (42), whereas in our hands, BE alone cultured in ALI produced CCL2. It is also important to mention that the HRV-related increased migration was not related to lower BE junction, since no HRV particle was observed in both co-culture supernatants and BSM cells. Moreover, the inflammatory response of BE was only due to HRV-16 infection since UV-inactivated HRV-16 did not trigger IL-6 production (**Figure S4**).

Several limitations can be discussed in this study. Firstly, BE cells were only obtained from control patients. Since the goal of the present study was to assess the role of BSM on monocyte migration induced by HRV-infected BE, we thus compared asthmatic and control BSM in a paired fashion. Moreover, when we previously evaluated the effects of BE on BSM cells, results were similar when asthmatic and control BE cells were used (21). Furthermore, the barrier function of the BE is compromised in asthma, which would improve the passage of viruses across the BE (43). Secondly, this study used primary cells, which are complex to obtain from patients and explain why we limited the study to a low number of patients. One can argue that we are not powered enough for non-significant results. Thirdly, we only used monocyte from asthmatic patients. Indeed, the current ethic protocol did not plan to use blood from control subjects. It has been shown that intermediate monocytes $CD14^{hi}$ $CD16^{+}$ were increased in severe asthmatic patients (44) which may impact the migration pattern of monocytes, as well as the differential chemokine receptor expression pattern. Fourth, we provided a mechanistic explanation for monocyte migration but further studies are required to understand how CCL5 production is increased in the asthmatic condition. Finally, our *in vitro* findings indicated that CCL5 may play a role in monocyte recruitment in rhinovirus-induced asthma exacerbation. This has to be confirmed with further *in vivo* or *ex vivo* studies.

In conclusion, this study highlighted a new role of BSM in asthma which altered BE response against HRV infection. These results were in agreement with the double association of, on the one hand, the increased exacerbation rate in asthmatic patients with increased BSM mass (15), and, on the other hand, the decreased exacerbation rate induced by bronchial thermoplasty, which decreased BSM mass (16, 17). However, whether or not BSM was directly involved in HRV-induced asthma exacerbation remained to be further elucidated.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the National Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BA and HL designed the research, performed the experiments, collected, analyzed and interpreted the data, and wrote the manuscript. PE, AC, and EM performed the experiments, analyzed the data, and revised the final manuscript. MT and PG provided the human samples from the Clinical Investigation Center of Bordeaux and revised the final manuscript. TT and PB designed the research, supervised the study, analyzed the data, and revised 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.2019.02998/full#supplementary-material

Figure S1 | Monocyte do not cross epithelium in a short migration assay. Illustration of monocyte migration through a reconstituted bronchial epithelium evaluated by micro-OCT. **(A)** At T 0 min, the epithelium is inverted and neutrophils (white arrows) **(B)** or monocytes **(C)** are added on the upper chamber and set to migrate for 4 h. (Scale bar = 100 μm).

Figure S2 | Chemokine expression in co-cultured epithelial cells after rhinovirus infection. Additional chemokines mRNA were quantified in epithelial cells by multiplex gene expression analysis. Data are presented as mean ± SEM values ($n = 3$ per group, one-way ANOVA, Newman-Keuls multiple comparisons test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

Figure S3 | Asthmatic bronchial smooth muscle cell co-culture increases inflammation in epithelial cells after rhinovirus infection. **(A)** Cytokines and **(B)** rhinovirus-mediated genes were assessed in epithelial cells by multiplex gene expression analysis. Data are presented as mean ± SEM values ($n = 3$ per group, one-way ANOVA, Newman-Keuls multiple comparisons test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

Figure S4 | Human rhinovirus infection induces IL-6 expression by bronchial epithelial cells. IL-6 was quantified in supernatants of BE cells infected by HRV or UV-inactivated HRV. Data are presented as mean ± SEM values ($n = 6$ per group, one-way ANOVA, Bonferroni's multiple comparisons test, $^{***}P < 0.001$).

Table S1 | Patients' characteristics for BSM.

Table S2 | Control subjects' characteristics for BE.

Table S3 | Asthmatic patients' characteristics for blood.

REFERENCES

1. Global Initiative for Asthma. *Global Strategy for Asthma Management and Prevention*. NIH Publication 1995 (2019). (accessed August 2019). Available online at: http://www.ginasthma.org
2. Silkoff PE, Flavin S, Gordon R, Loza MJ, Sterk PJ, Lutter R, et al. Toll-like receptor 3 blockade in rhinovirus-induced experimental asthma exacerbations: a randomized controlled study. *J Allergy Clin Immunol.* (2017) 141:1220–30. doi: 10.1016/j.jaci.2017.06.027
3. Holgate ST. Rhinoviruses in the pathogenesis of asthma: the bronchial epithelium as a major disease target. *J Allergy Clin Immunol.* (2006) 118:587–90. doi: 10.1016/j.jaci.2006.06.023
4. Shelfoon C, Shariff S, Traves SL, Kooi C, Leigh R, Proud D. Chemokine release from human rhinovirus-infected airway epithelial cells promotes fibroblast migration. *J Allergy Clin Immunol.* (2016) 138:114–22. doi: 10.1016/j.jaci.2015.12.1308
5. Bartemes KR, Kita H. Dynamic role of epithelium-derived cytokines in asthma. *Clin Immunol.* (2012) 143:222–35. doi: 10.1016/j.clim.2012.03.001
6. Zhou X, Zhu L, Lizarraga R, Chen Y. Human airway epithelial cells direct significant rhinovirus replication in monocytic cells by enhancing ICAM1 expression. *Am J Respir Cell Mol Biol.* (2017) 57:216–25. doi: 10.1165/rcmb.2016-0271OC
7. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol.* (2005) 5:953–64. doi: 10.1038/nri1733
8. Hung CH, Wang CC, Suen JL, Sheu CC, Kuo CH, Liao WT, et al. Altered pattern of monocyte differentiation and monocyte-derived TGF-beta1 in severe asthma. *Sci Rep.* (2018) 8:919. doi: 10.1038/s41598-017-19105-z
9. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol.* (2011) 11:762–74. doi: 10.1038/nri3070
10. Zhao L, Yang W, Yang X, Lin Y, Lv J, Dou X, et al. Chemerin suppresses murine allergic asthma by inhibiting CCL2 production and subsequent airway recruitment of inflammatory dendritic cells. *Allergy*. (2014) 69:763–74. doi: 10.1111/all.12408
11. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med.* (2006) 354:610–21. doi: 10.1056/NEJMra052723
12. Shi HZ, Xie ZF, Deng JM, Chen YQ, Xiao CQ. Soluble CD86 protein in serum samples of patients with asthma. *Thorax*. (2004) 59:870–5. doi: 10.1136/thx.2004.021840
13. Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzo H, et al. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. *J Allergy Clin Immunol.* (2009) 124:45–51.e1–4. doi: 10.1016/j.jaci.2009.03.049
14. Ramos-Barbon D, Fraga-Iriso R, Brienza NS, Montero-Martinez C, Verea-Hernando H, Olivenstein R, et al. T Cells localize with proliferating smooth

muscle alpha-actin+ cell compartments in asthma. *Am J Respir Crit Care Med*. (2010) 182:317–24. doi: 10.1164/rccm.200905-0745OC
15. Girodet PO, Allard B, Thumerel M, Begueret H, Dupin I, Ousova O, et al. Bronchial smooth muscle remodeling in nonsevere asthma. *Am J Respir Crit Care Med*. (2016) 193:627–33. doi: 10.1164/rccm.201507-1404OC
16. Pretolani M, Dombret MC, Thabut G, Knap D, Hamidi F, Debray MP, et al. Reduction of airway smooth muscle mass by bronchial thermoplasty in patients with severe asthma. *Am J Respir Crit Care Med*. (2014) 190:1452–4. doi: 10.1164/rccm.201407-1374LE
17. Cox G, Thomson NC, Rubin AS, Niven RM, Corris PA, Siersted HC, et al. Asthma control during the year after bronchial thermoplasty. *N Engl J Med*. (2007) 356:1327–37. doi: 10.1056/NEJMoa064707
18. Bara I, Ozier A, Girodet PO, Carvalho G, Cattiaux J, Begueret H, et al. Role of YKL-40 in bronchial smooth muscle remodeling in asthma. *Am J Respir Crit Care Med*. (2012) 185:715–22. doi: 10.1164/rccm.201105-0915OC
19. Allard B, Bara I, Gilbert G, Carvalho G, Trian T, Ozier A, et al. Protease activated receptor-2 expression and function in asthmatic bronchial smooth muscle. *PLoS ONE*. (2014) 9:e86945. doi: 10.1371/journal.pone.0086945
20. Trian T, Allard B, Ozier A, Maurat E, Dupin I, Thumerel M, et al. Selective dysfunction of p53 for mitochondrial biogenesis induces cellular proliferation in bronchial smooth muscle from asthmatic patients. *J Allergy Clin Immunol*. (2016) 137:1717–26.e13. doi: 10.1016/j.jaci.2015.10.031
21. Trian T, Allard B, Dupin I, Carvalho G, Ousova O, Maurat E, et al. House dust mites induce proliferation of severe asthmatic smooth muscle cells via an epithelium-dependent pathway. *Am J Respir Crit Care Med*. (2015) 191:538–46. doi: 10.1164/rccm.201409-1582OC
22. Trian T, Moir LM, Ge Q, Burgess JK, Kuo C, King NJ, et al. Rhinovirus-induced exacerbations of asthma: how is the {beta}2-adrenoceptor implicated? *Am J Respir Cell Mol Biol*. (2010) 43:227–33. doi: 10.1165/rcmb.2009-0126OC
23. Liu L, Gardecki JA, Nadkarni SK, Toussaint JD, Yagi Y, Bouma BE, et al. Imaging the subcellular structure of human coronary atherosclerosis using micro-optical coherence tomography. *Nat Med*. (2011) 17:1010–4. doi: 10.1038/nm.2409
24. Schneider D, Hong JY, Bowman ER, Chung Y, Nagarkar DR, McHenry CL, et al. Macrophage/epithelial cell CCL2 contributes to rhinovirus-induced hyperresponsiveness and inflammation in a mouse model of allergic airways disease. *Am J Physiol Lung Cell Mol Physiol*. (2013) 304:L162–9. doi: 10.1152/ajplung.00182.2012
25. Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol*. (2008) 8:142–52. doi: 10.1038/nri2236
26. Holt PG, Strickland DH, Sly PD. Virus infection and allergy in the development of asthma: what is the connection? *Curr Opin Allergy Clin Immunol*. (2012) 12:151–7. doi: 10.1097/ACI.0b013e3283520166
27. Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity*. (2013) 38:322–35. doi: 10.1016/j.immuni.2012.10.016
28. Robays LJ, Maes T, Lebecque S, Lira SA, Kuziel WA, Brusselle GG, et al. Chemokine receptor CCR2 but not CCR5 or CCR6 mediates the increase in pulmonary dendritic cells during allergic airway inflammation. *J Immunol*. (2007) 178:5305–11. doi: 10.4049/jimmunol.178.8.5305
29. Reeves SR, Kaber G, Sheih A, Cheng G, Aronica MA, Merrilees MJ, et al. Subepithelial accumulation of versican in a cockroach antigen-induced murine model of allergic asthma. *J Histochem Cytochem*. (2016) 64:364–80. doi: 10.1369/0022155416642989
30. Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R, et al. Tryptase-stimulated human airway smooth muscle cellsinduce cytokine synthesis and mast cell chemotaxis. *FASEB J*. (2003) 17:2139–41. doi: 10.1096/fj.03-0041fje
31. Sandblad KG, Jones P, Kostalla MJ, Linton L, Glise H, Winqvist O. Chemokine receptor expression on monocytes from healthy individuals. *Clin Immunol*. (2015) 161:348–53. doi: 10.1016/j.clim.2015.09.012
32. Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature*. (1990) 347:669–71. doi: 10.1038/347669a0
33. Alam R, Stafford S, Forsythe P, Harrison R, Faubion D, Lett-Brown MA, et al. RANTES is a chemotactic and activating factor for human eosinophils. *J Immunol*. (1993) 150:3442–8.
34. Giuffrida MJ, Valero N, Mosquera J, Duran A, Arocha F, Chacin B, et al. Increased systemic cytokine/chemokine expression in asthmatic and non-asthmatic patients with bacterial, viral or mixed lung infection. *Scand J Immunol*. (2017) 85:280–90. doi: 10.1111/sji.12532
35. Malavia NK, Raub CB, Mahon SB, Brenner M, Panettieri RA Jr, George SC. Airway epithelium stimulates smooth muscle proliferation. *Am J Respir Cell Mol Biol*. (2009) 41:297–304. doi: 10.1165/rcmb.2008-0358OC
36. Shariff S, Shelfoon C, Holden NS, Traves SL, Wiehler S, Kooi C, et al. Human rhinovirus infection of epithelial cells modulates airway smooth muscle migration. *Am J Respir Cell Mol Biol*. (2017) 56:796–803. doi: 10.1165/rcmb.2016-0252OC
37. Ozier A, Allard B, Bara I, Girodet PO, Trian T, Marthan R, et al. The pivotal role of airway smooth muscle in asthma pathophysiology. *J Allergy*. (2011) 2011:742710. doi: 10.1155/2011/742710
38. Singh SR, Sutcliffe A, Kaur D, Gupta S, Desai D, Saunders R, et al. CCL2 release by airway smooth muscle is increased in asthma and promotes fibrocyte migration. *Allergy*. (2014) 69:1189–97. doi: 10.1111/all.12444
39. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses. *Clin Microbiol Rev*. (2011) 24:210–29. doi: 10.1128/CMR.00014-10
40. Kieninger E, Vareille M, Kopf BS, Blank F, Alves MP, Gisler FM, et al. Lack of an exaggerated inflammatory response on virus infection in cystic fibrosis. *Eur Respir J*. (2012) 39:297–304. doi: 10.1183/09031936.00054511
41. Korpi-Steiner NL, Valkenaar SM, Bates ME, Evans MD, Gern JE, Bertics PJ. Human monocytic cells direct the robust release of CXCL10 by bronchial epithelial cells during rhinovirus infection. *Clin Exp Allergy*. (2010) 40:1203–13. doi: 10.1111/j.1365-2222.2010.03546.x
42. Schroth MK, Grimm E, Frindt P, Galagan DM, Konno SI, Love R, et al. Rhinovirus replication causes RANTES production in primary bronchial epithelial cells. *Am J Respir Cell Mol Biol*. (1999) 20:1220–8. doi: 10.1165/ajrcmb.20.6.3261
43. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, et al. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol*. (2011) 128:549–56.e1–12. doi: 10.1016/j.jaci.2011.05.038
44. Kowal K, Moniuszko M, Dabrowska M, Bodzenta-Lukaszyk A. Allergen challenge differentially affects the number of circulating monocyte subsets. *Scand J Immunol*. (2012) 75:531–9. doi: 10.1111/j.1365-3083.2012.02685.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Convergence of Inflammatory Pathways in Allergic Asthma and Sickle Cell Disease

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The underlying pathologies of sickle cell disease and asthma share many characteristics in terms of respiratory inflammation. The principal mechanisms of pulmonary inflammation are largely distinct, but activation of common pathways downstream of the initial inflammatory triggers may lead to exacerbation of both disease states. The altered inflammatory landscape of these respiratory pathologies can differentially impact respiratory pathogen susceptibility in patients with sickle cell disease and asthma. How these two distinct diseases behave in a comorbid setting can further exacerbate pulmonary complications associated with both disease states and impact susceptibility to respiratory infection. This review will provide a concise overview of how asthma distinctly affects individuals with sickle cell disease and how pulmonary physiology and inflammation are impacted during comorbidity.

Keywords: sickle cell disease (SCD), asthma, acute chest syndrome (ACS), respiratory infection, pulmonary inflammation

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INTRODUCTION

Red blood cells (RBCs) constitute the largest number of mobile cells in the human body (about 3×10^{12}) that perform the primary function of O_2 (and CO_2) transportation through hemoglobin (Hb). Alterations that occur in Hb through inherited genes can lead to a change in RBC morphology and function leading to sickle cell disease (SCD), a common inherited disorder leading to anemia, incidences of vaso-occlusive crises, acute chest syndrome (ACS), cumulative organ damage, and a number of additional chronic comorbidities (1). A large number of individuals carry the sickle cell trait, wherein a single sickle cell gene ("*S*") is inherited, and are mostly asymptomatic (2). However, a patient with two sickle genes are named to have the Hb*SS* form of SCD, while a patient who inherits one *S* gene and another abnormal hemoglobin gene (C, beta thalassemia, D, E, or O) will have alternate types of SCD such as HbSC or HbS beta thalassemia. Patients with SCD represent a significant health care burden in terms of cost, and despite a number of therapeutic strategies, life expectancy in this population remains decades premature compared to that of the general population (3–5). As the most commonly inherited blood disease, SCD affects >100,000 in the United States and millions more worldwide (6). With 1:13 babies born with the sickle cell trait and 1:365 patients having SCD, African Americans have the highest incidence of SCD in the U.S. (7). The high occurrence of pulmonary complications in SCD patients has led to the consideration of possible complications from other respiratory conditions that have similar symptomatologies, like asthma.

Asthma is a syndrome of the respiratory system that affects 26 million Americans and 300 million globally. Like SCD, the incidence of asthma is predicted to continue to increase as indicated by the 3.6% increase in prevalence since 2006 (8). Of note is the observation that individuals with SCD have an increased incidence of asthma when compared to the general population. In children, the incidence of asthma diagnosis is as high as 27% in individuals with SCD (9). Approximately 30–70% of patients with SCD also suffer from asthma (10, 11) leading to a poorer quality of life. Like SCD, African Americans (especially women) are more likely to have asthma and African American children have a much higher likelihood of dying from asthma compared to other ethnicities (12). While it is unclear why asthma incidence is disproportionately elevated in African American children with SCD, socioeconomic factors and perhaps even overdiagnosis of asthma in SCD patients may contribute to this bias. ACS, one of the most frequent complications of SCD, is correlated with the incidence of asthma in the SCD population (13–15). As such, gaining an understanding of the clinical and immunological consequences of asthma in the context of SCD is of critical importance for improving patient outcomes in this patient group.

Asthma and SCD share a number of similarities in terms of the immunological factors associated with their respective disease states. Both conditions result in inflammation and airway hyperreactivity, both conditions impact susceptibility to respiratory infections, and both require specific interventions to mitigate the complications associated with them. Despite the recognition that asthma in the context of SCD likely results in a comorbid condition distinct from the general population, there is relatively little mechanistic insight into how these two disease pathologies co-function. In this review we highlight the potential immunological synergies between asthma and SCD garnered from both clinical data and murine modeling studies to showcase how these conditions may exacerbate each other, thereby representing a unique comorbid condition in these high-risk patient populations.

IMMUNOLOGIC CONSEQUENCES OF ASTHMA IN SCD

The immunologic sequelae associated with SCD and asthma are complex but have some overlap. Given that both asthma and SCD impact inflammation in distinct ways, the interplay into how these two conditions function when present in a comorbid state raises important questions. Elevated IgE levels in children with SCD is much more common than in the general population and is associated with both asthma and increased morbidity in children (9). Increased serum IgE is a well-accepted biomarker of allergic asthma, and SCD patients have elevated IgE in sera which may occur as a result of non-specific immune activation in these patients, leading to a T_H2 bias and increased risk for asthma as a consequence. This enhanced serum IgE availability is also reflected in murine models, whereby the increase in total IgE in sensitized SCD mice is significantly greater than what is observed in sensitized wild type animals (16). Pulmonary function testing is often utilized to distinguish allergic asthma from other IgE mediated inflammatory conditions. Adult patients with SCD have a high incidence, up to 80%, of abnormal pulmonary function when tested (17). A similar, but less severe pattern is observed in children with SCD, with ∼50% of patients having abnormal results (18). Abnormal results were more prevalant in the asthmatic pediatric SCD patients, underscoring applicability of pulmonary function analysis as part of making an appropriate diagnosis of asthma in SCD patients (18). The utility of screening for respiratory disorders such as asthma in children and adults using pulmonary function tests has not been fully established and current guidelines suggest routine collection of a thorough respiratory history to identify pulmonary disease in patients with SCD. This is of particular importance in young children because pulmonary function tests can be unreliable in this population. While asthma represents a major and frequent health concern for patients with SCD, the mechanistic factors driving the development and immunological features of asthma in the context of SCD remain poorly elucidated and create barriers to appropriate asthma management in SCD patients.

Endothelial activation is considered to be a major pathway by which sickled RBCs contribute to vaso-occlusion. Sickled RBC binding to integrins on endothelial cells lead to injury via reactive oxygen species that also function in a feed-forward loop to continue to activate endothelial cells (19). This activation leads to the infiltration of other cells such as monocytes and neutrophils which contribute to uncontrolled cell adhesion that occurs in blood vessels of SCD patients (20, 21). Increased levels of pro-inflammatory cytokines such as IL-3, GM-CSF, and PGE2 have also been noted to occur in SCD patients (22–24). Steady state levels of TNF-α, IL-α, IL-1β, and IL-6 are all elevated in SCD (23–25). Elevated neutrophil counts are characteristic of SCD and can form neutrophil extracellular traps in the pulmonary vasculature, contributing to acute lung injury resulting from inflammatory cytokine signaling (26). Both the pulmonary and systemic responses to inflammatory stimuli are greatly elevated in the context of SCD with enhanced levels of TNF-α, IL-1β, s-VCAM-1 being observed following endotoxin treatment (27). This heightened inflammatory landscape contributes to multiple complications of SCD, ranging from pulmonary disfunction and infection susceptibility.

Airway inflammation is a canonical hallmark of asthma and eosinophils may dominate as the infiltrating leukocyte in severe allergic asthma. Endothelial activation is fundamental to the initiation of inflammation in asthma (28) wherein endothelial cells upregulate integrins and selectins in response to allergenic stimuli (29) and cytokines produced *in situ* by resident leukocytes (30). Markers of endothelial activation including ICAM-1, VCAM-1, P-selection, and E-selectin are also elevated in the context of SCD (24, 31). Similarly, IL-3 and GM-CSF can promote the allergic milieu (32) and support activation and survival of eosinophils in the airways (33). While the exact role of PGE2 in the lungs of asthmatics is still unclear, its elevation correlates with eosinophilia (34). Common inflammatory pathways between SCD and asthma may therefore lead to an asthma-like phenotype in patients with

SCD (**Figure 1**), or indeed, increase the likelihood of asthma pathogenesis in these patients.

MODELING ASTHMA AND SCD IN MICE

While SCD is a hereditary condition, asthma development is dependent on genetic and environmental components. Although rodents do not naturally develop asthma, asthma-like disease can be triggered in them through continuous exposure to natural aeroallergens or ovalbumin (OVA) (35). The possible overlap between asthma and SCD based on shared symptoms such as airway inflammation, hyperresponsiveness, and architectural damage has created a demand for animal models of asthma and SCD comorbidity, although only a few have been created to date using OVA and house dust mite (HDM) as triggering allergens.

Existing models of asthma in SCD mice after OVA sensitization and challenge suggest that mice with SCD respond more severely to allergen exposures (16, 36, 37). OVA exposure leads to the development of peribronchovascular inflammation (with active eosinophils) and inflammatory foci, elevated serum IgE, and bronchial epithelial hyperplasia in BERK SCD mice to equivalent levels as wild-type controls (16). However, more severe pathologic changes occur in SCD mice when OVA-challenge duration is prolonged causing death in about 30% of the animals (16), suggesting that the BERK SCD mice may have a lower threshold for asthma exacerbation. Using a bone marrow chimeric mouse model of SCD and shortened exposures to aerosolized OVA, Pritchard et al. demonstrated that OVA-induced allergic inflammation in these SCD mice correlates with a heightened T_H2 cytokine milieu and pulmonary tissue resistance marked by a decrease in lung tissue elasticity suggestive of greater alveolar occlusion (36). This trend in airway inflammation and general T_H2 skewing was recapitulated by Andemariam et al. using the BERK mouse model of SCD and a more standard model of OVA exposure (37). Of note, naïve BERK SCD have increased levels of T_H2-type cytokines in the bronchoalveolar lavage fluid and a higher number of T-lymphocytes in the lungs (37). Airway hyperresponsiveness is a shared hallmark of SCD and asthma (38, 39), that generally correlates with pulmonary inflammation. Despite heightened airway inflammation in response to OVA, BERK SCD mice had lower airway reactivity compared to wild-type mice even at very high doses of methacholine (37), suggesting that inflammation and airway hyperresponsiveness may be disjointed in SCD.

Although OVA is a commonly used trigger to induce allergic asthma-like disease in mice, due to its limitations as a clinically relevant aeroallergen, utilization of more relevant allergens such as HDM, cockroach, fungal, and viral antigens have gained popularity among investigators that model asthma in mice (40). Most recently, Jiang et al. found no differences between BERK SCD and wild-type mice in the inflammatory index, airway cytokines, or HDM-specific IgE levels after HDM exposure (41). These findings are exciting

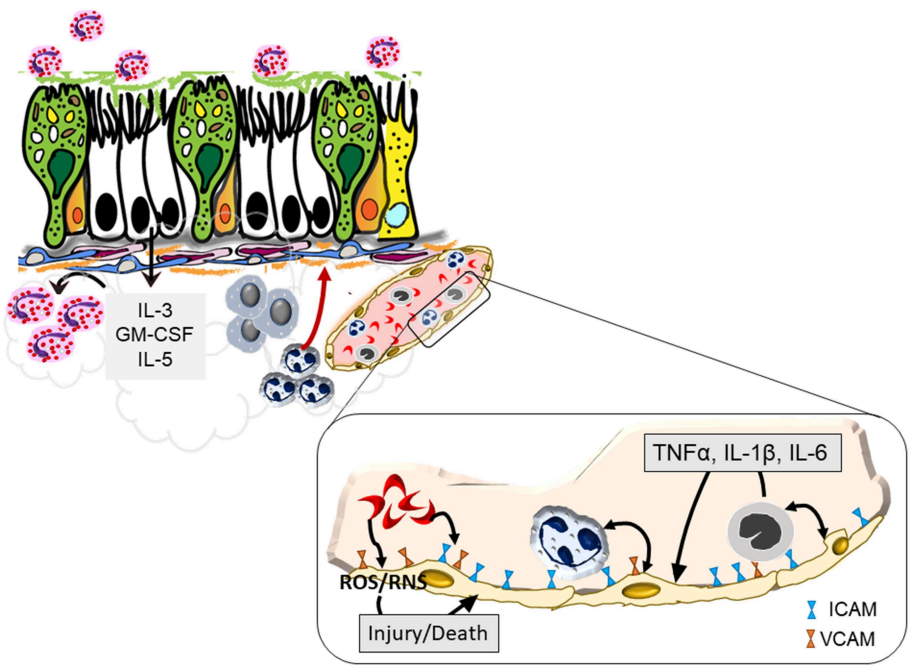

FIGURE 1 | Endothelial activation by sickled red blood cells that may exacerbate asthma. Sickled red cells induce endothelial upregulation of integrins, ICAM, and VCAM, that enhance attachment and subsequent infiltration of neutrophils and monocytes into the pulmonary tissue. Increased margination of these leukocytes trigger further interaction with endothelial cells through the production of pro-inflammatory cytokines that together with sickled red blood cells cause endothelial cell production of reactive oxygen and nitrogen species that can trigger blood vessel injury. Recruited cells further activate the bronchial epithelium leading to a positive feedback loop to promote heightened inflammation and airway hyperreactivity.

as they confirm that variation in outcome occurs based on the antigen, route of exposure, and adjuvants in SCD mice. Mouse models of SCD with asthma that can be used to decipher mechanisms that underlie asthma pathogenesis in patients with SCD is an important gap in technology to address.

TREATMENT OF ASTHMA IN INDIVIDUALS WITH SICKLE CELL DISEASE

Information amassed on the immunologic basis of asthma has resulted in the development of biologics targeted for patients with specific endotypes. However, since the efficacy of these therapeutics are quite low, corticosteroids are used to alleviate the symptoms during asthma attacks despite our knowledge of the long term negative impact of steroids on human health (42). Although the SCD-asthma comorbid condition is prevalent, relatively little evidence based models exist for its management. Current models for asthma management in SCD are based on NIH guidelines for the general population and include liberal use of inhaled steroids, despite the extensive literature recognizing SCD patients as a uniquely susceptible and vulnerable patient population (10, 43, 44). Use of inhaled steroids further increases the risk of colonization of *S. pneumoniae* which may increase the likelihood for the development of invasive disease to which the SCD population is particularly susceptible (45, 46). Inhaled corticosteroids have been proposed to be used to prevent additional episodes of vaso-occlusive crisis in pediatric patients, and recent studies have underscored the feasibility of this approach in young children (47). Inhaled steroids given to non-asthmatic patients with SCD have demonstrated considerable promise, with significant reductions in pain and sVCAM levels as well as inflammatory macrophage markers, underscoring the potential for targeting inflammation to improve health outcomes in these patients (48–50). Whether treatments for specific asthma endotypes can be extended to patients with SCD remains unclear, though given the underlying differences in inflammation tailored therapeutic strategies may be required.

SCD AND ASTHMA: INDEPENDENT PATHWAYS TO INFECTION SUSCEPTIBILITY

Asthma and SCD both fundamentally alter susceptibility and immune responses to respiratory infection. Patients with SCD are overwhelmingly susceptible to multiple respiratory pathogens, most importantly the pneumococcus (51, 52), and infectious diseases increase the development of ACS in these patients (53–55). The heightened sensitivity to infection is recapitulated in the murine model of SCD, whereby the SCD mice demonstrate dramatically enhanced susceptibility to both bacterial and viral respiratory infections (56–58). This issue can be further confounded by strains outside of vaccine coverage causing invasive disease in these patients, as is the case with *S. pneumoniae* (59). Immunogenic responses to vaccines in SCD patients may also be suboptimal to confer effective protection as the responses have been reported to wane more rapidly to a number of serotypes included in the current pneumococcal vaccines, an observation that has been recapitulated in murine models of SCD (60, 61). These underscore the importance of appropriate prophylactic strategies to mitigate infection risk in individuals with SCD.

Similar but distinct to what is observed in the context of SCD, allergic asthma dramatically also alters susceptibility to multiple respiratory pathogens including both viral and bacterial pathogens (62). However, unlike in the case of SCD, allergic asthma has been found to confer both sensitivity and resistance to subsequent respiratory infection, wherein outcomes are more likely to be dependent on the type of pathogen. Asthma exacerbations triggered by rhinoviruses and respiratory syncytial virus, for example, can be detrimental to the host (63), while asthma exacerbations triggered by influenza virus infection is tolerated by the host which also exhibit reduced signs of influenza morbidity and enhanced viral clearance (64, 65). Immune responses to viruses in hosts with asthma may be dependent on a multitude of factors including gender, age, virus strain and prior exposures, endotype of asthma, and environmental factors including pollution and nutrition. How these alterations in pulmonary inflammation during the asthma-SCD comorbid state differentially impact the risk of infection remains poorly understood, though given the distinct nature of these two disease settings, it may be anticipated that together they impact infection susceptibility in a manner distinct from the general population.

ANTIBIOTIC EXPOSURE AND ASTHMA

Due to the propensity of patients with SCD to develop fulminant lethal sepsis caused by *S. pneumoniae*, during childhood, penicillin prophylaxis is prescribed for all children with SCD until the age of 5 years, which has dramatically improved mortality in this patient population prior to the advent of the pneumococcal conjugate vaccine (66–70). There is considerable evidence linking antibiotic exposure to the development of childhood asthma and other allergic disease in the general population, though there are challenges in terms of confounding respiratory infections (71–79). Early antibiotic use is associated with allergic asthma in young children even when accounting for bias inherent from antibiotic prescriptions to treat early symptoms of asthma; this is predictable as bacterial colonization of the respiratory and gastrointestinal tract are critical mediators that shape susceptibility to allergic airway inflammation (80–82). These effects may be amplified in SCD patients due to the prolonged exposure to antibiotics.

Administering penicillin to patients with SCD eliminates several bacterial species from the nasal-oral microbiota (83). Exposure to antibiotics early in life can have long lasting consequences on the developing bacterial microbiome (84, 85). Bacterial colonization of the respiratory and gastrointestinal tract are critical mediators that can shape susceptibility to allergic airway inflammation (82). Numerous gaps in knowledge including alterations that may naturally occur in the microbiome of the SCD host (86), the relative impact of long-term penicillin

prophylaxis on the intestinal and respiratory flora of these patients, and whether this long-term prophylaxis also impacts the likelihood of subsequent asthma development preclude our understanding of disease pathogenesis in SCD patients and those that may develop asthma.

ARGININE DEFICIENCY: A COMMON CROSSROAD IN SCD AND ASTHMA PATHOPHYSIOLOGY

Arginine deficiency has long been recognized as an important aspect of SCD pathophysiology (87, 88). Low arginine bioavailability is associated with a multitude of complications in SCD including pulmonary hypertension and vaso-occlusive pain episodes (89–91). The decreased arginine availability in SCD is severe enough to impact the contribution of bacterial arginine biosynthesis and uptake pathways to virulence (57). Due to the multiple facets of host pathophysiology, arginine supplementation has been proposed as a therapeutic intervention for SCD (92, 93). Arginine supplementation has been suggested as a means by which to alleviate complications in patients with SCD and improve overall health (94, 95). Clinical trials further support the potential for arginine supplementation to confer benefit in SCD individuals in terms of endothelial function and to induce nitric oxide production during vaso-occlusive crisis (93, 96).

Arginine deficiency is a common feature underlying the pathophysiology of both allergic asthma and SCD (**Figure 2**). Murine models of allergic asthma have demonstrated that arginine deficiency to nitric oxide synthase (NOS) results in deficiencies in nitric oxide, a bronchodilator, in tandem with increased peroxynitrite, a pro-contractile molecule, both of which contribute to airway hyperresponsiveness in the context of asthma. Polycation secretion by eosinophils, which are dramatically elevated in allergic asthma, can inhibit arginine uptake via the y+ system (97, 98). In the context of SCD, there are additional mechanisms underlying arginine deficiency that are independent of pathways operative during asthma. The increased hemolysis of RBCs leads to the release of cellular arginase which can scavenge arginine prior to cellular uptake. In the context of asthma and SCD comorbidity, heightened baseline inflammation may lead to increased expression of both iNOS and arginase, thereby further depleting cellular arginine pools (99). As such, the extracellular arginase released by hemolysis coupled with the increased pulmonary eosinophil infiltrate inhibiting arginine uptake are likely to have an additive effect. Likewise, the increased arginase and iNOS activity resulting from increased levels of inflammatory cytokines inherent in both SCD and allergic asthma, are also potentially synergistic in terms of arginine depletion. Due to the divergence of many of these arginine depleting pathways, it would be expected that such deficiency may be synergistic in comorbid patients with SCD and asthma.

The benefits conferred by arginine supplementation may be most evident in comorbid patients with both SCD and asthma due to the non-overlapping mechanisms of arginine deficiency.

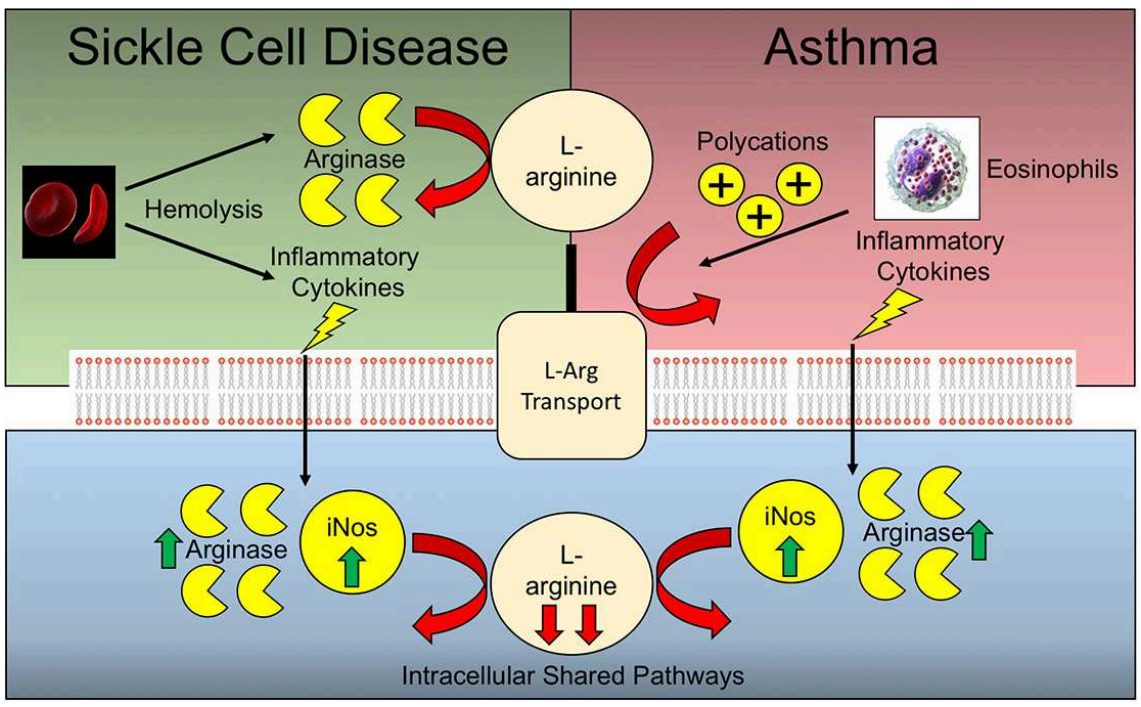

FIGURE 2 | Arginine deficiencies in Sickle cell disease and asthma. Sickle cell disease and asthma share complementary and potentially synergistic mechanisms of arginine deficiency. Many of the pathways operative extracellularly are different, while the intracellular pathways are shared.

DISCUSSION

SCD and asthma share similar manifestations in terms of airway hyperreactivity despite being immunologically distinct diseases. Experimental modeling and clinical data suggest that asthma impacts individuals with SCD in a specific manner distinct from the general population. Laying a mechanistic foundation for understanding pulmonary complications of sickle cell disease and how these complications can be rationally targeted in a SCD-specific manner may provide novel opportunities for treatment. Given the unique host pathophysiology that underlies SCD, these individuals may benefit from tailored interventions for the treatment of asthma.

AUTHOR CONTRIBUTIONS

JR and AS wrote the manuscript jointly.

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REFERENCES

1. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. *N Engl J Med*. (1994) 330:1639–44. doi: 10.1056/NEJM199406093302303
2. Ojodu J, Hulihan MM, Pope SN, Grant AM, Centers for Disease Control and Prevention. Incidence of sickle cell trait–United States, 2010. *MMWR Morb Mortal Wkly Rep*. (2014) 63:1155–8.
3. Kauf TL, Coates TD, Huazhi L, Mody-Patel N, Hartzema AG. The cost of health care for children and adults with sickle cell disease. *Am J Hematol.* (2009) 84:323–7. doi: 10.1002/ajh.21408
4. Elmariah H, Garrett ME, De Castro LM, Jonassaint JC, Ataga KI, Eckman JR, et al. Factors associated with survival in a contemporary adult sickle cell disease cohort. *Am J Hematol.* (2014) 89:530–5. doi: 10.1002/ajh.23683
5. Telen MJ, Malik P, Vercellotti GM. Therapeutic strategies for sickle cell disease: towards a multi-agent approach. *Nat Rev Drug Discov.* (2018) 18:139–58. doi: 10.1038/s41573-018-0003-2
6. Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. *Lancet.* (2010) 376:2018–31. doi: 10.1016/S0140-6736(10)61029-X
7. Centers for Disease Control Sickle Cell Disease (SCD) (2019). Available online at: https://www.cdc.gov/ncbddd/sicklecell/index.html (accessed October 22, 2019).
8. Network GA. *The Global Asthma Report 2018*. Auckland: Global Asthma Network (2018).
9. An P, Barron-Casella EA, Strunk RC, Hamilton RG, Casella JF, Debaun MR. Elevation of IgE in children with sickle cell disease is associated with doctor diagnosis of asthma and increased morbidity. *J Allergy Clin Immunol.* (2011) 127:1440–6. doi: 10.1016/j.jaci.2010.12.1114
10. Morris CR. Asthma management: reinventing the wheel in sickle cell disease. *Am J Hematol.* (2009) 84:234–41. doi: 10.1002/ajh.21359
11. Debaun MR, Strunk RC. The intersection between asthma and acute chest syndrome in children with sickle-cell anaemia. *Lancet.* (2016) 387:2545–53. doi: 10.1016/S0140-6736(16)00145-8
12. Akinbami LJ, Moorman JE, Liu X. Asthma prevalence, health care use, and mortality: United States, 2005–2009. *Natl Health Stat Report.* (2011) 32:1–14.
13. Boyd JH, Macklin EA, Strunk RC, Debaun MR. Asthma is associated with acute chest syndrome and pain in children with sickle cell anemia. *Blood.* (2006) 108:2923–7. doi: 10.1182/blood-2006-01-011072
14. Bernaudin F, Strunk RC, Kamdem A, Arnaud C, An P, Torres M, et al. Asthma is associated with acute chest syndrome, but not with an increased rate of hospitalization for pain among children in France with sickle cell anemia: a retrospective cohort study. *Haematologica.* (2008) 93:1917–8. doi: 10.3324/haematol.13090
15. Debaun MR, Rodeghier M, Cohen R, Kirkham FJ, Rosen CL, Roberts I, et al. Factors predicting future ACS episodes in children with sickle cell anemia. *Am J Hematol.* (2014) 89:E212–17. doi: 10.1002/ajh.23819
16. Nandedkar SD, Feroah TR, Hutchins W, Weihrauch D, Konduri KS, Wang J, et al. Histopathology of experimentally induced asthma in a murine model of sickle cell disease. *Blood.* (2008) 112:2529–38. doi: 10.1182/blood-2008-01-132506
17. Lunt A, Desai SR, Wells AU, Hansell DM, Mushemi S, Melikian N, et al. Pulmonary function, CT and echocardiographic abnormalities in sickle cell disease. *Thorax.* (2014) 69:746–51. doi: 10.1136/thoraxjnl-2013-204809
18. Intzes S, Kalpatthi RV, Short R, Imran H. Pulmonary function abnormalities and asthma are prevalent in children with sickle cell disease and are associated with acute chest syndrome. *Pediatr Hematol Oncol.* (2013) 30:726–32. doi: 10.3109/08880018.2012.756961
19. Canalli AA, Costa FF, Saad ST, Conran N. Granulocytic adhesive interactions and their role in sickle cell vaso-occlusion. *Hematology.* (2005) 10:419–25. doi: 10.1080/10245330500141259
20. Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM. Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. *Blood.* (2000) 96:2451–9. doi: 10.1182/blood.V96.7.2451
21. Belcher JD, Chen C, Nguyen J, Zhang P, Abdulla F, Nguyen P, et al. Control of oxidative stress and inflammation in sickle cell disease with the Nrf2 activator dimethyl fumarate. *Antioxid Redox Signal.* (2017) 26:748–62. doi: 10.1089/ars.2015.6571
22. Conran N, Saad ST, Costa FF, Ikuta T. Leukocyte numbers correlate with plasma levels of granulocyte-macrophage colony-stimulating factor in sickle cell disease. *Ann Hematol.* (2007) 86:255–61. doi: 10.1007/s00277-006-0246-6
23. Croizat H. Circulating cytokines in sickle cell patients during steady state. *Br J Haematol.* (1994) 87:592–7. doi: 10.1111/j.1365-2141.1994.tb08318.x
24. Graido-Gonzalez E, Doherty JC, Bergreen EW, Organ G, Telfer M, Mcmillen MA. Plasma endothelin-1, cytokine, and prostaglandin E2 levels in sickle cell disease and acute vaso-occlusive sickle crisis. *Blood.* (1998) 92:2551–5. doi: 10.1182/blood.V92.7.2551
25. Malave I, Perdomo Y, Escalona E, Rodriguez E, Anchustegui M, Malave H, et al. Levels of tumor necrosis factor alpha/cachectin (TNF alpha) in sera from patients with sickle cell disease. *Acta Haematol.* (1993) 90:172–6. doi: 10.1159/000204452
26. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS. Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. *Blood.* (2014) 123:3818–27. doi: 10.1182/blood-2013-10-529982
27. Holtzclaw JD, Jack D, Aguayo SM, Eckman JR, Roman J, Hsu LL. Enhanced pulmonary and systemic response to endotoxin in transgenic sickle mice. *Am J Respir Crit Care Med.* (2004) 169:687–95. doi: 10.1164/rccm.200302-224OC
28. Green CE, Turner AM. The role of the endothelium in asthma and chronic obstructive pulmonary disease (COPD). *Respir Res.* (2017) 18:20. doi: 10.1186/s12931-017-0505-1
29. Asosingh K, Weiss K, Queisser K, Wanner N, Yin M, Aronica M, et al. Endothelial cells in the innate response to allergens and initiation of atopic asthma. *J Clin Invest.* (2018) 128:3116–28. doi: 10.1172/JCI97720
30. Toews GB. Cytokines and the lung. *Eur Respir J Suppl.* (2001) 34:3s—17s. doi: 10.1183/09031936.01.00266001
31. Lebensburger JD, Howard T, Hu Y, Pestina TI, Gao G, Johnson M, et al. Hydroxyurea therapy of a murine model of sickle cell anemia inhibits the progression of pneumococcal disease by down-modulating E-selectin. *Blood.* (2012) 119:1915–21. doi: 10.1182/blood-2011-08-374447

32. Asquith KL, Ramshaw HS, Hansbro PM, Beagley KW, Lopez AF, Foster PS. The IL-3/IL-5/GM-CSF common receptor plays a pivotal role in the regulation of Th2 immunity and allergic airway inflammation. *J Immunol.* (2008) 180:1199–206. doi: 10.4049/jimmunol.180.2.1199
33. Tomaki M, Zhao LL, Sjostrand M, Linden A, Ichinose M, Lotvall J. Comparison of effects of anti-IL-3, IL-5 and GM-CSF treatments on eosinophilopoiesis and airway eosinophilia induced by allergen. *Pulm Pharmacol Ther.* (2002) 15:161–8. doi: 10.1006/pupt.2001.0333
34. Profita M, Sala A, Bonanno A, Riccobono L, Siena L, Melis MR, et al. Increased prostaglandin E2 concentrations and cyclooxygenase-2 expression in asthmatic subjects with sputum eosinophilia. *J Allergy Clin Immunol.* (2003) 112:709–16. doi: 10.1016/S0091-6749(03)01889-X
35. Aun MV, Bonamichi-Santos R, Arantes-Costa FM, Kalil J, Giavina-Bianchi P. Animal models of asthma: utility and limitations. *J Asthma Allergy.* (2017) 10:293–301. doi: 10.2147/JAA.S121092
36. Pritchard KA Jr., Feroah TR, Nandedkar SD, Holzhauer SL, Hutchins W, Schulte ML, et al. Effects of experimental asthma on inflammation and lung mechanics in sickle cell mice. *Am J Respir Cell Mol Biol.* (2012) 46:389–96. doi: 10.1165/rcmb.2011-0097OC
37. Andemariam B, Adami AJ, Singh A, Mcnamara JT, Secor ER, Guernsey LA, et al. The sickle cell mouse lung: proinflammatory and primed for allergic inflammation. *Transl Res.* (2015) 166:254–68. doi: 10.1016/j.trsl.2015.03.001
38. Field JJ, Stocks J, Kirkham FJ, Rosen CL, Dietzen DJ, Semon T, et al. Airway hyperresponsiveness in children with sickle cell anemia. *Chest.* (2011) 139:563–8. doi: 10.1378/chest.10-1243
39. Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. *Clin Exp Allergy.* (2015) 45:706–19. doi: 10.1111/cea.12506
40. Kumar RK, Herbert C, Foster PS. Mouse models of acute exacerbations of allergic asthma. *Respirology.* (2016) 21:842–9. doi: 10.1111/resp.12760
41. Jiang C, Gavitt TD, Szczepanek SM. House dust mite-induced allergic lung inflammation is not exacerbated in sickle cell disease Mice. *Int Arch Allergy Immunol.* (2019) 179:192–200. doi: 10.1159/000499043
42. Buchman AL. Side effects of corticosteroid therapy. *J Clin Gastroenterol.* (2001) 33:289–94. doi: 10.1097/00004836-200110000-00006
43. Sobota A, Graham DA, Heeney MM, Neufeld EJ. Corticosteroids for acute chest syndrome in children with sickle cell disease: variation in use and association with length of stay and readmission. *Am J Hematol.* (2010) 85:24–8. doi: 10.1002/ajh.21565
44. Gomez E, Morris CR. Asthma management in sickle cell disease. *Biomed Res Int.* (2013) 2013:604140. doi: 10.1155/2013/604140
45. Leikin SL, Gallagher D, Kinney TR, Sloane D, Klug P, Rida W. Mortality in children and adolescents with sickle cell disease. Cooperative Study of Sickle Cell Disease. *Pediatrics.* (1989) 84:500–8.
46. Zhang L, Prietsch SO, Mendes AP, Von Groll A, Rocha GP, Carrion L, et al. Inhaled corticosteroids increase the risk of oropharyngeal colonization by Streptococcus pneumoniae in children with asthma. *Respirology.* (2013) 18:272–7. doi: 10.1111/j.1440-1843.2012.02280.x
47. Vance Utset L, Ivy Z, Willen SM, Rodeghier M, Watt A, Schilling L, et al. Inhaled corticosteroid use to prevent severe vaso-occlusive episode recurrence in children between 1 and 4 years of age with sickle cell disease: a multicenter feasibility trial. *Am J Hematol.* (2018) 93:E101–3. doi: 10.1002/ajh.25033
48. Glassberg J, Minnitti C, Cromwell C, Cytryn L, Kraus T, Skloot GS, et al. Inhaled steroids reduce pain and sVCAM levels in individuals with sickle cell disease: a triple-blind, randomized trial. *Am J Hematol.* (2017) 92:622–31. doi: 10.1002/ajh.24742
49. Carden MA, Little J. Emerging disease-modifying therapies for sickle cell disease. *Haematologica.* (2019) 104:1710–9. doi: 10.3324/haematol.2018.207357
50. Langer AL, Leader A, Kim-Schulze S, Ginzburg Y, Merad M, Glassberg J. Inhaled steroids associated with decreased macrophage markers in nonasthmatic individuals with sickle cell disease in a randomized trial. *Ann Hematol.* (2019) 98:841–9. doi: 10.1007/s00277-019-03635-9
51. Gill FM, Sleeper LA, Weiner SJ, Brown AK, Bellevue R, Grover R, et al. Clinical events in the first decade in a cohort of infants with sickle cell disease. Cooperative Study of Sickle Cell Disease. *Blood.* (1995) 86:776–83. doi: 10.1182/blood.V86.2.776.bloodjournal862776
52. Overturf GD. Infections and immunizations of children with sickle cell disease. *Adv Pediatr Infect Dis.* (1999) 14:191–218.
53. Suara RO. Group A beta-haemolytic streptococcal acute chest event in a child with sickle cell anaemia. *Ann Trop Paediatr.* (2001) 21:175–8. doi: 10.1080/02724930120058278
54. Dean D, Neumayr L, Kelly DM, Ballas SK, Kleman K, Robertson S, et al. *Chlamydia pneumoniae* and acute chest syndrome in patients with sickle cell disease. *J Pediatr Hematol Oncol.* (2003) 25:46–55. doi: 10.1097/00043426-200301000-00010
55. Van Tuijn CF, Nur E, Van Beers EJ, Zaaijer HL, Biemond BJ. Acute chest syndrome in sickle cell disease due to the new influenza A (H1N1) virus infection. *Am J Hematol.* (2010) 85:303–4. doi: 10.1002/ajh.21638
56. Rosch JW, Boyd AR, Hinojosa E, Pestina T, Hu Y, Persons DA, et al. Statins protect against fulminant pneumococcal infection and cytolysin toxicity in a mouse model of sickle cell disease. *J Clin Invest.* (2010) 120:627–35. doi: 10.1172/JCI39843
57. Carter R, Wolf J, Van Opijnen T, Muller M, Obert C, Burnham C, et al. Genomic analyses of pneumococci from children with sickle cell disease expose host-specific bacterial adaptations and deficits in current interventions. *Cell Host Microbe.* (2014) 15:587–99. doi: 10.1016/j.chom.2014.04.005
58. Karlsson EA, Oguin TH, Meliopoulos V, Iverson A, Broadnax A, Yoon SW, et al. Vascular permeability drives susceptibility to influenza infection in a murine model of sickle cell disease. *Sci Rep.* (2017) 7:43308. doi: 10.1038/srep43308
59. Ellison AM, Ota KV, Mcgowan KL, Smith-Whitley K. Pneumococcal bacteremia in a vaccinated pediatric sickle cell disease population. *Pediatr Infect Dis J.* (2012) 31:534–6. doi: 10.1097/INF.0b013e3182480fed
60. Santoro JD, Myers L, Kanter J. Assessing the immunogenic response of a single center's pneumococcal vaccination protocol in sickle cell disease. *J Pediatr Hematol Oncol.* (2016) 38:e102–6. doi: 10.1097/MPH.0000000000000510
61. Szczepanek SM, Roberts S, Rogers K, Cotte C, Adami AJ, Bracken SJ, et al. Poor long-term efficacy of prevnar-13 in sickle cell disease mice is associated with an inability to sustain pneumococcal-specific antibody titers. *PLoS ONE.* (2016) 11:e0149261. doi: 10.1371/journal.pone.0149261
62. Torres A, Blasi F, Dartois N, Akova M. Which individuals are at increased risk of pneumococcal disease and why? Impact of COPD, asthma, smoking, diabetes, and/or chronic heart disease on community-acquired pneumonia and invasive pneumococcal disease. *Thorax.* (2015) 70:984–9. doi: 10.1136/thoraxjnl-2015-206780
63. Han M, Rajput C, Ishikawa T, Jarman CR, Lee J, Hershenson MB. Small animal models of respiratory viral infection related to asthma. *Viruses.* (2018) 10:E682. doi: 10.3390/v10120682
64. Samarasinghe AE, Woolard SN, Boyd KL, Hoselton SA, Schuh JM, Mccullers JA. The immune profile associated with acute allergic asthma accelerates clearance of influenza virus. *Immunol Cell Biol.* (2014) 92:449–59. doi: 10.1038/icb.2013.113
65. Furuya Y, Furuya AK, Roberts S, Sanfilippo AM, Salmon SL, Metzger DW. Prevention of influenza virus-induced immunopathology by TGF-beta produced during allergic asthma. *PLoS Pathog.* (2015) 11:e1005180. doi: 10.1371/journal.ppat.1005180
66. Gaston MH, Verter JI, Woods G, Pegelow C, Kelleher J, Presbury G, et al. Prophylaxis with oral penicillin in children with sickle cell anemia. A randomized trial. *N Engl J Med.* (1986) 314:1593–9. doi: 10.1056/NEJM198606193142501
67. Falletta JM, Woods GM, Verter JI, Buchanan GR, Pegelow CH, Iyer RV, et al. Discontinuing penicillin prophylaxis in children with sickle cell anemia. Prophylactic Penicillin Study II. *J Pediatr.* (1995) 127:685–90. doi: 10.1016/S0022-3476(95)70154-0
68. Adamkiewicz TV, Sarnaik S, Buchanan GR, Iyer RV, Miller ST, Pegelow CH, et al. Invasive pneumococcal infections in children with sickle cell disease in the era of penicillin prophylaxis, antibiotic resistance, and 23-valent pneumococcal polysaccharide vaccination. *J Pediatr.* (2003) 143:438–44. doi: 10.1067/S0022-3476(03)00331-7
69. Mccavit TL, Gilbert M, Buchanan GR. Prophylactic penicillin after 5 years of age in patients with sickle cell disease: a survey of sickle cell disease experts. *Pediatr Blood Cancer.* (2013) 60:935–9. doi: 10.1002/pbc.24395

70. Navalkele P, Ozgonenel B, Mcgrath E, Lephart P, Sarnaik S. Invasive pneumococcal disease in patients with sickle cell disease. *J Pediatr Hematol Oncol.* (2017) 39:341–4. doi: 10.1097/MPH.0000000000000858
71. Marra F, Lynd L, Coombes M, Richardson K, Legal M, Fitzgerald JM, et al. Does antibiotic exposure during infancy lead to development of asthma?: a systematic review and metaanalysis. *Chest.* (2006) 129:610–18. doi: 10.1378/chest.129.3.610
72. Reynolds LA, Finlay BB. A case for antibiotic perturbation of the microbiota leading to allergy development. *Expert Rev Clin Immunol.* (2013) 9:1019–30. doi: 10.1586/1744666X.2013.851603
73. Ortqvist AK, Lundholm C, Kieler H, Ludvigsson JF, Fall T, Ye W, et al. Antibiotics in fetal and early life and subsequent childhood asthma: nationwide population based study with sibling analysis. *BMJ.* (2014) 349:g6979. doi: 10.1136/bmj.g6979
74. Stokholm J, Sevelsted A, Bonnelykke K, Bisgaard H. Maternal propensity for infections and risk of childhood asthma: a registry-based cohort study. *Lancet Respir Med.* (2014) 2:631–7. doi: 10.1016/S2213-2600(14)70152-3
75. Pitter G, Ludvigsson JF, Romor P, Zanier L, Zanotti R, Simonato L, et al. Antibiotic exposure in the first year of life and later treated asthma, a population based birth cohort study of 143,000 children. *Eur J Epidemiol.* (2016) 31:85–94. doi: 10.1007/s10654-015-0038-1
76. Hirsch AG, Pollak J, Glass TA, Poulsen MN, Bailey-Davis L, Mowery J, et al. Early-life antibiotic use and subsequent diagnosis of food allergy and allergic diseases. *Clin Exp Allergy.* (2017) 47:236–44. doi: 10.1111/cea.12807
77. Yamamoto-Hanada K, Yang L, Narita M, Saito H, Ohya Y. Influence of antibiotic use in early childhood on asthma and allergic diseases at age 5. *Ann Allergy Asthma Immunol.* (2017) 119:54–8. doi: 10.1016/j.anai.2017.05.013
78. Adami AJ, Bracken SJ, Guernsey LA, Rafti E, Maas KR, Graf J, et al. Early-life antibiotics attenuate regulatory T cell generation and increase the severity of murine house dust mite-induced asthma. *Pediatr Res.* (2018) 84:426–34. doi: 10.1038/s41390-018-0031-y
79. Yoshida S, Ide K, Takeuchi M, Kawakami K. Prenatal and early-life antibiotic use and risk of childhood asthma: a retrospective cohort study. *Pediatr Allergy Immunol.* (2018) 29:490–5. doi: 10.1111/pai.12902
80. Murk W, Risnes KR, Bracken MB. Prenatal or early-life exposure to antibiotics and risk of childhood asthma: a systematic review. *Pediatrics.* (2011) 127:1125–38. doi: 10.1542/peds.2010-2092
81. Risnes KR, Belanger K, Murk W, Bracken MB. Antibiotic exposure by 6 months and asthma and allergy at 6 years: findings in a cohort of 1,401 US children. *Am J Epidemiol.* (2011) 173:310–8. doi: 10.1093/aje/kwq400
82. Ver Heul A, Planer J, Kau AL. The human microbiota and asthma. *Clin Rev Allergy Immunol.* (2018) 57:350–63. doi: 10.1007/s12016-018-8719-7
83. Fukuda JT, Sonis AL, Platt OS, Kurth S. Acquisition of mutans streptococci and caries prevalence in pediatric sickle cell anemia patients receiving long-term antibiotic therapy. *Pediatr Dent.* (2005) 27:186–90.
84. Zeissig S, Blumberg RS. Life at the beginning: perturbation of the microbiota by antibiotics in early life and its role in health and disease. *Nat Immunol.* (2014) 15:307–10. doi: 10.1038/ni.2847
85. Schulfer A, Blaser MJ. Risks of antibiotic exposures early in life on the developing microbiome. *PLoS Pathog.* (2015) 11:e1004903. doi: 10.1371/journal.ppat.1004903
86. Lim SH, Morris A, Li K, Fitch AC, Fast L, Goldberg L, et al. Intestinal microbiome analysis revealed dysbiosis in sickle cell disease. *Am J Hematol.* (2018) 93:E91–3. doi: 10.1002/ajh.25019
87. Enwonwu CO, Xu XX, Turner E. Nitrogen metabolism in sickle cell anemia: free amino acids in plasma and urine. *Am J Med Sci.* (1990) 300:366–71. doi: 10.1097/00000441-199012000-00005
88. Morris CR, Kato GJ, Poljakovic M, Wang X, Blackwelder WC, Sachdev V, et al. Dysregulated arginine metabolism, hemolysis-associated pulmonary hypertension, and mortality in sickle cell disease. *JAMA.* (2005) 294:81–90. doi: 10.1001/jama.294.1.81
89. Morris CR, Kuypers FA, Larkin S, Vichinsky EP, Styles LA. Patterns of arginine and nitric oxide in patients with sickle cell disease with vaso-occlusive crisis and acute chest syndrome. *J Pediatr Hematol Oncol.* (2000) 22:515–20. doi: 10.1097/00043426-200011000-00009
90. Lopez BL, Kreshak AA, Morris CR, Davis-Moon L, Ballas SK, Ma XL. L-arginine levels are diminished in adult acute vaso-occlusive sickle cell crisis in the emergency department. *Br J Haematol.* (2003) 120:532–4. doi: 10.1046/j.1365-2141.2003.04109.x
91. Morris CR, Kuypers FA, Lavrisha L, Ansari M, Sweeters N, Stewart M, et al. A randomized, placebo-controlled trial of arginine therapy for the treatment of children with sickle cell disease hospitalized with vaso-occlusive pain episodes. *Haematologica.* (2013) 98:1375–82. doi: 10.3324/haematol.2013.086637
92. Solomons C, Hathaway W, Cotton E. L-arginine, the sickling phenomenon, and cystic fibrosis. *Pediatrics.* (1972) 49:933.
93. Morris CR, Kuypers FA, Larkin S, Sweeters N, Simon J, Vichinsky EP, et al. Arginine therapy: a novel strategy to induce nitric oxide production in sickle cell disease. *Br J Haematol.* (2000) 111:498–500. doi: 10.1046/j.1365-2141.2000.02403.x
94. Morris CR. Alterations of the arginine metabolome in sickle cell disease: a growing rationale for arginine therapy. *Hematol Oncol Clin North Am.* (2014) 28:301–21. doi: 10.1016/j.hoc.2013.11.008
95. Bakshi N, Morris CR. The role of the arginine metabolome in pain: implications for sickle cell disease. *J Pain Res.* (2016) 9:167–75. doi: 10.2147/JPR.S55571
96. Cox SE, Ellins EA, Marealle AI, Newton CR, Soka D, Sasi P, et al. Ready-to-use food supplement, with or without arginine and citrulline, with daily chloroquine in Tanzanian children with sickle-cell disease: a double-blind, random order crossover trial. *Lancet Haematol.* (2018) 5:e147–60. doi: 10.1016/S2352-3026(18)30020-6
97. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J.* (1998) 12:929–37. doi: 10.1096/fasebj.12.11.929
98. Mcbrien CN, Menzies-Gow A. The biology of eosinophils and their role in asthma. *Front Med.* (2017) 4:93. doi: 10.3389/fmed.2017.00093
99. Rath M, Muller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: two competing arginine pathways in macrophages. *Front Immunol.* (2014) 5:532. doi: 10.3389/fimmu.2014.00532

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REVIEW
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Respiratory Barrier as a Safeguard and Regulator of Defense Against Influenza A Virus and *Streptococcus pneumoniae*

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The primary function of the respiratory system of gas exchange renders it vulnerable to environmental pathogens that circulate in the air. Physical and cellular barriers of the respiratory tract mucosal surface utilize a variety of strategies to obstruct microbe entry. Physical barrier defenses including the surface fluid replete with antimicrobials, neutralizing immunoglobulins, mucus, and the epithelial cell layer with rapidly beating cilia form a near impenetrable wall that separates the external environment from the internal soft tissue of the host. Resident leukocytes, primarily of the innate immune branch, also maintain airway integrity by constant surveillance and the maintenance of homeostasis through the release of cytokines and growth factors. Unfortunately, pathogens such as influenza virus and *Streptococcus pneumoniae* require hosts for their replication and dissemination, and prey on the respiratory tract as an ideal environment causing severe damage to the host during their invasion. In this review, we outline the host-pathogen interactions during influenza and post-influenza bacterial pneumonia with a focus on inter- and intra-cellular crosstalk important in pulmonary immune responses.

Keywords: co-infection, lung mucosa, epithelial cells, barrier defense, respiratory tract

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INTRODUCTION

The respiratory system is divided into the upper (nasal passages, pharynx, larynx) and lower (trachea, bronchial tree, lungs) components with a cumulative mucosal surface area that exceeds 140 m^2. The entire length of the system, roughly divided into the upper respiratory tract (URT) and the lower respiratory tract (LRT), contains a physical barrier made up of liquid and cell layers (**Figure 1**). The "one/united airway concept" was proposed to underscore the importance of considering changes that occur in the upper and lower airways concomitantly when investigating diseases that affect the respiratory tract like rhinitis and asthma (1). Approximately 2^{23} branches lined with epithelial cells make up the airways (2) within the soft lung tissue that handles ~10,000 L of inhaled air each day, placing this epithelial surface in contact with various noxious and innocuous material including environmentally disseminated viruses and bacteria.

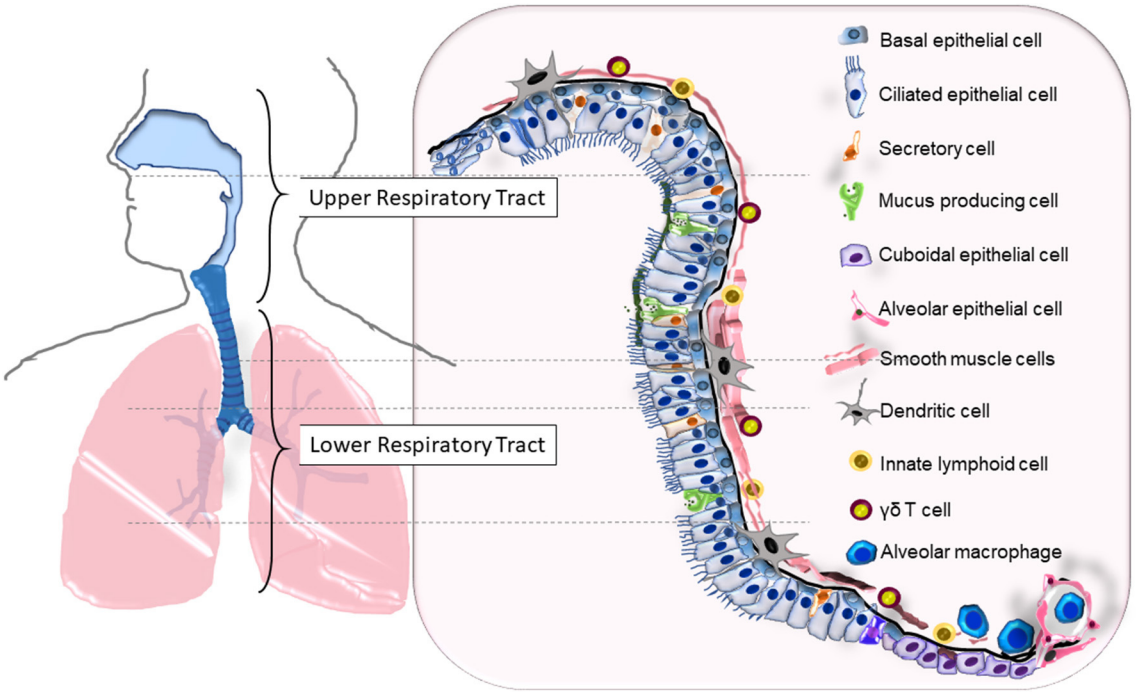

FIGURE 1 | The cellular composition of the upper and lower respiratory tracts that serves as the primary barrier. Epithelial cells (ECs) that span the entire length of the respiratory tract (RT) are lined with basal cells that are attached to the basement membrane. Squamous ECs make up the beginning (nasal) and ends (alveoli) of the RT, ciliated and non-ciliated columnar epithelia makeup the upper RT and the large bronchi, while cuboidal epithelia line the small bronchi and bronchioles. Surface liquid that overlays the ECs consists of mucus secreted from mucus producing cells, airway liquids secreted from secretory cells, neutralizing immunoglobulins, and antimicrobials. Resident leukocytes such as dendritic cells, γδ T cells, and innate lymphoid cells line the mucosa while alveolar macrophages are found in the lower airways and alveoli. The bronchial smooth muscle cells underlying the RT from the basal end provide structural support and elasticity to the airways.

As the primary point of contact, the epithelia of the respiratory system can be considered the regulatory point of immune responses at the respiratory mucosa. Made up of several types of epithelial cells, secretory cells, goblet cells and neuroendocrine cells, the mucosal barrier is multifunctional providing a physical barrier, secretory barrier, and immune defense (2, 3). Uniformity of upper and lower respiratory barrier components ensure multiple levels of filtration of air particles to safeguard the single-layer-thick alveolar spaces (**Figure 1**). When the secretory barrier consisting of mucus, antimicrobial proteins, neutralization antibodies, etc. is breached and epithelial cells come into contact with invading environmental pathogens, these cells become activated and begin communicating with resident leukocytes to participate in the inflammatory cascade and repair mechanisms that follow the invasion. In this review, we will discuss our current understanding of the barrier responses to two major respiratory pathogens, influenza A virus and *Streptococcus pneumoniae* in otherwise healthy hosts.

CROSSTALK WITHIN THE MUCOSAL BARRIER DURING INFLUENZA A VIRUS (IAV) INFECTION

Influenza is an infectious disease caused by influenza viruses belonging to the Orthomyxoviridae family. Of the four genera of influenza viruses, *influenza A* and *influenza B* are known to cause influenza in humans, with the former having a greater propensity to cause severe disease. Between 2010 and 2017, influenza illness in the United States affected 9–34 million persons and killed between 12,000–51,000 annually (4). As a segmented negative sense RNA virus, IAV is predisposed to genetic mutations and gene reassortment, the latter of which is supported by IAV's proclivity for zoonotic infections. Subtypes of IAV are based on the characteristics of surface expressed glycoproteins hemagglutinin (HA) and neuraminidase (NA) which also regulate viral binding and release during its life cycle within host cells. Although IAV has been shown to infect a variety of cell types (5), epithelial cells of both the upper and lower respiratory tracts are its primary target for replication (6, 7).

Mechanisms of Inter-epithelial Crosstalk During IAV Infection

Virus transmission is fundamental to IAV pathogenesis, and while its establishment in a new host is governed by HA molecules, environmental factors also play an important role in the distribution of mucosal secretions (large or small droplets and droplet nuclei) that contain infectious virions, as does human/animal behavior (8). Once IAV reaches the mucosa of the new host, it utilizes numerous strategies to overcome the hostile host environment for successful infection and pathogenesis.

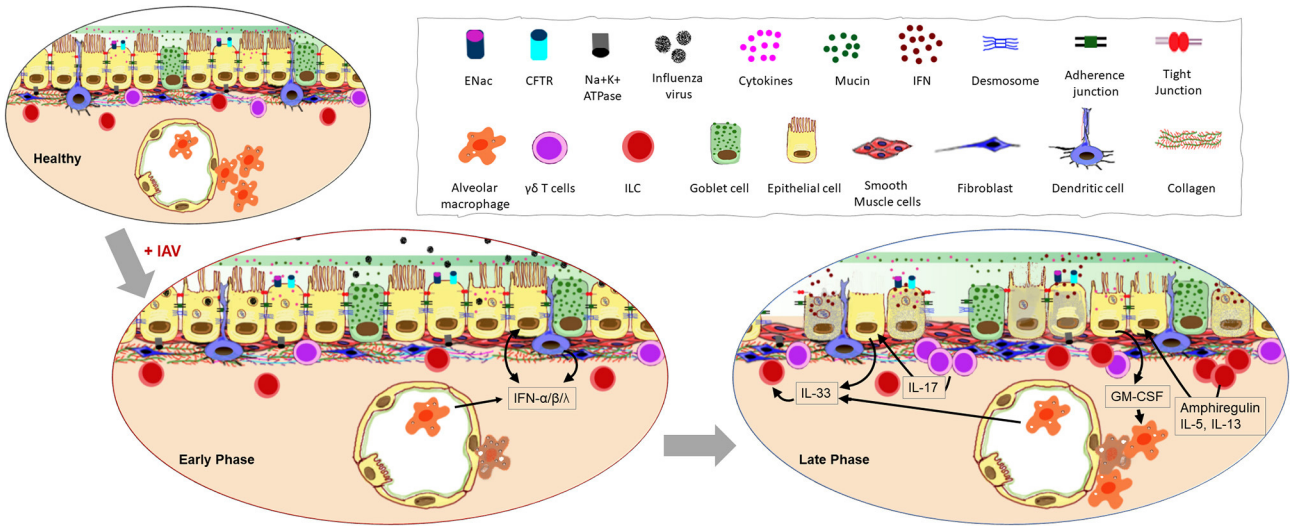

FIGURE 2 | Impact of influenza A virus (IAV) infection on the respiratory barrier. Early infection of epithelial cells that express the sialic acid receptors causes damage to the physical barrier as junctional proteins become compromised during cell death. Increased cellular secretions and loss of cilia slow mucociliary clearance. Resident cells respond to the infection with type I and type III interferon (IFN) production and response. Continuation of these processes lead to the loss of epithelial cells thereby exposing the basement membrane. Morphological changes to the remaining epithelia further compromise the barrier response inducing leakiness in junctional proteins, inflammation, and aberrant repair processes.

The airway epithelium consists of ciliated and non-ciliated cells overlaid by two layers of mucus (**Figure 2**); a bottom layer of less viscous periciliary liquid (PCL) which allows free ciliary movement and a top layer of gel-like mucus layer to which inhaled matter "sticks" (9). The mucus layer is also rich in various highly polymeric mucins (10), antimicrobial peptides (11), neutralizing antibodies (12), etc. that serve as a biochemical barrier to inhibit pathogen penetration (13). Most inhaled particles never gain access to the PCL as they bind to the gel layer and get brushed upward through the mucociliary escalator. Similarly, surfactant proteins that are abundant in lower airway secretions, bind to IAV and enhance viral clearance (14, 15). Virus attachment to the respiratory epithelia will be possible only for those infectious virions that bypass the upper gel barrier and gain access to the sol layer beneath. Viral HA protein facilitates its entry into the cell by binding to sialic acid receptors present on the apical side of epithelial cells. The linkage of sialic acid to the galactose could be either α-2,3 (recognized by avian viruses) or α-2,6 (recognized by human viruses) (16). Since sialic acid receptors are present as a heterogenous mix on epithelial cells in different species (17, 18), it is unclear how IAV selects its specificity and also why binding to sialic acids is usually limited to the URT epithelia (19) when these receptors are available throughout the airway epithelial barrier (17, 19, 20).

The physical manifestation of a barrier is afforded by three types of junctional proteins in the epithelia: tight junctions (TJ), adherens junctions (AJ), and desmosomes (**Figure 2**). Of these, the role of TJs is well-characterized during influenza virus pathogenesis. Three main transmembrane proteins [occludins, claudins, and junctional adhesion molecules (JAM)] are responsible for tightly sealing membranes of adjacent cells within the TJs. Peripheral membrane protein, zonula occludin (ZO), binds to these transmembrane proteins of the TJs to stabilize them in the cytoskeleton and mediate signaling (21–23). IAV infection disrupts the epithelial barrier by causing reduced expression of occludin, claudin-4, and JAM soon after infection (24). The non-structural protein 1 (NS1) of IAV plays a key role in virulence as the PDZ-binding motif (PBM) of NS1 binds to the PDZ domain present in TJ proteins (25) which then destabilizes junctional integrity through the rearrangement of ZO-1 and occludin (25).

During an active infection, the ability for host cells to communicate with one another is essential in order to warn surrounding cells of the threat and to initiate immune responses (**Figure 2**). Various strategies are employed by airway epithelia for this purpose including the release of interferons (IFNs) and other cytokines, antimicrobial peptides, nitric oxide (26), and the more recently described extracellular vesicles (27). The main viral countermeasure to these epithelial responses is the induction of epithelial cell death (28). Infection-induced production of type I IFN is known to trigger the expression of a variety of death-associated molecules in epithelia including Fas, TRAIL receptor, and caspases (29), causing epithelial cell death during the early phase of infection (30). The release of pro-inflammatory cytokines such as IL-1β initiated through inflammasome activation by IAV (31) can lead to pyroptosis (32). Virus-mediated epithelial cell death occurs early after infection with >50% death within 72 h (28), and since cell death increases permeability of the epithelial layer (33), productive infection of the respiratory epithelium is detrimental to barrier potency. Additionally, infected epithelia that present viral antigen-loaded MHC-I molecules are targeted by antigen-specific $CD8^+$ T

cells for destruction (34) which is a major mechanism of viral clearance in the lungs (35). Interestingly however, some ciliated and alveolar epithelial cells downregulate MHC-I and evade $CD8^+$ T cell-mediated death to survive the IAV infection, showcasing a mechanism used by the immune system to reduce host pathology during influenza (36).

Epithelial cells of the lower respiratory tract terminate in the alveoli as squamous type I and type II pneumocytes (**Figure 1**). Since these cells are the primary site for gas exchange, they are bathed in a thin layer of fluid rich in surfactant proteins to reduce the surface tension with the adjoining capillary network of the lungs. One of the important functions of the alveolar epithelium is to remove fluid from the alveolar lumen with the help of ion channels such as amiloride-sensitive epithelial sodium channels (ENaCs), present on the apical surface of the pneumocytes (37, 38) and Na,K-ATPase present at the basolateral membranes (38, 39). Alveolar epithelia are also susceptible to IAV infection which leads to barrier destruction (40) thereby disrupting the intricate balance of ion transport and fluid maintenance causing edema, hypoxemia and pneumonia (38). In fact, IAV matrix protein 2 can inhibit ENaC to cause edema and respiratory insufficiency during influenza (41). Further evidence suggests that there is a cumulative downregulation of ENaC, CFTR, and Na,K-ATPase on epithelial cells during early stages of IAV infection (42). Interestingly, type I IFNs released by epithelia during the late phase of IAV infection, causes the upregulation of TRAIL on alveolar macrophages (AMs) which in turn causes epithelial cell Na,K-ATPase downregulation and edema (43). Alterations to the airway fluid dynamics affect all neighboring cells, infected or not, thereby influencing their functions. Similarly, epithelial cell-derived transforming growth factor (TGF)-β can be activated by viral NA (44) and can reduce the activity of Na,K-ATPase (45).

Epithelial-Resident Leukocyte Crosstalk During Early IAV Infection

The respiratory mucosal barrier contains sentinel cells comprised of AMs, dendritic cells (DCs), γδ T-cells, and innate lymphoid cells (ILCs) which support the antiviral immune response at early and late phases of IAV infection as recently reviewed by us (46). While functional responses in each of these cells during influenza has been investigated, their interactions with the epithelium during an ongoing infection is not fully explored. Indirect communication between the epithelia and these resident leukocytes by means of cytokines may be of greater significance than direct interaction during IAV infection (**Figure 2**). Early release of cytokines from the infected epithelial cells regulate the tone of the immune response through activation of these resident cells.

Epithelial cells become aware of virus invasion mainly through three families of pattern recognition receptors; retinoic acid-inducible gene-like receptor (RLRs) (47), nucleotide-binding domain and leucine-rich-repeat-containing proteins (NLRs) (48) and toll-like receptors (TLR) (49), which, when stimulated, trigger the production of a variety of cytokines and chemokines including IFNs (**Figure 2**). While all three types of IFNs (type I, type II, and type III), are important in antiviral defense against IAV, type I and III are produced by the epithelia (50). The type I IFN receptor (IFNAR) is expressed on a variety of leukocytes in addition to the airway epithelial cells (AECs) allowing them to be responsive to IFNα and IFNβ (51, 52). Since the type III IFN receptor (IFNLR) is predominantly expressed on AECs, they are the most responsive to this cytokine (53). However, the discovery of the IFNLR on neutrophils and DCs suggests a more broad function for this cytokine during respiratory pathogen to protect the barrier response (54). Type II IFN is largely secreted by natural killer (NK) cells (55) and recruited $CD8^+$ T cells (56) in response to IAV infection, and IFNγ signals the local macrophage populations that express the receptor IFNGR to promote phagocytosis, reactive bursts, and the production of proinflammatory cytokines (57).

Immediately following IAV infection, AMs contribute to the first wave of type I and type III IFNs, which are essential for the protection of the LRT from viral progression and dissemination (58, 59) and the virus needs to overcome this wave of IFNs if it is to establish a successful infection (60). Additional pro-inflammatory cytokines produced by AMs in response to IAV including TNFα, IFNγ, IL-1α, IL-1β, and IL-18 also contribute to enhanced viral clearance through the activation of antiviral defense mechanisms in surrounding immune and epithelial cells (61–64). However, a sudden and excessive production of cytokines (as are sometimes triggered by highly virulent strains of IAV), can cause alveolar hemorrhage, pulmonary edema, bronchopneumonia, and acute respiratory distress syndrome through damage to the mucosal epithelia (65–68).

The importance of AMs to all stages of respiratory immunity during influenza was highlighted by Ghoneim et al. wherein a virus-induced depletion of AMs in the lungs left the host vulnerable to invading opportunistic bacteria (69). Mice deficient in AMs are more susceptible to severe influenza due to increased infection of type I pneumocytes and diffuse alveolar damage (70). One critical growth factor for the differentiation, proliferation and activation of AMs is GM-CSF (71–73) which is largely produced by type II alveolar epithelial cells during influenza (74, 75) and mice deficient in GM-CSF ($Csf2^{-/-}$), or its receptor ($Csf2rb^{-/-}$) have increased morbidity and mortality during influenza similar to animals that are devoid of AMs (76) (**Figure 2**). Macrophages maintain environmental homeostasis through the removal of apoptotic cells and debris. As such, AMs are also important during the tissue repair phase that follows an active infection by IAV through the efferocytosis of dying epithelia and neutrophils (77). Epithelial cell proliferation and repair after influenza is promoted by AM products such as hepatocyte growth factor (78), TGF-α (79), and TGF-β (80).

Epithelial cell TLRs can guide the adaptive immune responses to IAV through molding the activation of DCs (81). Serving as a bridge between innate and adaptive immunity, DCs intersperse the epithelial barrier to sample inhaled air through dendrites. The majority of reports investigating the function of DCs during influenza have focused on their interaction with immune effectors that are recruited during the late phase of the immune response. Therefore, very little is known about the interaction of DCs with mucosal resident cells. Plasmacytoid DCs (pDCs) are known to produce high amounts of type I IFN during

IAV infection through the TLR7/MyD88 pathway (82, 83). Human primary bronchial epithelial cells enhanced type I IFN production and the upregulation of IFN response genes in pDCs when co-cultured (84) showcasing crosstalk between the structural cells and local immune cells through cytokines. Similar crosstalk occurs between pDCs and AMs wherein pDCs control the number and cytokine profile of the AMs (85).

The airway epithelial barrier also contains a small percentage of γδ T cells that are considered to function in barrier defense. In murine models of IAV infection, γδ T cells increased during the late phase of disease (86), and produced immunoregulatory cytokines IL-2, IL-4, and IFN-γ (87). However, depletion of γδ T cells did not have any impact on viral clearance or IFN-γ production in a neonatal model of IAV infection in mice (88). Highly pathogenic H5N1 IAV can directly activate γδ T cells inducing the upregulation of CD69 expression and enhancing IFN-γ secretion (89). Similarly, γδ T cells produce IL-17A in response to IAV that triggers the release of IL-33 by AECs which in turn mediates ILC2s and Treg cells (88). These data indicate that γδ T cells are critical in maintenance of lung homeostasis and tissue repair during the viral clearance phase.

Additional protection and regulation to the mucosal barrier is provided by ILCs that are characterized by the absence of both T- and B-cell receptors. Like T-cells, ILCs have also been categorized according to cytokine production profile (90), of which ILC2 is the most investigated subset in the context of influenza. ILC2 is classically known to produce IL-5 and IL-13 in response to epithelial cytokines IL-25, IL-33, and TSLP (91). Infection of wild type as well as *Rag1*$^{-/-}$ mice with IAV led to ILC accumulation in the lung (92) although there is no direct evidence that IAV-mediated ILC accumulation is dependent on AEC-derived cytokines. Furthermore, it has been reported that IAV infection induced AMs to produce IL-33 which promotes IL-13-dependent airway hyperreactivity (93). Its role in tissue homeostasis is implied in studies wherein ILC depletion was shown to impact lung function, epithelial integrity and tissue remodeling (92). The high amounts of type I and type II IFNs produced during the early phase of IAV infection have been shown to inhibit ILC2 function and proliferation (94). Conversely, IFN-γ deficiency leads to host protection through increased production of IL-5 and amphiregulin by ILC2 (94). Both NKT-cells and AMs have also been shown to produce IL-33 in response to IAV signaling ILCs to produce IL-5 (95), and increased levels of IL-5 during the viral clearance phase may help recruit eosinophils to the airway mucosal barrier (95) which can enhance cellular immune responses (96) and perhaps necessary for tissue repair (97).

OPPORTUNISTIC *STREPTOCOCCUS PNEUMONIAE* INFECTIONS

In some instances, virus-induced inflammation and dysregulated communication with the lung framework can leave the host vulnerable to secondary bacterial infections. This is exemplified by the increased susceptibility of an individual with IAV infection to the acquisition of *Streptococcus pneumoniae* (pneumococcus) (98, 99), resulting in a convergence that provokes far greater morbidity and mortality than infection with either pathogen alone (100, 101). The host remains susceptible to *S. pneumoniae* infection even after the virus itself has been cleared (102), suggesting that a compromised immune milieu and structural barrier contribute to increased bacterial pathogenesis. Although IAV can enhance *S. pneumoniae* pathogenesis directly, for instance by exposing cryptic binding sites through epithelial damage (103) or by liberating sialic acid and sialylated mucin that can be catabolized by *S. pneumoniae* (104), influenza virus can also modify interactions between the epithelium and inflammatory components, creating an environment that can be subverted by the pneumococcus.

Impact of Influenza-Mediated Alterations to Epithelial Crosstalk on Pneumococcal Infection

Surface expressed TLRs on epithelial cells can sense *S. pneumoniae* by recognition of numerous bacterial components, including TLR2 agonists type 1 pilus, peptidoglycan, lipoteichoic acid and bacterial lipoproteins, and the TLR4 agonist pneumolysin (105–110). Although IAV is not directly recognized by either TLR2 or TLR4, the regulation and activation of TLRs during influenza has been shown to enhance susceptibility to secondary bacterial infection. Increased TLR2 signaling during IAV/*S. pneumoniae* co-infection results in heightened production of IL-1β, augmenting inflammation and morbidity (111). Additionally, IAV infection positively regulates TLR3 on pulmonary epithelial cells (112), which recognizes double-stranded RNA and impairs the clearance of *S. pneumoniae* from the lungs following activation by poly I:C (113). Stimulation of TLR3 also leads to early production of IFNβ by AECs (114), contributing to the type I IFN response elicited during influenza, which is a key factor in host susceptibility to secondary pneumococcal infection, as discussed later in this review.

A crucial initial step in pneumococcal pathogenesis is bacterial adherence to the respiratory epithelium. Initially, *S. pneumoniae* establishes itself in the host by colonizing the nasopharynx, which is considered a necessary precursor to pneumococcal disease (115). IAV-induced epithelial cell death may expose the basement membrane to which *S. pneumoniae* can bind to and use as a shortcut to the bloodstream (116, 117). Pneumococcus surface proteins including PavA and PavB, PfbA and PfbB, PepO and pilus subunit RgrA all have the ability to bind basement membrane components fibronectin, laminin, and collagen (118–122).

From the URT, pneumococci can migrate to the lungs and establish symptomatic infections such as pneumonia and bacteremia (123). In a healthy individual, most wayward pneumococci in the airways are expelled by the mucociliary escalator before reaching the LRT (13). However, a recent IAV infection reduces the velocity of ciliary beating and causes death of ciliated tracheal cells, providing pneumococci an opportunity to bind to the epithelium observed as early as 2 h after challenge in mice (116, 124). In addition to increased access, IAV regulates binding receptors for *S. pneumoniae* on the epithelial surface (**Figure 3**). Numerous viruses, including IAV, can increase the prevalence of host platelet activating factor receptor (PAFr), which binds phosphorylcholine moieties in the

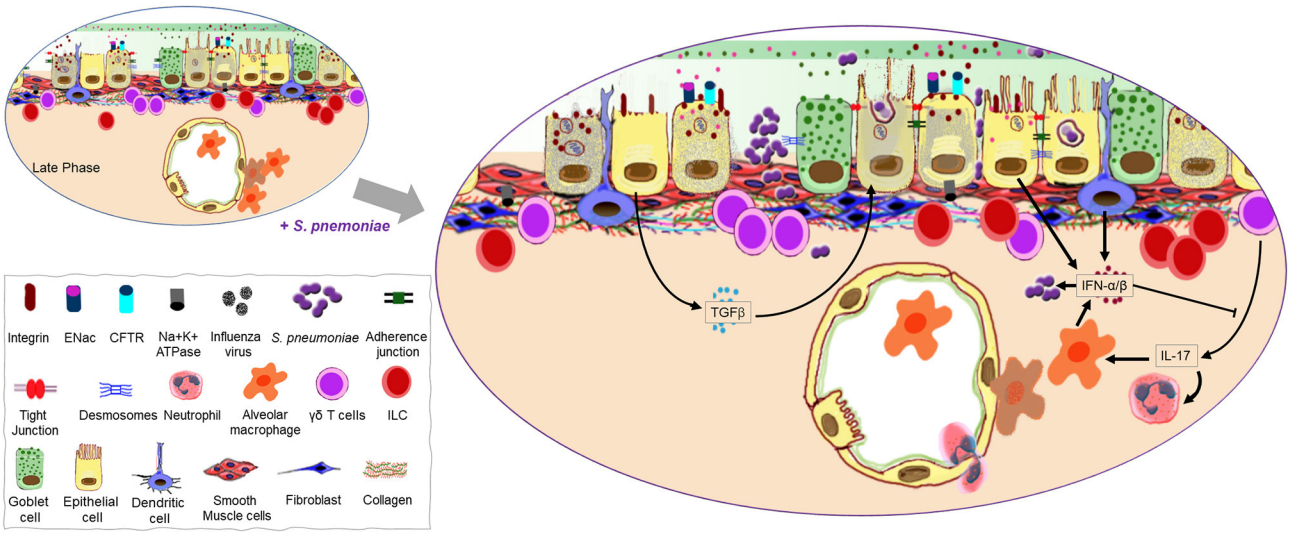

FIGURE 3 | Continuation of the mucosal damage cascade permitting opportunistic infection. A host recovering from virus-induced damage to the lung mucosa is highly susceptible to *Streptococcus pneumoniae* infection possibly due to exposed binding partners on the host cells as well as an open barrier (gate) due to significant loss of epithelial cells. A second wave of type I IFNs may help promote bacterial colonization as it negates the positive influence interleukin (IL)-17 has on the recruitment of phagocytes. Transforming growth factor (TGF)-β produced during the late phase of influenza as a repair mechanism may also promote bacterial adherence to the mucosal surface.

pneumococcal cell wall (125–127). The activation of latent TGFβ by IAV NA present in the airways during influenza primes the epithelium for bacterial adherence by stimulating cells to upregulate bacterial receptors such as integrins (128). In the absence of TGFβ signaling, IAV-infected epithelial cells lose their increased vulnerability to pneumococcal colonization (129).

Although invasive disease is arguably not a favorable outcome for an extracellular respiratory bacterium like *S. pneumoniae* where optimal infection doesn't extend past the airways, prior influenza can promote its migration from the lungs to the bloodstream (130, 131). Under homeostatic conditions, however, the strict maintenance of TJs between cells in the epithelial and endothelial barriers prevents pneumococcal migration by physically restricting the movement of bacteria between cells and masking receptors. The disruption of TJs during influenza permits *S. pneumoniae* to migrate from the airways to the bloodstream. Pneumococci can also enter the blood from the airways by transmigrating through epithelial and endothelial cells (132–134). Following the binding of cell wall phosphorylcholine moieties to host PAFr, pneumococci can be internalized when the receptor is recycled (132, 135, 136) (**Figure 3**). Alternatively, invasion may be facilitated by the interaction of polymeric immunoglobulin receptor (pIgR) with pneumococcal CbpA and RrgA pillus subunit, the latter of which is involved in pIgR-mediated invasion of the brain microvascular endothelium, a mechanism that may also be applicable to nasopharyngeal epithelial cells (134, 137, 138). While levels of epithelial surface activation markers associated with bacterial defense EpCAM, IL-22Rα1, HLA-DR, CD40, CD54, and CD107a are not altered during pneumococcal colonization of the URT, bacterial uptake by pharyngeal epithelial cells is associated with strain-dependent changes to the transcriptome (139). While invasive strains like TIGR4 induce the upregulation of more genes compared to strains typically associated with carriage, the regulated pathways common to both colonizing and disease-causing *S. pneumoniae* strains are those associated with the innate immune response, such as NFκB and MAP kinase activation, toll receptor and cytokine signaling (139, 140) and correspond to hypersecretion of IL-6, and IL-8 (139). Furthermore, the most profound changes to the transcriptome following pneumococcal infection coincide with clearance of colonizing bacteria in an experimental human pneumococcal carriage model (139), suggesting that transmigration to the bloodstream may be an unintentional consequence during the innate resolution of pneumococcal carriage.

Epithelial-Leukocyte Crosstalk During Pneumococcal Infection

During pneumococcal infection, IL-17 is produced by γδ T-cells (predominant source of IL-17 during pneumococcal pneumonia) and later by T_H17 $CD4^+$ T-cells. IL-17 and a T_H17 response at the mucosal epithelium participate in pneumococcal clearance in the nasopharynx and lungs by recruiting monocytes and neutrophils, and offer protection against reinfection (141–144). However, the induction of type I IFN during influenza inhibits T_H17 defense during secondary pneumococcal infection and suppresses the expression of IL-17 by pulmonary γδ T-cells, resulting in impaired recruitment of these phagocytes (129, 141, 145) (**Figure 3**). Furthermore, type I IFN also reduces the production of CCL2, leading to fewer recruited macrophages in the airways during a concurrent pneumococcal infection and increased colonization of the URT (146). Mice recovering from influenza are also unable to mount an effective KC and MIP-2 response following infection with *S. pneumoniae*,

which stunts neutrophil recruitment (147). Macrophages and neutrophils are major components of the innate cell response against extracellular bacteria, controlling bacterial infection by phagocytosis, direct killing, and recruitment/activation of other inflammatory cells (148, 149). Early induction of type I IFN by AMs, DCs and AECs is of fundamental importance to antiviral immunity during influenza (150–154), but, can be detrimental during pneumococcal infection by disrupting the recruitment of cells that are important in controlling bacterial outgrowth (147). Accordingly, mice lacking IFNAR signaling have fewer bacteria in the lungs, lower levels of bacteremia and a better outcome following IAV-*S. pneumoniae* co-infection (147).

Mononuclear cells and neutrophils that are recruited to the airways during influenza contribute to damage of the respiratory epithelium. Recruited macrophages cause significant TRAIL-dependent apoptosis and leakage through the AECs (155). The increase in recruited macrophages is paralleled by a loss of AMs, hampering the host's ability to restrict a secondary pneumococcal infection which rapidly progresses to pneumonia (69). Neutrophil extracellular traps released in response to IAV are potentially damaging to the epithelium and are ineffective against secondary pneumococcal infection (156).

Pneumococci that enter the post-influenza RT not only are presented with an environment harboring reduced numbers of resident macrophages (69), but also encounter lymphocytes that are in a state of immunological exhaustion and unable to appropriately respond to the infection (157). Type I IFN produced by epithelial cells and others during IAV infection causes polyclonal activation of T- and B-cells which, despite the cells returning to a "baseline" state several days after infection, prevents activation by subsequent exposure to type I IFN. This state of exhaustion lasts for several days, during which the host is particularly vulnerable to secondary infections (157).

IAV infection is not solely good news for *S. pneumoniae*, with the host response to the viral infection also promoting protection against secondary bacterial infection in some instances. For example, while type I IFN disrupts cell recruitment during pneumococcal infection, its induction also restricts *S. pneumoniae* pathogenesis by up-regulating the expression of TJ proteins (ZO-1, claudin 4, claudin 5, claudin 18, and E-cadherin) and decreasing PAFr levels in epithelial and endothelial lung cells (158). Adenosine is present in the extracellular environment during stress and inflammation, and has been shown to be released by respiratory epithelial cells amongst others (159). During IAV infection of mice, ATP levels in the airways are elevated due to increased *de novo* synthesis and poor alveolar fluid clearance (160, 161), which can be sequentially hydrolyzed to generate adenosine (162, 163). The activation of A1-adenosine receptors by extracellular adenosine decreases expression of the PAFr on the lung epithelium (164) and promotes the recruitment of neutrophils, monocytes and lymphocytes during influenza (161), which contribute to protection against secondary infection with *S. pneumoniae* (164, 165).

IL-22 is produced during influenza by pulmonary NK cells (166) and RORγ$^+$ αβ, and γδ T cells (167) and binds IL-22Rα1 on AECs and endothelial cells (168–170), an interaction that can be antagonized by its soluble form, IL-22BP (171, 172). Human endothelial cells respond to IL-22 by increasing production of CCL2 and CCL20 (169), which are chemoattractants for cells involved in the resolution of bacterial infection such as monocytes, dendritic cells, and lymphocytes. IL-22 is critical to epithelial repair following infection with A/PR/8/1934 (173), and in its absence, mice sustain significantly higher lung injury and loss of airway epithelial integrity during sublethal IAV infection followed by *S. pneumoniae* co-infection (167). Administration of exogenous IL-22 to mice with influenza causes the upregulation of genes encoding proteins involved in cell-cell adhesion such as *Cldn24* and *Pcdh15* (encoding claudin 24 and protocadherin 15, respectively) in the lungs, and reduces systemic dissemination of *S. pneumoniae* during secondary bacterial infection (174). Interestingly, although mice lacking the IL-22 decoy IL-22BP have significantly reduced bacterial outgrowth in the lungs during co-infection, dissemination is unaffected (175).

Impact of IAV-Pneumococci Co-infection on Immune Defense at the Respiratory Barrier

The mucoepithelial barrier is one of the most important host respiratory defenses against encroaching bacterial pathogens. However, local damage and the inflammatory milieu occasioned during influenza can compromise the efficacy of the physical barrier and its interactions with other components of the inflammatory repertoire. It is interesting that many aspects of the post-influenza lung microenvironment known to exacerbate pneumococcal infection, are also targeted by *S. pneumoniae* in order to avoid immune clearance and establish infection. The pneumococcal cytotoxin, pneumolysin, disrupts TJs and reduces cilia organization and prevalence with negligible impact on ciliary beating (117, 176). In addition, *S. pneumoniae* causes cell damage and loss of planar epithelial architecture at the mucosal surface (117, 176). Pneumococci are able to evade neutrophils by expressing a polysaccharide capsule that also physically reduces deposition of complement and antibodies (177, 178), and by molecular mimicry wherein bacterial phosphorylcholine moieties bind PAFr, preventing PAF from initiating neutrophil phagocytosis and bactericidal activities (135, 179–181). In this respect, IAV is a perfect partner for *S. pneumoniae*, providing it with a compromised mucosal epithelial barrier that is permissive for it to establish infection, while at the same time dampening antibacterial host responses.

In reported *in vivo* models of co-infection, animals are commonly challenged with *S. pneumoniae* 3–7 days after IAV, corresponding to the most pronounced changes to morbidity and mortality (100, 182). However, influenza still predisposes mice to *S. pneumoniae* infection at later times of challenge, and clinically there are positive correlations between influenza and severe pneumococcal pneumonia with up to 4 weeks separating the two infectious agents, suggesting the IAV imparts long term effects in the host (183, 184). This is predictable, as IAV causes profound destruction of type II pneumocytes causing impaired regeneration after disease resolution, and also infects EpCam^{high}CD24^{low}integrin(α6β4)^{high}CD200$^+$ epithelial stem/progenitor cells thereby reducing renewal

of cells at the respiratory barrier (185, 186). Influenza that precedes a pneumococcal infection may also affect the immune response during reinfection with *S. pneumoniae*. T_H17 immunity promotes accelerated bacterial clearance in the URT following a secondary infection with *S. pneumoniae* (144). Considering that type I IFN inhibits T_H17 activation (145) and thus the generation of memory cells, influenza may prevent T_H17-mediated protection against subsequent infections with the same or heterologous pneumococcal serotypes (144, 187, 188).

TARGETING IAV AND *S. PNEUMONIAE* AT THE MUCOSAL BARRIER

Clinical influenza disease commonly manifests as an uncomplicated upper respiratory infection with fever, malaise, headache, cough, and myalgias. Symptomatic treatment consists of over the counter anti-inflammatory and pain medications. The mainstay of current influenza antiviral medications are the NA inhibitors: oseltamivir, zanamivir, and peramivir. The sialic acid cleavage activity of NA is required for release of virions from infected epithelial cells and also facilitates migration through the epithelial mucin layer (189, 190). Benefit from NA inhibitors is primarily restricted to uncomplicated disease where treatment is instituted within the first 48 h of symptoms with a modest reduction in duration of illness (191, 192). A recently approved antiviral, baloxavir marboxil, acts as a selective inhibitor of influenza cap endonuclease (193). Similar to NA inhibitors, baloxavir marboxil has proven benefit in early treatment of uncomplicated influenza cases (193). Additionally, there was an observation of rapid development of resistance in outpatient trials raising concern for its long-term usage (194). Nitazoxanide is an antiprotozoal drug used to treat *Cryptosporidium* and *Giardia* infections. *In vitro* data demonstrate antiviral activity against influenza A and B strains (195, 196). It acts by inhibiting influenza HA trafficking through the epithelial endoplasmic reticulum and Golgi apparatus and preventing maturation by blocking HA terminal glycosylation (197). A phase 2b/3 trial of nitazoxanide in uncomplicated influenza was well-tolerated and showed reduced symptoms and viral loads (198). A randomized placebo-controlled phase III trial was completed in March 2019 and remains currently unpublished (196). If approved, this drug, through its primary targeting of the virus, will also affect the local immune responses to the virus initiated by the respiratory epithelial cells as detailed above.

Severe influenza can lead to respiratory failure and acute respiratory distress syndrome (ARDS) which has a mortality rate of 27–45% (199). Epithelial barrier disruption and pronounced pulmonary edema are hallmarks of ARDS and since there are no directed treatments that counteract these effects at present, and care remains predominantly supportive with mechanical ventilation, secretion clearance, and extracorporeal membrane oxygenation when necessary. As such, there is an evident need for additional influenza therapies, particularly for hospitalized patients with severe disease. As the primary site of infection, the respiratory epithelium represents an important area of focus for disease treatment. Fludase is a recombinant sialidase that cleaves the sialic acid receptor for IAV on AECs preventing viral entry into cells (200). Pre-clinical trials show broad *in vitro* influenza antiviral activity and protective effects in animal models (200, 201). In phase I and II trials, Fludase was well-tolerated and led to decreased viral load and shedding (202, 203). However, Fludase liberation of sialic acid raises interesting questions regarding *S. pneumoniae* co-infection as sialic acid has been shown to facilitate its colonization during IAV infection (104). *S. pneumoniae* infection of Fludase-treated mice with influenza did not alter bacterial colonization or mortality (204). The effects of continued Fludase treatment with concurrent *S. pneumoniae* colonization/infection are not fully elucidated.

As detailed above, late influenza infection leads to significant TRAIL-mediated apoptosis contributing to continued pathogenesis even as the viral load subsides. Pre-clinical data show that IAV-infected mice treated with anti-TRAIL sera had attenuated lung epithelial apoptosis, lung leakage and increased survival after IAV infection (155). Moreover, anti-TRAIL treatment was able to reduce bacterial load and protect against *S. pneumoniae* coinfection (205). Alternatively, Bcl-2 inhibitors which were developed to treat certain cancers are anti-apoptotic and have been suggested as potential treatment for influenza. *In vitro* data showed decreased viral replication and spread due to these agents (206, 207). Maintenance of the epithelial barrier and induction of antiviral mechanisms involve IFN signaling during influenza. Interferon-lambda treatment in mice leads to reduced viral load and improved survival without inducing a pro-inflammatory cytokine release (208). In another study, IFNλ treatment was able to prevent viral spread from the nasal passages to the lungs and confer resistance to IAV in mice for up to 6 days (209). However, in a model of IAV and methicillin resistant *Staphylococcus aureus*/Streptococcal superinfection, increased INFλ in IAV-infected mice lead to increased bacterial burden due to decreased bacterial uptake by neutrophils (210). It remains to be seen if any of these potential therapies will prove beneficial in treating human influenza.

Corticosteroids are routinely used for their anti-inflammatory properties in chronic conditions such as asthma and chronic obstructive pulmonary disease (COPD). Because influenza and ARDS manifests with a severe pro-inflammatory response, appropriately blunting that response may be beneficial during clinical illness. Additionally, corticosteroids have direct effects on the respiratory epithelium that may be protective. *In vitro* steroid treatment led to decreased epithelial permeability through the action of claudin-8 and occludin recruitment to TJs (211). However, corticosteroids were not found to be of benefit to patients during IAV infections (212, 213). A Cochrane review and another meta-analysis highlighted significant heterogeneity in published studies and did not show benefit but instead had a trend toward increased mortality (214, 215), and therefore, their efficacy as a therapy during influenza remains controversial.

S. pneumoniae is typically susceptible to many commonly used β-lactam antibiotics like penicillin. However, their resistance to multiple antibiotic classes is growing (216). Current vaccines for pneumococcal disease include 13-valent pneumococcal conjugate and 23-valent polysaccharide vaccines (217). Despite

broad immunization practices however, invasive pneumococcal disease remains common with high morbidity and mortality. Similar to influenza, targeting the microbe-host interaction could provide novel treatment strategies for pneumococcal disease. One example is *S*-carboxymethylcysteine (S-CMC) which is a mucolytic agent used in COPD which has been shown to inhibit adherence to both pharyngeal and alveolar epithelia (218, 219).

CONCLUSION

As a mucosal organ system with a large surface area and unremitting exposure to the external environment, protection of the respiratory barrier is of utmost importance to human health. Since barrier breach is a necessary first step for environmental pathogens to gain a foothold in the RT, maintaining the integrity of the mucosal barrier is a focus point of host defense and redundant mechanisms/pathways may be utilized to ensure its subsistence. Herein, we reviewed findings that pertain to crosstalk between structural cells and local leukocytes that play a role in immune defenses against IAV and *S. pneumoniae*. Although not covered here, the endogenous microbiome is likely to play an important role as a mediator of pulmonary immune responses during infection. The crosstalk at the interface of microbial pathogens and human host epithelium presents multiple opportunities for the development of clinically relevant therapies. Targeting host mechanisms may provide less opportunities for the emergence of pathogen resistance, and if used in combination with direct antimicrobial medications may prove superior to monotherapy.

As these pathogens evolve, it is imperative that additional information is garnered on interactions that occur between host cells and these agents as well as cell-cell crosstalk in order to discover more effective therapeutic strategies to overcome infection when the mucosal barrier is breached. It is also of importance to determine how these primary mechanisms relate to an individual with underlying chronic lung disease such as asthma, COPD, and interstitial pulmonary fibrosis, as the immune and structural architecture as well as the microbiome of these hosts are fundamentally different which likely leads to alterations in the defense mechanisms during respiratory infections.

AUTHOR CONTRIBUTIONS

All authors participated in writing and editing the paper and approved the final submission. Figures were drawn by AS (**Figure 1**) and MT (**Figures 2**, **3**).

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REFERENCES

1. Giavina-Bianchi P, Aun MV, Takejima P, Kalil J, Agondi RC. United airway disease: current perspectives. *J Asthma Allergy.* (2016) 9:93–100. doi: 10.2147/JAA.S81541
2. Crystal RG, Randell SH, Engelhardt JF, Voynow J, Sunday ME. Airway epithelial cells: current concepts and challenges. *Proc Am Thorac Soc.* (2008) 5:772–7. doi: 10.1513/pats.200805-041HR
3. Ganesan S, Comstock AT, Sajjan US. Barrier function of airway tract epithelium. *Tissue Barriers.* (2013) 1:e24997. doi: 10.4161/tisb.24997
4. CDC. *Influenza (Flu)*. CDC (2017).
5. Inglot A, Davenport FM. Studies on the role of leukocytes in infection with influenza virus. *J Immunol.* (1962) 88:55–65.
6. Chen ZG, Wang ZN, Yan Y, Liu J, He TT, Thong KT, et al. Upregulation of cell-surface mucin MUC15 in human nasal epithelial cells upon influenza A virus infection. *BMC Infect Dis.* (2019) 19:622. doi: 10.1186/s12879-019-4213-y
7. Slepushkin VA, Staber PD, Wang G, Mccray PB Jr, Davidson BL. Infection of human airway epithelia with H1N1, H2N2, and H3N2 influenza A virus strains. *Mol Ther.* (2001) 3:395–402. doi: 10.1006/mthe.2001.0277
8. Killingley B, Nguyen-Van-Tam J. Routes of influenza transmission. *Influenza Other Respir Viruses.* (2013) 7:42–51. doi: 10.1111/irv.12080
9. Rubin BK. Physiology of airway mucus clearance. *Respir Care.* (2002) 47:761–8.
10. Zanin M, Baviskar P, Webster R, Webby R. The interaction between respiratory pathogens and mucus. *Cell Host Microbe.* (2016) 19:159–68. doi: 10.1016/j.chom.2016.01.001
11. Gaudreault E, Gosselin J. Leukotriene B4 induces release of antimicrobial peptides in lungs of virally infected mice. *J Immunol.* (2008) 180:6211–21. doi: 10.4049/jimmunol.180.9.6211
12. Wang YY, Harit D, Subramani DB, Arora H, Kumar PA, Lai SK. Influenza-binding antibodies immobilise influenza viruses in fresh human airway mucus. *Eur Respir J.* (2017) 49:1601709. doi: 10.1183/13993003.01709-2016
13. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med.* (2010) 363:2233–47. doi: 10.1056/NEJMra0910061
14. Kerr MH, Paton JY. Surfactant protein levels in severe respiratory syncytial virus infection. *Am J Respir Crit Care Med.* (1999) 159:1115–8. doi: 10.1164/ajrccm.159.4.9709065
15. Levine AM, Whitsett JA, Hartshorn KL, Crouch EC, Korfhagen TR. Surfactant protein D enhances clearance of influenza A virus from the lung *in vivo*. *J Immunol.* (2001) 167:5868–73. doi: 10.4049/jimmunol.167.10.5868
16. Van Riel D, Munster VJ, De Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, et al. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *Am J Pathol.* (2007) 171:1215–23. doi: 10.2353/ajpath.2007.070248
17. Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, et al. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J Virol.* (2006) 80:7469–80. doi: 10.1128/JVI.02677-05
18. Thompson CI, Barclay WS, Zambon MC, Pickles RJ. Infection of human airway epithelium by human and avian strains of influenza A virus. *J Virol.* (2006) 80:8060–8. doi: 10.1128/JVI.00384-06
19. Gagneux P, Cheriyan M, Hurtado-Ziola N, Van Der Linden ECB, Anderson D, Mcclure H, et al. Human-specific regulation of α2–6-linked sialic acids. *J Biol Chem.* (2003) 278:48245–50. doi: 10.1074/jbc.M309813200
20. Baum LG, Paulson JC. Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. *Acta Histochem Suppl.* (1990) 40:35–8.
21. Wittekindt OH. Tight junctions in pulmonary epithelia during lung inflammation. *Pflugers Arch.* (2017) 469:135–47. doi: 10.1007/s00424-016-1917-3

22. Van Itallie CM, Anderson JM. Architecture of tight junctions and principles of molecular composition. *Semin Cell Dev Biol.* (2014) 36:157–65. doi: 10.1016/j.semcdb.2014.08.011
23. Garcia MA, Nelson WJ, Chavez N. Cell–cell junctions organize structural and signaling networks. *Cold Spring Harb Perspect Biol.* (2018) 10:a029181. doi: 10.1101/cshperspect.a029181
24. Short KR, Kasper J, Van Der Aa S, Andeweg AC, Zaaraoui-Boutahar F, Goeijenbier M, et al. Influenza virus damages the alveolar barrier by disrupting epithelial cell tight junctions. *Eur Respir J.* (2016) 47:954–66. doi: 10.1183/13993003.01282-2015
25. Golebiewski L, Liu H, Javier RT, Rice AP. The avian influenza virus NS1 ESEV PDZ binding motif associates with Dlg1 and Scribble to disrupt cellular tight junctions. *J Virol.* (2011) 85:10639–48. doi: 10.1128/JVI.05070-11
26. Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses. *Clin Microbiol Rev.* (2011) 24:210–29. doi: 10.1128/CMR.00014-10
27. Lee H, Abston E, Zhang D, Rai A, Jin Y. Extracellular vesicle: an emerging mediator of intracellular crosstalk in lung inflammation and injury. *Front Immunol.* (2018) 9:924. doi: 10.3389/fimmu.2018.00924
28. Lee S, Hirohama M, Noguchi M, Nagata K, Kawaguchi A. Influenza A virus infection triggers pyroptosis and apoptosis of respiratory epithelial cells through the type I interferon signaling pathway in a mutually exclusive manner. *J Virol.* (2018) 92:e00396-18. doi: 10.1128/JVI.00396-18
29. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, et al. *Apoptosis* and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis.* (2003) 8:237–49. doi: 10.1023/A:1023668705040
30. Fujikura D, Miyazaki T. Programmed cell death in the pathogenesis of influenza. *Int J Mol Sci.* (2018) 19:E2065. doi: 10.3390/ijms19072065
31. Chen IY, Ichinohe T. Response of host inflammasomes to viral infection. *Trends Microbiol.* (2015) 23:55–63. doi: 10.1016/j.tim.2014.09.007
32. Man SM, Karki R, Kanneganti TD. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol Rev.* (2017) 277:61–75. doi: 10.1111/imr.12534
33. Armstrong SM, Wang C, Tigdi J, Si X, Dumpit C, Charles S, et al. Influenza infects lung microvascular endothelium leading to microvascular leak: role of apoptosis and claudin-5. *PLoS ONE.* (2012) 7:e47323. doi: 10.1371/journal.pone.0047323
34. Nguyen HH, Boyaka PN, Moldoveanu Z, Novak MJ, Kiyono H, Mcghee JR, et al. Influenza virus-infected epithelial cells present viral antigens to antigen-specific $CD8^+$ cytotoxic T lymphocytes. *J Virol.* (1998) 72:4534–6. doi: 10.1128/JVI.72.5.4534-4536.1998
35. Sun J, Braciale TJ. Role of T cell immunity in recovery from influenza virus infection. *Curr Opin Virol.* (2013) 3:425–9. doi: 10.1016/j.coviro.2013.05.001
36. Fiege JK, Stone IA, Dumm RE, Waring BM, Fife BT, Agudo J, et al. Long-term surviving influenza infected cells evade $CD8^+$ T cell mediated clearance. *PLoS Pathog.* (2019) 15:e1008077. doi: 10.1371/journal.ppat.1008077
37. Matalon S, Bartoszewski R, Collawn JF. Role of epithelial sodium channels in the regulation of lung fluid homeostasis. *Am J Physiol Lung Cell Mol Physiol.* (2015) 309:L1229–38. doi: 10.1152/ajplung.00319.2015
38. Matalon S, O'brodovich H. Sodium channels in alveolar epithelial cells: molecular characterization, biophysical properties, and physiological significance. *Annu Rev Physiol.* (1999) 61:627–61. doi: 10.1146/annurev.physiol.61.1.627
39. Looney MR, Sartori C, Chakraborty S, James PF, Lingrel JB, Matthay MA. Decreased expression of both the α1-and α2-subunits of the Na-K-ATPase reduces maximal alveolar epithelial fluid clearance. *Am J Physiol Lung Cell Mol Physiol.* (2005) 289:L104–10. doi: 10.1152/ajplung.00464.2004
40. Travanty E, Zhou B, Zhang HB, Di YP, Alcorn JF, Wentworth DE, et al. Differential susceptibilities of human lung primary cells to H1N1 influenza viruses. *J Virol.* (2015) 89:11935–44. doi: 10.1128/JVI.01792-15
41. Lazrak A, Iles KE, Liu G, Noah DL, Noah JW, Matalon SJTFJ. Influenza virus M2 protein inhibits epithelial sodium channels by increasing reactive oxygen species. *FASEB J.* (2009) 23:3829–42. doi: 10.1096/fj.09-135590
42. Brand JD, Lazrak A, Trombley JE, Shei RJ, Adewale AT, Tipper JL, et al. Influenza-mediated reduction of lung epithelial ion channel activity leads to dysregulated pulmonary fluid homeostasis. *JCI Insight.* (2018) 3:123467. doi: 10.1172/jci.insight.123467
43. Peteranderl C, Morales-Nebreda L, Selvakumar B, Lecuona E, Vadasz I, Morty RE, et al. Macrophage-epithelial paracrine crosstalk inhibits lung edema clearance during influenza infection. *J Clin Invest.* (2016) 126:1566–80. doi: 10.1172/JCI83931
44. Carlson CM, Turpin EA, Moser LA, O'brien KB, Cline TD, Jones JC, et al. Transforming growth factor-beta: activation by neuraminidase and role in highly pathogenic H5N1 influenza pathogenesis. *PLoS Pathog.* (2010) 6:e1001136. doi: 10.1371/journal.ppat.1001136
45. Wujak LA, Becker S, Seeger W, Morty RE. TGF-β regulates Na, K-ATPase activity by changing the regulatory subunit stoichiometry of the Na, K-ATPase complex. *FASEB J.* (2011) 25.
46. Lamichhane PP, Samarasinghe AE. The role of innate leukocytes during influenza virus infection. *J Immunol Res.* (2019) 2019:8028725. doi: 10.1155/2019/8028725
47. Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, et al. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature.* (2006) 441:101–5. doi: 10.1038/nature04734
48. Allen IC, Scull MA, Moore CB, Holl EK, Mcelvania-Tekippe E, Taxman DJ, et al. The NLRP3 inflammasome mediates *in vivo* innate immunity to influenza A virus through recognition of viral RNA. *Immunity.* (2009) 30:556–65. doi: 10.1016/j.immuni.2009.02.005
49. Leiva-Juarez MM, Kolls JK, Evans SE. Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense. *Mucosal Immunol.* (2018) 11:21–34. doi: 10.1038/mi.2017.71
50. Killip MJ, Fodor E, Randall RE. Influenza virus activation of the interferon system. *Virus Res.* (2015) 209:11–22. doi: 10.1016/j.virusres.2015.02.003
51. Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G, et al. Distribution of interferon-gamma receptor in human tissues. *Eur J Immunol.* (1992) 22:2403–12. doi: 10.1002/eji.1830220933
52. Klinkhammer J, Schnepf D, Ye L, Schwaderlapp M, Gad HH, Hartmann R, et al. IFN-λ prevents influenza virus spread from the upper airways to the lungs and limits virus transmission. *eLife.* (2018) 7:e33354. doi: 10.7554/eLife.33354.018
53. Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, et al. An important role for type III interferon (IFN-λ/IL-28) in TLR-induced antiviral activity. *J Immunol.* (2008) 180:2474–85. doi: 10.4049/jimmunol.180.4.2474
54. Zanoni I, Granucci F, Broggi A. Interferon (IFN)-lambda takes the Helm: immunomodulatory roles of type III IFNs. *Front Immunol.* (2017) 8:1661. doi: 10.3389/fimmu.2017.01661
55. Hwang I, Scott JM, Kakarla T, Duriancik DM, Choi S, Cho C, et al. Activation mechanisms of natural killer cells during influenza virus infection. *PLoS ONE.* (2012) 7:e51858. doi: 10.1371/journal.pone.0051858
56. Wiley JA, Cerwenka A, Harkema JR, Dutton RW, Harmsen AG. Production of interferon-gamma by influenza hemagglutinin-specific CD8 effector T cells influences the development of pulmonary immunopathology. *Am J Pathol.* (2001) 158:119–30. doi: 10.1016/S0002-9440(10)63950-8
57. Lee AJ, Ashkar AA. The dual nature of type I and type II interferons. *Front Immunol.* (2018) 9:2061. doi: 10.3389/fimmu.2018.02061
58. Goritzka M, Makris S, Kausar F, Durant LR, Pereira C, Kumagai Y, et al. Alveolar macrophage-derived type I interferons orchestrate innate immunity to RSV through recruitment of antiviral monocytes. *J Exp Med.* (2015) 212:699–714. doi: 10.1084/jem.20140825
59. Wang J, Nikrad MP, Travanty EA, Zhou B, Phang T, Gao B, et al. Innate immune response of human alveolar macrophages during influenza A infection. *PLoS ONE.* (2012) 7:e29879. doi: 10.1371/journal.pone.0029879
60. Helft J, Manicassamy B, Guermonprez P, Hashimoto D, Silvin A, Agudo J, et al. Cross-presenting $CD103^+$ dendritic cells are protected from influenza virus infection. *J Clin Invest.* (2012) 122:4037–47. doi: 10.1172/JCI60659
61. Sakabe S, Iwatsuki-Horimoto K, Takano R, Nidom CA, et al. Cytokine production by primary human macrophages infected with highly pathogenic H5N1 or pandemic H1N1 2009 influenza viruses. *J Gen Virol.* (2011) 92:1428. doi: 10.1099/vir.0.030346-0
62. Monteerarat Y, Sakabe S, Ngamurulert S, Srichatraphimuk S, Jiamtom W, Chaichuen K, et al. Induction of TNF-alpha in human macrophages by avian and human influenza viruses. *Arch Virol.* (2010) 155:1273–9. doi: 10.1007/s00705-010-0716-y

63. Geiler J, Michaelis M, Sithisarn P, Cinatl J Jr. Comparison of pro-inflammatory cytokine expression and cellular signal transduction in human macrophages infected with different influenza A viruses. *Med Microbiol Immunol.* (2011) 200:53–60. doi: 10.1007/s00430-010-0173-y
64. Nguyen TH, Maltby S, Tay HL, Eyers F, Foster PS, Yang M. Identification of IFN-gamma and IL-27 as critical regulators of respiratory syncytial virus-induced exacerbation of allergic airways disease in a mouse model. *J Immunol.* (2018) 200:237–47. doi: 10.4049/jimmunol.1601950
65. Us D. Cytokine storm in avian influenza. *Mikrobiyol Bul.* (2008) 42:365–80.
66. Ostler T, Davidson W, Ehl S. Virus clearance and immunopathology by CD8(+) T cells during infection with respiratory syncytial virus are mediated by IFN-gamma. *Eur J Immunol.* (2002) 32:2117–23. doi: 10.1002/1521-4141(200208)32:8<2117::AID-IMMU2117>3.0.CO;2-C
67. Choi J, Callaway Z, Kim HB, Fujisawa T, Kim CK. The role of TNF-alpha in eosinophilic inflammation associated with RSV bronchiolitis. *Pediatr Allergy Immunol.* (2010) 21:474–9. doi: 10.1111/j.1399-3038.2009.00908.x
68. Van Riel D, Leijten LM, Van Der Eerden M, Hoogsteden HC, Boven LA, Lambrecht BN, et al. Highly pathogenic avian influenza virus H5N1 infects alveolar macrophages without virus production or excessive TNF-alpha induction. *PLoS Pathog.* (2011) 7:e1002099. doi: 10.1371/journal.ppat.1002099
69. Ghoneim HE, Thomas PG, Mccullers JA. Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *J Immunol.* (2013) 191:1250–9. doi: 10.4049/jimmunol.1300014
70. Cardani A, Boulton A, Kim TS, Braciale TJ. Alveolar macrophages prevent lethal influenza pneumonia by inhibiting infection of type-1 alveolar epithelial cells. *PLoS Pathog.* (2017) 13:e1006140. doi: 10.1371/journal.ppat.1006140
71. Shibata Y, Berclaz P-Y, Chroneos ZC, Yoshida M, Whitsett JA, Trapnell BC. GM-CSF regulates alveolar macrophage differentiation and innate immunity in the lung through PU.1. *Immunity.* (2001) 15:557–67. doi: 10.1016/S1074-7613(01)00218-7
72. Akagawa K, Kamoshita K, Tokunaga T. Effects of granulocyte-macrophage colony-stimulating factor and colony-stimulating factor-1 on the proliferation and differentiation of murine alveolar macrophages. *J Immunol.* (1988) 141:3383–90.
73. Chen BD, Mueller M, Chou TH. Role of granulocyte/macrophage colony-stimulating factor in the regulation of murine alveolar macrophage proliferation and differentiation. *J Immunol.* (1988) 141:139–44.
74. Ito Y, Correll K, Zemans RL, Leslie CC, Murphy RC, Mason RJ. Influenza induces IL-8 and GM-CSF secretion by human alveolar epithelial cells through HGF/c-Met and TGF-α/EGFR signaling. *Am J Physiol Lung Cell Mol Physiol.* (2015) 308:L1178–88. doi: 10.1152/ajplung.00290.2014
75. Hennet T, Ziltener HJ, Frei K, Peterhans E. A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. *J Immunol.* (1992) 149:932–9.
76. Schneider C, Nobs SP, Heer AK, Kurrer M, Klinke G, Van Rooijen N, et al. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLOS Pathog.* (2014) 10:e1004053. doi: 10.1371/journal.ppat.1004053
77. Wong CK, Smith CA, Sakamoto K, Kaminski N, Koff JL, Goldstein DR. Aging impairs alveolar macrophage phagocytosis and increases influenza-induced mortality in mice. *J Immunol.* (2017) 199:1060–8. doi: 10.4049/jimmunol.1700397
78. Narasaraju T, Ng HH, Phoon MC, Chow VTK. MCP-1 antibody treatment enhances damage and impedes repair of the alveolar epithelium in influenza pneumonitis. *Am J Respir Cell Mol Biol.* (2010) 42:732–43. doi: 10.1165/rcmb.2008-0423OC
79. Eierhoff T, Hrincius ER, Rescher U, Ludwig S, Ehrhardt C. The epidermal growth factor receptor (EGFR) promotes uptake of influenza A viruses (IAV) into host cells. *PLoS Pathog.* (2010) 6:e1001099. doi: 10.1371/journal.ppat.1001099
80. Allard B, Panariti A, Martin JG. Alveolar macrophages in the resolution of inflammation, tissue repair, and tolerance to infection. *Front Immunol.* (2018) 9:1777. doi: 10.3389/fimmu.2018.01777
81. Tipping PG. Toll-like receptors: the interface between innate and adaptive immunity. *J Am Soc Nephrol.* (2006) 17:1769–71. doi: 10.1681/ASN.2006050489
82. Colonna M, Trinchieri G, Liu Y-J. Plasmacytoid dendritic cells in immunity. *Nat Immunol.* (2004) 5:1219–26. doi: 10.1038/ni1141
83. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci USA.* (2004) 101:5598–603. doi: 10.1073/pnas.0400937101
84. Rahmatpanah F, Agrawal S, Jaiswal N, Nguyen HM, Mcclelland M, Agrawal A. Airway epithelial cells prime plasmacytoid dendritic cells to respond to pathogens via secretion of growth factors. *Mucosal Immunol.* (2019) 12:77–84. doi: 10.1038/s41385-018-0097-1
85. Soloff AC, Weirback HK, Ross TM, Barratt-Boyes SM. Plasmacytoid dendritic cell depletion leads to an enhanced mononuclear phagocyte response in lungs of mice with lethal influenza virus infection. *Comp Immunol Microbiol Infect Dis.* (2012) 35:309–17. doi: 10.1016/j.cimid.2012.01.012
86. Carding SR, Allan W, Kyes S, Hayday A, Bottomly K, Doherty PC. Late dominance of the inflammatory process in murine influenza by gamma/delta + T cells. *J Exp Med.* (1990) 172:1225–31. doi: 10.1084/jem.172.4.1225
87. Carding SR, Allan W, Mcmickle A, Doherty PC. Activation of cytokine genes in T cells during primary and secondary murine influenza pneumonia. *J Exp Med.* (1993) 177:475–82. doi: 10.1084/jem.177.2.475
88. Guo X-ZJ, Dash P, Crawford JC, Allen EK, Zamora AE, Boyd DF, et al. Lung γδ T cells mediate protective responses during neonatal influenza infection that are associated with type 2 immunity. *Immunity.* (2018) 49:531–44.e536. doi: 10.1016/j.immuni.2018.07.011
89. Dong P, Ju X, Yan Y, Zhang S, Cai M, Wang H, et al. gammadelta T cells provide protective function in highly pathogenic avian H5N1 influenza A virus infection. *Front Immunol.* (2018) 9:2812. doi: 10.3389/fimmu.2018.02812
90. Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells—a proposal for uniform nomenclature. *Nat Rev Immunol.* (2013) 13:145–9. doi: 10.1038/nri3365
91. Kabata H, Moro K, Koyasu S. The group 2 innate lymphoid cell (ILC2) regulatory network and its underlying mechanisms. *Immunol Rev.* (2018) 286:37–52. doi: 10.1111/imr.12706
92. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
93. Chang Y-J, Kim HY, Albacker LA, Baumgarth N, Mckenzie ANJ, Smith DE, et al. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol.* (2011) 12:631–8. doi: 10.1038/ni.2045
94. Califano D, Furuya Y, Roberts S, Avram D, Mckenzie ANJ, Metzger DW. IFN-γ increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. *Mucosal Immunol.* (2018) 11:209–19. doi: 10.1038/mi.2017.41
95. Gorski SA, Hahn YS, Braciale TJ. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. *PLoS Pathog.* (2013) 9:e1003615. doi: 10.1371/journal.ppat.1003615
96. Samarasinghe AE, Melo RC, Duan S, Lemessurier KS, Liedmann S, Surman SL, et al. Eosinophils promote antiviral immunity in mice infected with influenza A virus. *J Immunol.* (2017) 198:3214–26. doi: 10.4049/jimmunol.1600787
97. Lee JJ, Jacobsen EA, Mcgarry MP, Schleimer RP, Lee NA. Eosinophils in health and disease: the LIAR hypothesis. *Clin Exp Allergy.* (2010) 40:563–75. doi: 10.1111/j.1365-2222.2010.03484.x
98. Diavatopoulos DA, Short KR, Price JT, Wilksch JJ, Brown LE, Briles DE, et al. Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. *FASEB J.* (2010) 24:1789–98. doi: 10.1096/fj.09-146779
99. Mccullers JA, Mcauley JL, Browall S, Iverson AR, Boyd KL, Henriques Normark B. Influenza enhances susceptibility to natural acquisition of and disease due to *Streptococcus pneumoniae* in ferrets. *J Infect Dis.* (2010) 202:1287–95. doi: 10.1086/656333
100. Mccullers JA, Rehg JE. Lethal synergism between influenza virus and *Streptococcus pneumoniae*: characterization of a mouse model and the

role of platelet-activating factor receptor. *J Infect Dis.* (2002) 186:341–50. doi: 10.1086/341462
101. Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis.* (2008) 198:962–70. doi: 10.1086/591708
102. Sharma-Chawla N, Sender V, Kershaw O, Gruber AD, Volckmar J, Henriques-Normark B, et al. Influenza A virus infection predisposes hosts to secondary infection with different *Streptococcus pneumoniae* serotypes with similar outcome but serotype-specific manifestation. *Infect Immun.* (2016) 84:3445–57. doi: 10.1128/IAI.00422-16
103. Peltola VT, Murti KG, Mccullers JA. Influenza virus neuraminidase contributes to secondary bacterial pneumonia. *J Infect Dis.* (2005) 192:249–57. doi: 10.1086/430954
104. Siegel SJ, Roche AM, Weiser JN. Influenza promotes pneumococcal growth during coinfection by providing host sialylated substrates as a nutrient source. *Cell Host Microbe.* (2014) 16:55–67. doi: 10.1016/j.chom.2014.06.005
105. Basset A, Zhang F, Benes C, Sayeed S, Herd M, Thompson C, et al. Toll-like receptor (TLR) 2 mediates inflammatory responses to oligomerized RrgA pneumococcal pilus type 1 protein. *J Biol Chem.* (2013) 288:2665–75. doi: 10.1074/jbc.M112.398875
106. Droemann D, Goldmann T, Branscheid D, Clark R, Dalhoff K, Zabel P, et al. Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung. *Histochem Cell Biol.* (2003) 119:103–8. doi: 10.1007/s00418-003-0497-4
107. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, et al. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proc Natl Acad Sci USA.* (2003) 100:1966–71. doi: 10.1073/pnas.0435928100
108. Schroder NW, Morath S, Alexander C, Hamann L, Hartung T, Zahringer U, et al. Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J Biol Chem.* (2003) 278:15587–94. doi: 10.1074/jbc.M212829200
109. Tomlinson G, Chimalapati S, Pollard T, Lapp T, Cohen J, Camberlein E, et al. TLR-mediated inflammatory responses to *Streptococcus pneumoniae* are highly dependent on surface expression of bacterial lipoproteins. *J Immunol.* (2014) 193:3736–45. doi: 10.4049/jimmunol.1401413
110. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol.* (1999) 163:1–5.
111. Rodriguez AE, Bogart C, Gilbert CM, Mccullers JA, Smith AM, Kanneganti TD, et al. Enhanced IL-1beta production is mediated by a TLR2-MYD88-NLRP3 signaling axis during coinfection with influenza A virus and *Streptococcus pneumoniae*. *PLoS ONE.* (2019) 14:e0212236. doi: 10.1371/journal.pone.0212236
112. Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, et al. Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem.* (2005) 280:5571–80. doi: 10.1074/jbc.M410592200
113. Tian X, Xu F, Lung WY, Meyerson C, Ghaffari AA, Cheng G, et al. Poly I:C enhances susceptibility to secondary pulmonary infections by gram-positive bacteria. *PLoS ONE.* (2012) 7:e41879. doi: 10.1371/journal.pone.0041879
114. Ioannidis I, Ye F, Mcnally B, Willette M, Flano E. Toll-like receptor expression and induction of type I and type III interferons in primary airway epithelial cells. *J Virol.* (2013) 87:3261–70. doi: 10.1128/JVI.01956-12
115. Selinger DS, Reed WP. Pneumococcal adherence to human epithelial cells. *Infect Immun.* (1979) 23:545–8. doi: 10.1128/IAI.23.2.545-548.1979
116. Plotkowski MC, Puchelle E, Beck G, Jacquot J, Hannoun C. Adherence of type I Streptococcus pneumoniae to tracheal epithelium of mice infected with influenza A/PR8 virus. *Am Rev Respir Dis.* (1986) 134:1040–4. doi: 10.1164/arrd.1986.134.5.1040
117. Rayner CF, Jackson AD, Rutman A, Dewar A, Mitchell TJ, Andrew PW, et al. Interaction of pneumolysin-sufficient and -deficient isogenic variants of *Streptococcus pneumoniae* with human respiratory mucosa. *Infect Immun.* (1995) 63:442–7. doi: 10.1128/IAI.63.2.442-447.1995
118. Hilleringmann M, Giusti F, Baudner BC, Masignani V, Covacci A, Rappuoli R, et al. Pneumococcal pili are composed of protofilaments exposing adhesive clusters of Rrg A. *PLoS Pathog.* (2008) 4:e1000026. doi: 10.1371/journal.ppat.1000026
119. Nelson AL, Ries J, Bagnoli F, Dahlberg S, Falker S, Rounioja S, et al. RrgA is a pilus-associated adhesin in *Streptococcus pneumoniae*. *Mol Microbiol.* (2007) 66:329–40. doi: 10.1111/j.1365-2958.2007.05908.x
120. Paterson GK, Orihuela CJ. Pneumococcal microbial surface components recognizing adhesive matrix molecules targeting of the extracellular matrix. *Mol Microbiol.* (2010) 77:1–5. doi: 10.1111/j.1365-2958.2010.07190.x
121. Van Der Flier M, Chhun N, Wizemann TM, Min J, Mccarthy JB, Tuomanen EI. Adherence of Streptococcus pneumoniae to immobilized fibronectin. *Infect Immun.* (1995) 63:4317–22. doi: 10.1128/IAI.63.11.4317-4322.1995
122. Yamaguchi M, Terao Y, Mori Y, Hamada S, Kawabata S. PfbA, a novel plasmin- and fibronectin-binding protein of *Streptococcus pneumoniae*, contributes to fibronectin-dependent adhesion and antiphagocytosis. *J Biol Chem.* (2008) 283:36272–9. doi: 10.1074/jbc.M807087200
123. Van Der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet.* (2009) 374:1543–56. doi: 10.1016/S0140-6736(09)61114-4
124. Pittet LA, Hall-Stoodley L, Rutkowski MR, Harmsen AG. Influenza virus infection decreases tracheal mucociliary velocity and clearance of *Streptococcus pneumoniae*. *Am J Respir Cell Mol Biol.* (2010) 42:450–60. doi: 10.1165/rcmb.2007-0417OC
125. Golda A, Malek N, Dudek B, Zeglen S, Wojarski J, Ochman M, et al. Infection with human coronavirus NL63 enhances streptococcal adherence to epithelial cells. *J Gen Virol.* (2011) 92:1358–68. doi: 10.1099/vir.0.028381-0
126. Ishizuka S, Yamaya M, Suzuki T, Takahashi H, Ida S, Sasaki T, et al. Effects of rhinovirus infection on the adherence of *Streptococcus pneumoniae* to cultured human airway epithelial cells. *J Infect Dis.* (2003) 188:1928–39. doi: 10.1086/379833
127. Van Der Sluijs KF, Van Elden LJ, Nijhuis M, Schuurman R, Florquin S, Shimizu T, et al. Involvement of the platelet-activating factor receptor in host defense against *Streptococcus pneumoniae* during postinfluenza pneumonia. *Am J Physiol Lung Cell Mol Physiol.* (2006) 290:L194–9. doi: 10.1152/ajplung.00050.2005
128. Schultz-Cherry S, Hinshaw VS. Influenza virus neuraminidase activates latent transforming growth factor beta. *J Virol.* (1996) 70:8624–9. doi: 10.1128/JVI.70.12.8624-8629.1996
129. Li W, Moltedo B, Moran TM. Type I interferon induction during influenza virus infection increases susceptibility to secondary Streptococcus pneumoniae infection by negative regulation of gammadelta T cells. *J Virol.* (2012) 86:12304–12. doi: 10.1128/JVI.01269-12
130. Damjanovic D, Lai R, Jeyanathan M, Hogaboam CM, Xing Z. Marked improvement of severe lung immunopathology by influenza-associated pneumococcal superinfection requires the control of both bacterial replication and host immune responses. *Am J Pathol.* (2013) 183:868–80. doi: 10.1016/j.ajpath.2013.05.016
131. Wolter N, Cohen C, Tempia S, Madhi SA, Venter M, Moyes J, et al. HIV and influenza virus infections are associated with increased blood pneumococcal load: a prospective, hospital-based observational study in South Africa, 2009-2011. *J Infect Dis.* (2014) 209:56–65. doi: 10.1093/infdis/jit427
132. Ring A, Weiser JN, Tuomanen EI. Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. *J Clin Invest.* (1998) 102:347–60. doi: 10.1172/JCI2406
133. Talbot UM, Paton AW, Paton JC. Uptake of Streptococcus pneumoniae by respiratory epithelial cells. *Infect Immun.* (1996) 64:3772–7. doi: 10.1128/IAI.64.9.3772-3777.1996
134. Zhang JR, Mostov KE, Lamm ME, Nanno M, Shimida S, Ohwaki M, et al. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell.* (2000) 102:827–37. doi: 10.1016/S0092-8674(00)00071-4
135. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. *Nature.* (1995) 377:435–8. doi: 10.1038/377435a0
136. Radin JN, Orihuela CJ, Murti G, Guglielmo C, Murray PJ, Tuomanen EI. beta-Arrestin 1 participates in platelet-activating factor receptor-mediated

endocytosis of *Streptococcus pneumoniae*. *Infect Immun.* (2005) 73:7827–35. doi: 10.1128/IAI.73.12.7827-7835.2005
137. Brock SC, Mcgraw PA, Wright PF, Crowe JE Jr. The human polymeric immunoglobulin receptor facilitates invasion of epithelial cells by Streptococcus pneumoniae in a strain-specific and cell type-specific manner. *Infect Immun.* (2002) 70:5091–95. doi: 10.1128/IAI.70.9.5091-5095.2002
138. Iovino F, Engelen-Lee JY, Brouwer M, Van De Beek D, Van Der Ende A, Valls Seron M, et al. pIgR and PECAM-1 bind to pneumococcal adhesins RrgA and PspC mediating bacterial brain invasion. *J Exp Med.* (2017) 214:1619–30. doi: 10.1084/jem.20161668
139. Weight CM, Venturini C, Pojar S, Jochems SP, Reine J, Nikolaou E, et al. Microinvasion by *Streptococcus pneumoniae* induces epithelial innate immunity during colonisation at the human mucosal surface. *Nat Commun.* (2019) 10:3060. doi: 10.1038/s41467-019-11005-2
140. Sandgren A, Sjostrom K, Olsson-Liljequist B, Christensson B, Samuelsson A, Kronvall G, et al. Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. *J Infect Dis.* (2004) 189:785–96. doi: 10.1086/381686
141. Cao J, Wang D, Xu F, Gong Y, Wang H, Song Z, et al. Activation of IL-27 signalling promotes development of postinfluenza pneumococcal pneumonia. *EMBO Mol Med.* (2014) 6:120–40. doi: 10.1002/emmm.201302890
142. Hoe E, Boelsen LK, Toh ZQ, Sun GW, Koo GC, Balloch A, et al. Reduced IL-17A secretion is associated with high levels of pneumococcal nasopharyngeal carriage in Fijian children. *PLoS ONE.* (2015) 10:e0129199. doi: 10.1371/journal.pone.0129199
143. Lu YJ, Gross J, Bogaert D, Finn A, Bagrade L, Zhang Q, et al. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* (2008) 4:e1000159. doi: 10.1371/journal.ppat.1000159
144. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest.* (2009) 119:1899–909. doi: 10.1172/JCI36731
145. Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, et al. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol.* (2011) 186:1666–74. doi: 10.4049/jimmunol.1002194
146. Nakamura S, Davis KM, Weiser JN. Synergistic stimulation of type I interferons during influenza virus coinfection promotes *Streptococcus pneumoniae* colonization in mice. *J Clin Invest.* (2011) 121:3657–65. doi: 10.1172/JCI57762
147. Shahangian A, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY, et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest.* (2009) 119:1910–20. doi: 10.1172/JCI35412
148. Eddens T, Kolls JK. Host defenses against bacterial lower respiratory tract infection. *Curr Opin Immunol.* (2012) 24:424–30. doi: 10.1016/j.coi.2012.07.005
149. Kaufmann SHE, Dorhoi A. Molecular determinants in phagocyte-bacteria interactions. *Immunity.* (2016) 44:476–91. doi: 10.1016/j.immuni.2016.02.014
150. Crotta S, Davidson S, Mahlakoiv T, Desmet CJ, Buckwalter MR, Albert ML, et al. Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. *PLoS Pathog.* (2013) 9:e1003773. doi: 10.1371/journal.ppat.1003773
151. Jewell NA, Vaghefi N, Mertz SE, Akter P, Peebles RS Jr., et al. Differential type I interferon induction by respiratory syncytial virus and influenza a virus *in vivo*. *J Virol.* (2007) 81:9790–800. doi: 10.1128/JVI.00530-07
152. Richman DD, Murphy BR, Baron S, Uhlendorf C. Three strains of influenza A virus (H3N2): interferon sensitivity *in vitro* and interferon production in volunteers. *J Clin Microbiol.* (1976) 3:223–6.
153. Ronni T, Matikainen S, Sareneva T, Melen K, Pirhonen J, Keskinen P, et al. Regulation of IFN-alpha/beta, MxA, 2len K, Pirhonen J, Keskinen P, et al.al type I interferon induction by respiratory syncytial virus and influe*J Immunol.* (1997) 158:2363–74.
154. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, et al. Functional role of type I and type II interferons in antiviral defense. *Science.* (1994) 264:1918–21. doi: 10.1126/science.8009221
155. Herold S, Steinmueller M, Von Wulffen W, Cakarova L, Pinto R, Pleschka S, et al. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. *J Exp Med.* (2008) 205:3065–77. doi: 10.1084/jem.20080201
156. Narayana Moorthy A, Narasaraju T, Rai P, Perumalsamy R, Tan KB, Wang S, et al. *In vivo* and *in vitro* studies on the roles of neutrophil extracellular traps during secondary pneumococcal pneumonia after primary pulmonary influenza infection. *Front Immunol.* (2013) 4:56. doi: 10.3389/fimmu.2013.00056
157. Alsharifi M, Regner M, Blanden R, Lobigs M, Lee E, Koskinen A, et al. Exhaustion of type I interferon response following an acute viral infection. *J Immunol.* (2006) 177:3235–41. doi: 10.4049/jimmunol.177.5.3235
158. Lemessurier KS, Hacker H, Chi L, Tuomanen E, Redecke V. Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung. *PLoS Pathog.* (2013) 9:e1003727. doi: 10.1371/journal.ppat.1003727
159. Burnstock G, Brouns I, Adriaensen D, Timmermans JP. Purinergic signaling in the airways. *Pharmacol Rev.* (2012) 64:834–68. doi: 10.1124/pr.111.005389
160. Wolk KE, Lazarowski ER, Traylor ZP, Yu EN, Jewell NA, Durbin RK, et al. Influenza A virus inhibits alveolar fluid clearance in BALB/c mice. *Am J Respir Crit Care Med.* (2008) 178:969–76. doi: 10.1164/rccm.200803-455OC
161. Aeffner F, Woods PS, Davis IC. Activation of A1-adenosine receptors promotes leukocyte recruitment to the lung and attenuates acute lung injury in mice infected with influenza A/WSN/33 (H1N1) virus. *J Virol.* (2014) 88:10214–27. doi: 10.1128/JVI.01068-14
162. Yegutkin GG, Jankowski J, Jalkanen S, Gunthner T, Zidek W, Jankowski V. Dinucleotide polyphosphates contribute to purinergic signalling via inhibition of adenylate kinase activity. *Biosci Rep.* (2008) 28:189–94. doi: 10.1042/BSR20080052
163. Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC, et al. Crucial role for ecto-5K, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC, et*J Exp Med.* (2004) 200:1395–405. doi: 10.1084/jem.20040915
164. Bhalla M, Yeoh JH, Lamneck C, Herring SE, Tchalla EYI, Leong JM, et al. A1 adenosine receptor signaling reduces *Streptococcus pneumoniae* adherence to pulmonary epithelial cells by targeting expression of platelet-activating factor receptor. *Cell Microbiol.* (2019) doi: 10.1111/cmi.13141. [Epub ahead of print].
165. Bou Ghanem EN, Clark S, Roggensack SE, Mciver SR, Alcaide P, Haydon PG, et al. Extracellular adenosine protects against *Streptococcus pneumoniae* lung infection by regulating pulmonary neutrophil recruitment. *PLoS Pathog.* (2015) 11:e1005126. doi: 10.1371/journal.ppat.1005126
166. Guo H, Topham DJ. Interleukin-22 (IL-22) production by pulmonary Natural Killer cells and the potential role of IL-22 during primary influenza virus infection. *J Virol.* (2010) 84:7750–9. doi: 10.1128/JVI.00187-10
167. Ivanov S, Renneson J, Fontaine J, Barthelemy A, Paget C, Fernandez EM, et al. Interleukin-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection. *J Virol.* (2013) 87:6911–24. doi: 10.1128/JVI.02943-12
168. Whittington HA, Armstrong L, Uppington KM, Millar AB. Interleukin-22: a potential immunomodulatory molecule in the lung. *Am J Respir Cell Mol Biol.* (2004) 31:220–6. doi: 10.1165/rcmb.2003-0285OC
169. He X, Li H, Chen Y, Chen A, Shan K, Chen J, et al. The Effects of IL-22 on the inflammatory mediator production, proliferation, and barrier function of HUVECs. *Inflammation.* (2016) 39:1099–107. doi: 10.1007/s10753-016-0341-3
170. Wu Z, Hu Z, Cai X, Ren W, Dai F, Liu H, et al. Interleukin 22 attenuated angiotensin II induced acute lung injury through inhibiting the apoptosis of pulmonary microvascular endothelial cells. *Sci Rep.* (2017) 7:2210. doi: 10.1038/s41598-017-02056-w
171. Kotenko SV, Izotova LS, Mirochnitchenko OV, Esterova E, Dickensheets H, Donnelly RP, et al. Identification, cloning, and characterization of a novel soluble receptor that binds IL-22 and neutralizes its activity. *J Immunol.* (2001) 166:7096–103. doi: 10.4049/jimmunol.166.12.7096
172. Xu W, Presnell SR, Parrish-Novak J, Kindsvogel W, Jaspers S, Chen Z, et al. A soluble class II cytokine receptor, IL-22RA2, is a naturally

occurring IL-22 antagonist. *Proc Natl Acad Sci USA*. (2001) 98:9511–6. doi: 10.1073/pnas.171303198
173. Pociask DA, Scheller EV, Mandalapu S, Mchugh KJ, Enelow RI, Fattman CL, et al. IL-22 is essential for lung epithelial repair following influenza infection. *Am J Pathol.* (2013) 182:1286–96. doi: 10.1016/j.ajpath.2012.12.007
174. Barthelemy A, Sencio V, Soulard D, Deruyter L, Faveeuw C, Le Goffic R, et al. Interleukin-22 immunotherapy during severe influenza enhances lung tissue integrity and reduces secondary bacterial systemic invasion. *Infect Immun.* (2018) 86:e00706-17. doi: 10.1128/IAI.00706-17
175. Abood RN, Mchugh KJ, Rich HE, Ortiz MA, Tobin JM, Ramanan K, et al. IL-22-binding protein exacerbates influenza, bacterial superinfection. *Mucosal Immunol.* (2019) 12:1231–43. doi: 10.1038/s41385-019-0188-7
176. Fliegauf M, Sonnen AF, Kremer B, Henneke P. Mucociliary clearance defects in a murine *in vitro* model of pneumococcal airway infection. *PLoS ONE.* (2013) 8:e59925. doi: 10.1371/journal.pone.0059925
177. Gordon DL, Johnson GM, Hostetter MK. Ligand-receptor interactions in the phagocytosis of virulent *Streptococcus pneumoniae* by polymorphonuclear leukocytes. *J Infect Dis.* (1986) 154:619–26. doi: 10.1093/infdis/154.4.619
178. Melin M, Jarva H, Siira L, Meri S, Kayhty H, Vakevainen M. Streptococcus pneumoniae capsular serotype 19F is more resistant to C3 deposition and less sensitive to opsonophagocytosis than serotype 6B. *Infect Immun.* (2009) 77:676–84. doi: 10.1128/IAI.01186-08
179. Hergott CB, Roche AM, Naidu NA, Mesaros C, Blair IA, Weiser JN. Bacterial exploitation of phosphorylcholine mimicry suppresses inflammation to promote airway infection. *J Clin Invest.* (2015) 125:3878–90. doi: 10.1172/JCI81888
180. Kitchen E, Rossi AG, Condliffe AM, Haslett C, Chilvers ER. Demonstration of reversible priming of human neutrophils using platelet-activating factor. *Blood.* (1996) 88:4330–7. doi: 10.1182/blood.V88.11.4330.bloodjournal88114330
181. Shaw JO, Pinckard RN, Ferrigni KS, Mcmanus LM, Hanahan DJ. Activation of human neutrophils with 1-O-hexadecyl/octadecyl-2-acetyl-sn-glycerol-3-phosphorylcholine (platelet activating factor). *J Immunol.* (1981) 127:1250–5. doi: 10.1159/000232884
182. Wu Y, Tu W, Lam KT, Chow KH, Ho PL, Guan Y, et al. Lethal coinfection of influenza virus and *Streptococcus pneumoniae* lowers antibody response to influenza virus in lung and reduces numbers of germinal center B cells, T follicular helper cells, and plasma cells in mediastinal lymph node. *J Virol.* (2015) 89:2013–23. doi: 10.1128/JVI.02455-14
183. Van Der Sluijs KF, Van Elden LJ, Nijhuis M, Schuurman R, Pater JM, Florquin S, et al. IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *J Immunol.* (2004) 172:7603–9. doi: 10.4049/jimmunol.172.12.7603
184. O'Brien KL, Walters MI, Sellman J, Quinlisk P, Regnery H, Schwartz B, et al. Severe pneumococcal pneumonia in previously healthy children: the role of preceding influenza infection. *Clin Infect Dis.* (2000) 30:784–9. doi: 10.1086/313772
185. Loosli CG, Stinson SF, Ryan DP, Hertweck MS, Hardy JD, Serebrin R. The destruction of type 2 pneumocytes by airborne influenza PR8-A virus; its effect on surfactant and lecithin content of the pneumonic lesions of mice. *Chest.* (1975) 67:7s–14s. doi: 10.1378/chest.67.2_Supplement.7S
186. Quantius J, Schmoldt C, Vazquez-Armendariz AI, Becker C, El Agha E, Wilhelm J, et al. Influenza virus infects epithelial stem/progenitor cells of the distal lung: impact on Fgfr2b-driven epithelial repair. *PLoS Pathog.* (2016) 12:e1005544. doi: 10.1371/journal.ppat.1005544
187. Wang Y, Jiang B, Guo Y, Li W, Tian Y, Sonnenberg GF, et al. Cross-protective mucosal immunity mediated by memory Th17 cells against *Streptococcus pneumoniae* lung infection. *Mucosal Immunol.* (2017) 10:250–9. doi: 10.1038/mi.2016.41
188. Kuipers K, Jong WSP, Van Der Gaast-De Jongh CE, Houben D, Van Opzeeland F, Simonetti E, et al. Th17-mediated cross protection against pneumococcal carriage by vaccination with a variable antigen. *Infect Immun.* (2017) 85:e00281-17. doi: 10.1128/IAI.00281-17
189. Wagner R, Wolff T, Herwig A, Pleschka S, Klenk HD. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. *J Virol.* (2000) 74:6316–23. doi: 10.1128/JVI.74.14.6316-6323.2000
190. Cohen M, Zhang XQ, Senaati HP, Chen HW, Varki NM, Schooley RT, et al. Influenza A penetrates host mucus by cleaving sialic acids with neuraminidase. *Virol J.* (2013) 10:321. doi: 10.1186/1743-422X-10-321
191. Dobson J, Whitley RJ, Pocock S, Monto AS. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. *Lancet.* (2015) 385: 1729–37. doi: 10.1016/S0140-6736(14)62449-1
192. Malosh RE, Martin ET, Heikkinen T, Brooks WA, Whitley RJ, Monto AS. Efficacy and safety of oseltamivir in children: systematic review and individual patient data meta-analysis of randomized controlled trials. *Clin Infect Dis.* (2018). 66:1492–500. doi: 10.1093/cid/cix1040
193. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, et al. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. *N Engl J Med.* (2018) 379:913–23. doi: 10.1056/NEJMoa1716197
194. Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, Kawai M, et al. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci Rep.* (2018) 8:9633. doi: 10.1038/s41598-018-27890-4
195. Tilmanis D, van Baalen C, Oh DY, Rossignol JF, Hurt AC. The susceptibility of circulating human influenza viruses to tizoxanide, the active metabolite of nitazoxanide. *Antiviral Res.* (2017) 147:142–8. doi: 10.1016/j.antiviral.2017.10.002
196. Stachulski AV, Santoro MG, Piacentini S, Belardo G, Frazia S, Pidathala C, et al. Second-generation nitazoxanide derivatives: thiazolides are effective inhibitors of the influenza A virus. *Future Med Chem.* (2018) 10:851–62. doi: 10.4155/fmc-2017-0217
197. Rossignol JF, La Frazia S, Chiappa L, Ciucci A, Santoro MG. Thiazolides, a new class of anti-influenza molecules targeting viral hemagglutinin at the post-translational level. *J Biol Chem.* (2009) 284:29798–808. doi: 10.1074/jbc.M109.029470
198. Haffizulla J, Hartman A, Hoppers M, Resnick H, Samudrala S, Ginocchio C, et al. Effect of nitazoxanide in adults and adolescents with acute uncomplicated influenza: a double-blind, randomised, placebo-controlled, phase 2b/3 trial. *Lancet Infect Dis.* (2014) 14:609–18. doi: 10.1016/S1473-3099(14)70717-0
199. Khemani RG, Smith LS, Zimmerman JJ, Erickson S. Pediatric acute respiratory distress syndrome: definition, incidence, and epidemiology: proceedings from the Pediatric Acute Lung Injury Consensus Conference. *Pediatr Crit Care Med.* (2015) 16(5 Suppl. 1):S23–40. doi: 10.1097/PCC.0000000000000432
200. Malakhov MP, Aschenbrenner LM, Smee DF, Wandersee MK, Sidwell RW, Gubareva LV, et al. Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. *Antimicrob Agents Chemother.* (2006) 50:1470–9. doi: 10.1128/AAC.50.4.1470-1479.2006
201. Triana-Baltzer GB, Gubareva LV, Nicholls JM, Pearce MB, Mishin VP, Belser JA, et al. Novel pandemic influenza A(H1N1) viruses are potently inhibited by DAS181, a sialidase fusion protein. *PLoS ONE.* (2009) 4:e7788. doi: 10.1371/journal.pone.0007788
202. Moss RB, Hansen C, Sanders RL, Hawley S, Li T, Steigbigel RT. A phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection. *J Infect Dis.* (2012) 206:1844–51. doi: 10.1093/infdis/jis622
203. Zenilman JM, Fuchs EJ, Hendrix CW, Radebaugh C, Jurao R, Nayak SU, et al. Phase 1 clinical trials of DAS181, an inhaled sialidase, in healthy adults. *Antiviral Res.* (2015) 123:114–9. doi: 10.1016/j.antiviral.2015.09.008
204. Hedlund M, Aschenbrenner LM, Jensen K, Larson JL, Fang F. Sialidase-based anti-influenza virus therapy protects against secondary pneumococcal infection. *J Infect Dis.* (2010) 201:1007–15. doi: 10.1086/651170
205. Ellis GT, Davidson S, Crotta S, Branzk N, Papayannopoulos V, Wack A. TRAIL+ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-Streptococcus pneumoniae coinfection. *EMBO Rep.* (2015) 16:1203–18. doi: 10.15252/embr.201540473
206. Bulanova D, Ianevski A, Bugai A, Akimov Y, Kuivanen S, Paavilainen H, et al. Antiviral properties of chemical inhibitors of cellular anti-apoptotic Bcl-2 proteins. *Viruses.* (2017) 9:E271. doi: 10.3390/v9100271
207. Shim JM, Kim J, Tenson T, Min JY, Kainov DE. Influenza virus infection, interferon response, viral counter-response, and apoptosis. *Viruses.* (2017) 9:E223. doi: 10.20944/preprints201705.0209.v1
208. Davidson S, McCabe TM, Crotta S, Gad HH, Hessel EM, Beinke S, et al. IFNλ is a potent anti-influenza therapeutic without the inflammatory

side effects of IFNα treatment. *EMBO Mol Med.* (2016) 8:1099–112. doi: 10.15252/emmm.201606413
209. Klinkhammer J, Schnepf D, Ye L, Schwaderlapp M, Gad HH, Hartmann R, et al. IFN-λ prevents influenza virus spread from the upper airways to the lungs and limits virus transmission. *Elife*. (2018) 7:e33354. doi: 10.7554/eLife.33354
210. Rich HE, McCourt CC, Zheng WQ, McHugh KJ, Robinson KM, Wang J, et al. Interferon lambda inhibits bacterial uptake during influenza superinfection. *Infect Immun.* (2019) 87:e00114-19. doi: 10.1128/IAI.00114-19
211. Kielgast F, Schmidt H, Braubach P, Winkelmann VE, Thompson KE, Frick M, et al. Glucocorticoids regulate tight junction permeability of lung epithelia by modulating claudin 8. *Am J Respir Cell Mol Biol.* (2016) 54:707–17. doi: 10.1165/rcmb.2015-0071OC
212. Zhang Y, Sun W, Svendsen ER, Tang S, MacIntyre RC, Yang P, et al. Do corticosteroids reduce the mortality of influenza A (H1N1) infection? A meta-analysis. *Crit Care.* (2015) 19:46. doi: 10.1186/s13054-015-0764-5
213. Nedel WL, Nora DG, Salluh JI, Lisboa T, Póvoa P. Corticosteroids for severe influenza pneumonia: a critical appraisal. *World J Crit Care Med*. (2016) 5:89–95. doi: 10.5492/wjccm.v5.i1.89
214. Lansbury L, Rodrigo C, Leonardi-Bee J, Nguyen-Van-Tam J, Lim WS. Corticosteroids as adjunctive therapy in the treatment of influenza. *Cochrane Database Syst Rev*. (2019) 2:CD010406. doi: 10.1002/14651858.CD010406.pub3
215. Ni YN, Chen G, Sun J, Liang BM, Liang ZA. The effect of corticosteroids on mortality of patients with influenza pneumonia: a systematic review and meta-analysis. *Crit. Care.* (2019) 23:99. doi: 10.1186/s13054-019-2395-8
216. Cherazard R, Epstein M, Doan TL, Salim T, Bharti S, Smith MA. Antimicrobial resistant *Streptococcus pneumoniae*: prevalence, mechanisms, and clinical implications. *Am J Ther*. (2017) 24:e361–9. doi: 10.1097/MJT.0000000000000551
217. van Werkhoven CH, Huijts SM. Vaccines to prevent pneumococcal community-acquired pneumonia. *Clin Chest Med*. (2018) 39:733–52. doi: 10.1016/j.ccm.2018.07.007
218. Cakan G, Turkoz M, Turan T, Ahmed K, Nagatake T. S-carboxymethylcysteine inhibits the attachment of Streptococcus pneumoniae to human pharyngeal epithelial cells. *Microb Pathog.* (2003) 34:261–5. doi: 10.1016/S0882-4010(03)00048-2
219. Sumitomo T, Nakata M, Yamaguchi M, Terao Y, Kawabata S. S-carboxymethylcysteine inhibits adherence of *Streptococcus pneumoniae* to human alveolar epithelial cells. *J Med Microbiol.* (2012) 61(Pt 1):101–8. doi: 10.1099/jmm.0.033688-0

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MINI REVIEW
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Inflammation as a Modulator of Host Susceptibility to Pulmonary Influenza, Pneumococcal, and Co-Infections

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Bacterial and viral pathogens are predominant causes of pulmonary infections and complications. Morbidity and mortality from these infections is increased in populations that include the elderly, infants, and individuals with genetic disorders such as Down syndrome. Immune senescence, concurrent infections, and other immune alterations occur in these susceptible populations, but the underlying mechanisms that dictate increased susceptibility to lung infections are not fully defined. Here, we review unique features of the lung as a mucosal epithelial tissue and aspects of inflammatory and immune responses in model pulmonary infections and co-infections by influenza virus and *Streptococcus pneumoniae*. In these models, lung inflammatory responses are a double-edged sword: recruitment of immune effectors is essential to eliminate bacteria and virus-infected cells, but inflammatory cytokines drive changes in the lung conducive to increased pathogen replication. Excessive accumulation of inflammatory cells also hinders lung function, possibly causing death of the host. Some animal studies have found that targeting host modulators of lung inflammatory responses has therapeutic or prophylactic effects in these infection and co-infection models. However, conflicting results from other studies suggest microbiota, sequence of colonization, or other unappreciated aspects of lung biology also play important roles in the outcome of infections. Regardless, a predisposition to excessive or aberrant inflammatory responses occurs in susceptible human populations. Hence, in appropriate contexts, modulation of inflammatory responses may prove effective for reducing the frequency or severity of pulmonary infections. However, there remain limitations in our understanding of how this might best be achieved—particularly in diverse human populations.

Keywords: pulmonary inflammation, viral infection, bacterial infection, innate immunity, down syndrome

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INTRODUCTION

Pulmonary disease constitutes four of the ten leading causes of death in the human population [chronic obstructive pulmonary disease (COPD), lung cancers, pneumonias, and tuberculosis][1]. Each of these conditions is also associated with inflammatory reactions. Therefore, a better understanding of lung biology and the control of inflammation in the lungs during infection has potential to substantially impact human health.

[1]https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death.

The lungs are a vital organ that facilitate efficient transfer of oxygen and carbon dioxide. Their large surface area is comprised of small terminal air sacs called alveoli. In the alveoli, a single layer of epithelial cells separates inhaled air from underlying small capillaries. Maintenance of the alveolar structure and function is thus crucial for proper functioning of the lungs. Breathing exposes the upper respiratory tract and lung alveolar surface to microbes and other environmental substances. At an average of 15 breaths each minute, more than 10,000 L of air passes over airway mucosal surfaces in the course of a day (1). Each liter of air contains hundreds of thousands or even millions of microbes, thus nasal tissues and the lung alveoli may contact upwards of 10^9 inhaled microbes each day (2). Commensal microbes also inhabit these tissues (3). To protect the lungs from overgrowth or invasion by microbes, the upper respiratory tract is coated with a mucus layer containing antimicrobial peptides and proteins. Mucus traps many inhaled microbes, which are then cleared from the respiratory tract through the activity of ciliated cells (4). The lung luminal (environmental) alveolar surface is similarly coated with a thin layer of liquid surfactant with dissolved proteins and lipids (3, 4). This surfactant adsorbs at the air/water interface to reduce surface tension, maintain lung elasticity, and capture particles from the air. Beneath the surfactant, alveolar macrophages (AMs) patrol the apical surface of epithelial cells to engulf and remove inhaled microbes (1). Though generally effective, certain pathogens can overcome these upper and lower airway defenses. Infection by such pathogens elicits innate immune cell activation and the initiation of inflammatory responses. In this review, we focus on these innate immune players in the context of lung infections.

Bacterial and viral pathogens are common causes of human pulmonary infections and will be the focus of this review. Bacteria that commonly cause human lung infections include *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (5). Viruses that commonly cause human lung infections include Respiratory syncytial virus (RSV) and influenza viruses (5, 6). Co-infections with these bacteria and viruses is also common and is generally associated with more severe disease and a higher incidence of mortality (5). The current review provides an update and expands on elements previously reviewed by others [e.g., (7)]. Fungal pathogens also cause lung infections and co-infections with bacteria or viruses—particularly in immunocompromised individuals and individuals with polymorphisms in innate immune detection systems (8, 9). However, due to space limitations, fungal infections will not be further discussed in this review.

In this review, we provide an overview of the events that occur when innate lung defenses are overwhelmed by viral and/or bacterial pathogens. Our focus is on innate immune players in animal models of influenza A virus (IAV) and/or *S. pneumoniae* infection, though some relevant human subject studies are also mentioned. The available data support the hypothesis that the nature and magnitude of the inflammatory response contributes to host susceptibility and thus can drive overwhelmingly severe lung infection.

INFLAMMATORY RESPONSES TO PULMONARY INFECTION

The healthy lung houses both epithelial and resident immune cell populations. Resident immune cells typically found in the healthy lung include neutrophils, monocytes, macrophages, dendritic cells, natural killer (NK), and other innate lymphocyte (ILC) populations, as well as B and T cells (10). Of these cell populations, resident AMs are most abundant (**Figure 1A**). When an invasive pathogen overwhelms AMs and has established an active infection, pathogen-associated molecular pattern (PAMPs) and damage-associated molecular patterns (DAMPs) can engage pattern recognition receptors (PRRs) on these cell populations (10). Ligation of PRRs leads to activation of cellular signaling pathways and the production of soluble interferons (IFNs) which drive expression of IFN-stimulated genes (ISGs) that act in a cell-intrinsic manner to prevent or limit replication of invading pathogens (10). Simultaneously, PRR ligation induces the expression and production of cytokines and chemokines which regulate the activation and recruitment of additional immune and inflammatory cell populations in the lung (10). Recognition of PAMPs by specific PRRs [e.g., toll-like receptors (TLRs) 2 and 4] thus has substantial impact on disease susceptibility and pathogen transmission. In the initial stages of infections, recruited neutrophils, monocytes, and resident AMs are considered the primary effectors of pathogen clearance (**Figure 1**). The influx of inflammatory myeloid and other immune cells is necessary to contain and kill invasive microbes. However, the recruitment and activities of these cells can also impair gas exchange and cause damage to the lung epithelium. Thus, fine-tuning of these responses is essential for efficient pathogen clearance and to reduce host damage associated with severe lung infections (10). When accumulation of inflammatory cells and fluid in the lung alveoli disrupts their ability to mediate gas exchange, the clinical condition known as pneumonia ensues. Pneumonias occur with increased frequency in infants, the elderly, and/or individuals who are immunocompromised or have specific genetic conditions that include Down syndrome[2] (11). This suggests these groups have an inherent impaired ability to combat lung pathogens and/or to constrain the inflammation associated with these infections.

INFLUENZA VIRUS INFECTION IN THE LUNG

One of the most common viral pathogens associated with lung disease in humans is influenza virus. Influenza virus is a segmented RNA virus belonging to the *Orthomyxoviridae* family (12). Influenza, like other RNA viruses have high genetic variability due to poor proofreading activity during replication

[2]https://www.cdc.gov/pneumococcal/about/facts.html.

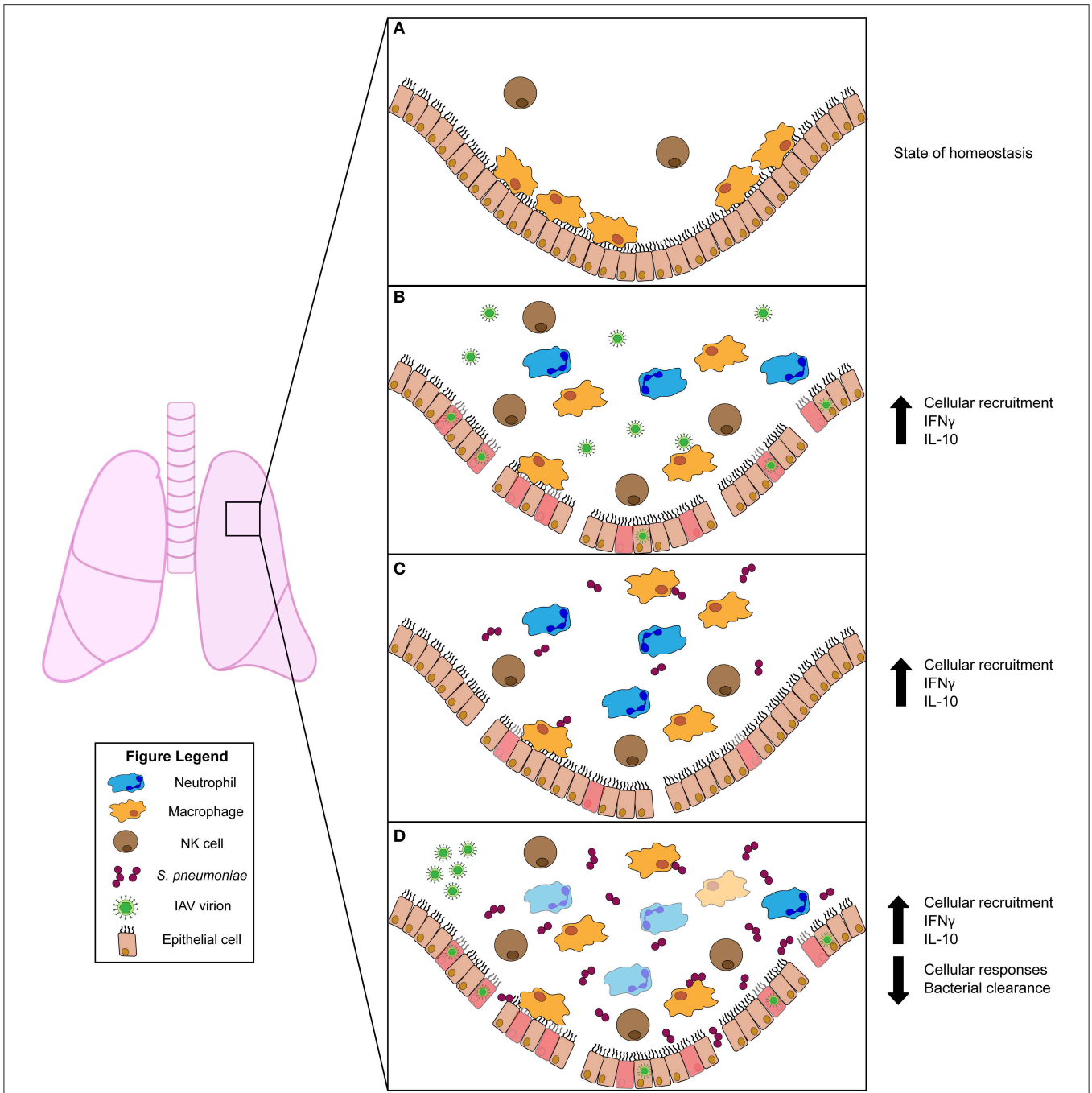

FIGURE 1 | Innate immune responses to viral and/or bacterial infection in the lung. **(A)** Uninfected lung in homeostatic state harbors resident alveolar macrophages (AMs) and natural killer (NK) cells. **(B)** Influenza A virus (IAV) infection activates AMs and NK cells. Infected cells produce chemokines that recruit inflammatory phagocytic cells (such as neutrophils) to aid in clearance. The pro-inflammatory cytokine IFNγ is induced during IAV infection but has conflicting roles in animal studies. The anti-inflammatory cytokine IL-10 is produced and plays a role in regulating lung inflammation. **(C)** *Streptococcus pneumoniae* infection also stimulates activation of AM and NK cells. NK cells produce IFNγ, which plays a protective role for the host. IL-10 is also induced and modulates the influx of neutrophils into the lung. **(D)** Primary IAV infection predisposes the host to secondary *S. pneumoniae* infection. This co-infection drives increased bacterial burdens in the lung. Increased burdens correlate with diminished bacterial clearance by AMs, which is attributed to stimulation of AMs by IFNγ. Similar to *S. pneumoniae* single infection, IL-10 is also induced during co-infection. This suppresses excessive neutrophil accumulation and subsequent lung damage.

(12). In addition, co-infection by different influenza viruses increases genetic diversity through reassortment of viral genome segments (12). These, and other factors result in antigenic and pathogenic diversity which limits effectiveness of vaccination. Consequently, influenza viruses continue to pose a serious health risk to the human population with over 3,000,000 cases and up to

650,000 deaths per year globally (average of 25,000–36,000 in the US)[3] (6, 13).

Upon surpassing the host's initial physical and chemical barriers to infection (i.e., mucosal layer), influenza virus invades and replicates in lung epithelial cells. Mucus production offers some resistance to IAV infection, but resolution of infection requires innate immune responses (14). Indeed, viral replication in lung epithelial cells leads to cytokine production and activation of AMs that contribute to the initial control of infection (**Figure 1B**). Thus, mice lacking AMs had increased pulmonary viral burdens and mortality (15, 16). However, AMs are significantly reduced by 4 days after IAV infection (17, 18). Such reductions may partly be driven by cytolytic NK cells, which can recognize and target IAV-infected cells (19, 20). Indeed, activating natural cytotoxicity (NCRs) and other receptors on NK cells have been shown to bind IAV hemagglutinin proteins. When these proteins are expressed at the surface of virus-infected cells, this recognition can induce NK cells to lyse the infected target cell (19–21). Such lysis, and possibility other NK cell effector functions, contribute to early protection, since NK cell depletion increases lung damage and mortality (22). Chemokines produced by IAV-infected cells, such as CCL-1 (MCP), further protect the host by recruiting inflammatory phagocytes that help control infection (23, 24). Recruitment of neutrophils to the lung is further induced by IL-1 and plays an important role in reducing viral replication (25). Thus, effective induction of innate immune responses is critical for host resistance to IAV.

Excessive, inflammatory cell recruitment and the induction of a "cytokine storm" are hallmarks of more severe IAV infection and lung disease. These responses can be exacerbated by polymorphisms in TLRs or other PRRs. The cytokine proteins contributing to this storm include the type I IFNS, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-15, and the only type II IFN, IFNγ (25–28). Several of these cytokines appear to be beneficial to the host by contributing to host resistance. For example, mice deficient for the interferon alpha/beta receptor, IFNAR, that mediates cellular responses to type I IFNs show increased morbidity and mortality (26, 29). However, excessive production of specific cytokines drives excessive and detrimental inflammation. In particular, IFNγ can exacerbate disease severity during IAV infection. Indeed, mice lacking either expression of IFNγ or the ligand-binding subunit of its receptor, IFNGR1, showed increased survival following IAV challenge (18, 30) The increased survival in mice lacking IFNγ was shown to be associated with reduced immunopathology due to increased activity of type II innate lymphoid cells (ILC2s), which produce IL-5 and amphiregulin to promote tissue homeostasis (30). In this context, IL-5 elicited eosinophils and was required for enhanced survival of the mice lacking IFNγ. In mice lacking IFNGR1, inflammatory infiltrates and cytokine production were also reduced (18). However, in mice lacking IFNGR1 expression viral titers were reduced at 6-8 days after infection, whereas no differences in viral burden were observed up to 9 days after infection in these mice (18, 30) The reasons for this discrepancy are not clear, but could reflect the use of different IAV strains and infection timelines in these studies. Regardless, this collective data support the conclusion that IFNγ drives increased inflammation and lung damage during IAV infection (**Figure 1B**). Yet, other reports showed therapeutic effects of administering recombinant IFNγ early during IAV infection and demonstrated important protective roles for endogenous IFNγ against IAV during a recall infection or in mice lacking *Nos2* expression (31–33). In one study, this protection was attributed to improved NK cell responses (33). Thus, IFNγ signaling to specific cell types and/or in specific settings can have both beneficial or detrimental roles in the response to IAV infection.

Given the potential detrimental effects of pro-inflammatory cytokines such as IFNγ, it is not surprising that anti-inflammatory cytokines are also key regulators of lung damage during IAV infection. IL-10 is a key anti-inflammatory cytokine implicated during IAV infection. A variety of immune cell types can produce IL-10 and respond to this cytokine through expression of the cognate receptor. IL-10 signaling activates STAT3 and other signaling pathways to suppress production of pro-inflammatory factors such as IL-12 and IFNγ (34, 35). In the context of murine IAV infection, IL-10 is important for dampening the pro-inflammatory cytokine response and subsequent pulmonary damage to increase survival of IAV-infected mice (26) (**Figure 1B**). Thus, the balance of IFNγ and IL-10 responses could be a key determinant of the outcome during IAV infection, with too little IL-10 tipping the balance to excessive IFNγ, inflammation and increased disease severity.

STREPTOCOCCUS PNEUMONIAE BACTERIAL INFECTION IN THE LUNG

Pulmonary infections are caused by both pathobiont (i.e., asymptomatically residing bacteria with pathogenic potential) and pathogenic (invasive) bacterial species, such as *S. pneumoniae*. *S. pneumoniae* (aka pneumococcus) transiently colonizes the nasopharynx asymptomatically in healthy humans with colonization rates highest in children[3,4] However, this Gram-positive pathobiont causes ∼50% of otitis media cases and is the most common cause of bacterial pneumonia in humans (36). *S. pneumoniae* can also establish invasive septicemia and meningitis with high mortality rates. In developed countries, pneumoccocal disease rates have dropped considerably in recent years due to vaccination. Nevertheless, nearly 900,000 people develop pneumococcal pneumonia each year in the United States and this remains an important cause of morbidity and mortality globally seen in immune compromised, elderly adults and particularly causing nearly 810,000 deaths in children under 5[3,4].

Pneumonia occurs when a colonizing *S. pneumoniae* strain gains access to the lower respiratory tract. Such access is promoted by inflammatory events, which likely contribute to increased density of colonizing *S. pneumoniae*. Consistent with this, polymorphisms in PRRs has been associated with increased colonization and/or invasive infection by *S. pneumoniae* (37, 38). Inflammation is thought to reflect an increased nutrient

[3]https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal).

[4]https://www.who.int/en/news-room/fact-sheets/detail/pneumonia.

availability following inflammation-driven epithelial damage and increased access of bacteria to adhesion receptors such as those for platelet-activating factor (PAFr) or polymeric immunoglobulin (pIgR), which are upregulated in response to inflammatory cytokines (39–41). Inflammation thus increases the density of *S. pneumoniae* in the nasopharynx and thus provides an opportunity for increased aerosolization of bacteria into the lungs (for host-derived pneumonia) and environment (for transmission). These or other effects of inflammation may partly explain the increased incidence of pneumococcal pneumonia in individuals with a primary respiratory viral infection, elderly individuals, or other populations (see further information below) (42).

Despite the evidence that aspects of inflammation promote *S. pneumoniae* colonization, murine infection models have demonstrated protective roles for certain inflammation-associated responses. Two studies reported that the type I IFN response protects mice from colonization and invasive infection following intranasal infection by a serotype 2 strain of *S. pneumoniae* (43, 44). However, a third report using the different bacterial serotype 3 strain correlated type I IFNs with increased lung bacterial burdens (29). Whether these differing results indicate distinct roles for type I IFNs in protection of distinct tissues or reflect use of distinct *S. pneumoniae* isolates remains unclear. IFNγ also appears to protect mice against pulmonary *S. pneumoniae.* Early work found that mice lacking IFNγ were more susceptible to bacteremia and mortality following intranasal infection (45). Treatment with IL-12 was subsequently shown to induce NK cell production of IFNγ and protect mice against pulmonary *S. pneumoniae* (46). However, the overall impact of NK cells in this setting may not be beneficial as NK cell depletion lowered lung bacterial burdens in infected *Scid* mice with no effect on burdens in controls (47). NK cell depletion likewise reduced survival of mice infected systemically with another streptococcus strain, *S. suis* (48). Yet, a more recent study using a genetic diptheria toxin (DT)-based approach to deplete NKp46+ NK cells found that this manipulation reduced mouse survival following pulmonary *S. pneumoniae* infection (49). Effects of the DT-induced NK cell depletion on bacterial burdens was not reported in the latter study, but the authors showed a transfer of wildtype NK cells improved survival in mice lacking the four-and-a-half LIM-only protein 2 (FHL2) significantly better than transfer of IFNγ-deficient NK cells. Though NK cell specific IFNγ improved survival in this setting, the impact of NK cell IFNγ in wildtype mice is not yet clear. Overall, IFNγ appears to play important roles during *S. pneumoniae* infection (**Figure 1C**).

In humans and in murine models, vaccination against pneumococcal capsular polysaccharides or killed bacteria reduces colonization and transmission of *S. pneumoniae*. In mice, vaccine-induced protection was shown to be mediated by antibody or T cell immune responses (50, 51). Together with complement, opsonizing antibodies increase the ability of neutrophils and other phagocytes to engulf and kill encapsulated pneumococci. Consistent with the importance of neutrophils, IL-17 production and neutrophil recruitment to the lungs reduce bacterial burdens (52). Moreover, protection in IL-12 treated mice correlates with increased neutrophil recruitment or survival in *S. pneumoniae*-infected lungs (46). Nevertheless, excessive neutrophil recruitment can damage lung function and increase mortality. IL-10 has been shown to dampen the influx of these inflammatory cells, as well as production of pro-inflammatory cytokines such as TNF-α, to reduce tissue damage and mortality during infection (53, 54) (**Figure 1C**). Supporting the interpretation that this is a key role for IL-10, mortality was increased in mice deficient for IL-10 despite reduced bacterial burdens in the lung and reduced bacterial dissemination (53). Thus, therapeutic strategies that mimic or induce IL-10 may reduce damage to lungs or other vital host tissues, though at risk of increasing bacterial burdens.

VIRAL-BACTERIAL CO-INFECTION IN THE LUNG

Influenza virus infection predisposes the host to severe disease outcomes during co-infection with *S. pneumoniae*. In this context, viral infection appears to both increase the incidence and the severity of secondary bacterial infections clinically. They are associated with high morbidity and mortality in the context of seasonal flu and were a major correlate of death during 1918 Spanish Flu and 2009 H1N1 pandemics (55–57).

A number of studies have modeled IAV/*S. pneumoniae* co-infection in mice. Importantly, IAV enhances susceptibility to multiple *S. pneumoniae* serotypes with more virulent strains exhibiting the highest susceptibility (58). Results of these studies suggest diverse mechanisms contribute to the enhanced susceptibility to secondary bacterial challenge following IAV infection. These mechanisms likely include damage to the lung epithelial barrier, which can permit increased bacterial crossing of the epithelium and may both increase nutrient availability and expose host adhesions such as PAFr or pIgR to increase bacterial numbers (59–61) (**Figure 1D**). A recent study additionally proposed that viral adherence to the *S. pneumoniae* surface promotes adhesion to respiratory epithelia (62). Other possible detrimental effects of IAV infection on airway physiology include altered mucus production, reduced ciliary beating and alteration of the host microbiome (63–66). Albeit, there have been conflicting results regarding the impact of influenza infection on the respiratory tract microbiome (67, 68). Yet, both murine and recent human studies agree that an initial influenza (or live-attenuated vaccine) exposure increases susceptibility to secondary (or colonizing) *S. pneumoniae* (69, 70).

IAV-driven alteration of lung immune defenses have also been implicated in increased susceptibility to *S. pneumoniae*. Susceptibility and severity of secondary bacterial infections might be impacted by TNF and IL-1β production, which increase expression of *S. pneumoniae* adhesion receptors such as PAFr and pIgR. However, in a mouse model system, pneumococci administered 7 days after IAV (when reductions in viral burdens were first observed) induced less TNF and IL1β compared to non-IAV-infected mice (71). This study attributed increased susceptibility to an impaired early bacterial clearance from the lung by AMs (71). Following IAV infection, these and other phagocytes showed reduced effectiveness at engulfing bacteria that correlated with the onset of T cell-dependent IFNγ

production. Further, burdens of *S. pneumoniae* at 4 h after infection (9 days after IAV) were ∼50% lower in lungs of IFNγ or IFNGR1-deficient mice than in co-infected control mice. Genetic deficiency for IFNγ or IFNGR1 or neutralization of IFNγ also improved survival from secondary pneumococcal infection. These effects correlated with IFNγ-dependent reductions in staining for MARCO on lavaged CD11c+ cells. The MARCO scavenger receptor was previously implicated in the engulfment of non-opsonized *S. pneumoniae* bacteria by AMs (72). However, while IFNγ stimulation of myeloid cells has been associated with increased phagocytic and bactericidal activity of other bacteria, it has not been demonstrated that detrimental effects of IFNγ in the IAV/*S. pneumoniae* co-infection model were due to IFNγ targeting of myeloid cells (73–75). Still, there are other lines of evidence supporting the notion that suppression of myeloid cell activity is an important mechanism driving increased susceptibility in IAV-infected animals. Specifically, the increased susceptibility correlates with increases in expression of CD200R, a negative regulator of myeloid cell function (63). Additionally, co-infection induces production of anti-inflammatory IL-10 that suppresses excessive neutrophil accumulation and host resistance (76) (**Figure 1D**). Type I IFNs also significantly increased the bacterial burdens following secondary exposure to *S. pneumoniae*, with little to no effect on viral burdens (29). Here, IFNAR expression correlated with reduced production of chemoattractant CXCL2 and impaired recruitment of neutrophils to the lungs. Thus, inflammatory responses elicited by IAV infection and the induction of endogenous mechanisms for dampening these responses may collectively impair myeloid cell antibacterial activity to exacerbate pneumococcal infections.

INFLAMMATION AND PREDISPOSITION TO LUNG INFECTIONS

Altered or constitutive inflammatory responses are observed in elderly individuals and in individuals with genetic predispositions such as Down syndrome (DS). These responses may contribute to the high frequency and severity of IAV and *S. pn*eumoniae infections in these populations (11, 77–80). Indeed, DS appears to accelerate aging-associated cellular processes and the health phenotype of individuals with DS overlaps with that of older non-DS individuals (81). Thus, improved understanding of inflammatory responses to lung infections in the context of DS may also provide insights into the causes and possible treatments for these infections in the elderly.

In the context of DS, production of inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8, IFNα, and IFNγ were elevated in blood samples from DS vs. sibling donors following an *ex vivo* treatment with IAV (82). In this study, expression of anti-inflammatory IL-10 was not altered in the DS cohort (82). However, IL-10 production was greater in DS blood cells following an *ex vivo* stimulation with *S. pneumoniae* (83). Thus, DS may predispose toward elevated IL-10 production during *S. pneumoniae* infection, which could dampen myeloid cell antibacterial functions and contribute to elevated bacterial burdens in DS patients, similarly observed in mice (54). However, IFNγ was also found to be increased in individuals with DS at specific time points following IAV stimulation (82, 84). This correlates with a trend toward basally increased IFNγ in DS individuals. Nevertheless, elevated production of IFNγ or other cytokines could have detrimental effects on resistance as described in the murine IAV-*S. pneumoniae* co-infection studies discussed above (**Figure 1**). Impairment of neutrophil function has also been shown in otherwise healthy individuals with DS (85) Thus, altered inflammatory responses could contribute to susceptibility to lung infections in DS (and elderly) individuals. However, it should be noted that other non-immune mechanisms may contribute to the susceptibility in the DS (and elderly) populations. For example, in the context of DS congenital abnormalities of the respiratory tract and altered ciliary function have also been reported (86).

CONCLUSIONS

Pulmonary infections caused by bacterial or viral pathogens are a serious clinical problem to the global human population. This clinical problem is even more concerning for specific susceptible groups including children, the elderly, and individuals with underlying genetic conditions that include Down syndrome. Co-infections of viral and bacterial pathogens can also increase susceptibility and disease severity in the broader immune-competent human population. Exacerbated or altered innate immune and inflammatory responses are characteristic of the above-mentioned susceptible groups and likely play important roles in defining disease outcome. Conflicting results and the difficulty of extrapolating from animal models of infection to human therapy remain and should be considered in the context of efforts to identify and implement specific and effective treatments. Thus, better defining the regulation of lung innate immune responses in susceptible populations and in the context of complex environmental elements (such as the microbiota) are needed to provide avenues for development of new treatments. It is also important to keep in mind the need to appropriately tune the immune and inflammatory mechanisms to minimize damage to lung tissue while ensuring adequate resistance to infections by various pathogen types.

AUTHOR CONTRIBUTIONS

EA and LL wrote and edited the manuscript.

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REFERENCES

1. Hasenberg M, Stegemann-Koniszewski S, Gunzer M. Cellular immune reactions in the lung. *Immunol. Rev.* (2013) 251:189–214. doi: 10.1111/imr.12020
2. Tong Y, Lighthart B. The annual bacterial particle concentration and size distribution in the ambient atmosphere in a rural area of the Willamette Valley, Oregon. *Aerosol Sci Technol*. (2000) 32:393–403. doi: 10.1080/027868200303533
3. Huffnagle GB, Dickson RP. The bacterial microbiota in inflammatory lung diseases. *Clin Immunol*. (2015) 159:177–82. doi: 10.1016/j.clim.2015.05.022
4. Bustamante-Marin XM, Ostrowski LE. Cilia and mucociliary clearance. *Cold Spring Harbor Persp Biol*. (2017) 9:a028241. doi: 10.1101/cshperspect.a028241
5. Morris DE, Cleary DW, Clarke SC. Secondary bacterial infections associated with influenza pandemics. *Front Microbiol*. (2017) 8:1041. doi: 10.3389/fmicb.2017.01041
6. Thompson WW, Shay DK, Weintraub E, Cox N, Anderson LJ, Fukuda K. Mortality associated with influenza and respiratory syncytial virus in the United States. *J Am Med Assoc*. (2003) 289:179–86. doi: 10.1001/jama.289.2.179
7. McCullers JA. The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat. Rev Microbiol*. (2014) 12:252–62. doi: 10.1038/nrmicro3231
8. Alshabani K, Haq A, Miyakawa R, Palla M, Soubani AO. Invasive pulmonary aspergillosis in patients with influenza infection: Report of two cases and systematic review of the literature. *Exp Rev Resp Med*. (2015) 9:89–96. doi: 10.1586/17476348.2015.996132
9. Kesh S, Mensah NY, Peterlongo P, Jaffe D, Hsu K, Van Den Brink M, et al. TLR1 and TLR6 polymorphisms are associated with susceptibility to invasive aspergillosis after allogeneic stem cell transplantation. *Ann N Y Acad Sci*. (2005) 1062:95–103. doi: 10.1196/annals.1358.012
10. Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. *Nat Rev Immunol*. (2014) 14:315–28. doi: 10.1038/nri3665
11. Hilton JM, Fitzgerald DA, Cooper DM. Respiratory morbidity of hospitalized children with Trisomy 21. *J Paediatr Child Health*. (1999) 35:383–6. doi: 10.1046/j.1440-1754.1999.00386.x
12. Taubenberger JK, Morens DM. The pathology of influenza virus infections. *Ann Rev Pathol*. (2008) 3:499–522. doi: 10.1146/annurev.pathmechdis.3.121806.154316
13. Thompson WW, Weintraub E, Dhankhar P, Cheng PY, Brammer L, Meltzer MIMI, et al. (2009). Estimates of US influenza-associated deaths made using four different methods. *Influ Other Respir Viruses*. (2008) 3:37–49. doi: 10.1111/j.1750-2659.2009.00073.x
14. Ehre C, Worthington EN, Liesman RM, Grubb BR, Barbier D, O'Neal WK, et al. Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs. *Proc Natl Acad Sci USA*. (2012) 109:16528–33. doi: 10.1073/pnas.1206552109
15. Schneider C, Nobs SP, Heer AK, Kurrer M, Klinke G, van Rooijen N, et al. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLoS Pathog*. (2014) 10:1004053. doi: 10.1371/journal.ppat.1004053
16. Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ, et al. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. *J Virol*. (2005) *79*:14933–44. doi: 10.1128/JVI.79.23.14933-14944.2005
17. Ghoneim HE, Thomas PG, McCullers JA. Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections. *J Immunol*. (2013) 191:1250–9. doi: 10.4049/jimmunol.1300014
18. Nicol MQ, Campbell GM, Shaw DJ, Dransfield I, Ligertwood Y, Beard PM, et al. (2019). Lack of IFNγ signaling attenuates spread of influenza A virus *in vivo* and leads to reduced pathogenesis. *Virology*, *526*, 155–164. doi: 10.1016/j.virol.2018.10.017
19. Achdout H, Meningher T, Hirsh S, Glasner A, Bar-On Y, Gur C, et al. Killing of avian and swine influenza virus by natural killer cells. *J Virol*. (2010) 84:3993–4001. doi: 10.1128/JVI.02289-09
20. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, Bushkin Y, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. (2001) 409:1055–60. doi: 10.1038/35059110
21. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Euro J Immunol*. (2001) 31:2680–9. doi: 10.1002/1521-4141(200109)31:9<2680::AID-IMMU2680>3.0.CO;2-A
22. Stein-Streilein J, Guffee J. *In vivo* treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *J Immunol*. (1986) 136:1435–41.
23. Dawson TC, Beck MA, Kuziel WA, Henderson F, Maeda N. Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. *Am J Pathol*. (2000) 156:1951–9. doi: 10.1016/S0002-9440(10)65068-7
24. Herold S, von Wulffen W, Steinmueller M, Pleschka S, Kuziel WA, Mack M, et al. Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. *J Immunol*. (2006) 77:1817–24. doi: 10.4049/jimmunol.177.3.1817
25. Schmitz N, Kurrer M, Bachmann MF, and Kopf M. Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. *J Virol*. (2005) 79:6441–8. doi: 10.1128/JVI.79.10.6441-6448.2005
26. Arimori Y, Nakamura R, Yamada H, Shibata K, Maeda N, Kase T, et al. Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral Res*. (2013) 99:230–7. doi: 10.1016/j.antiviral.2013.05.007
27. Imai Y, Kuba K, Neely GG, Yaghubian-Malhami R, Perkmann T, van Loo G, et al. Identification of oxidative stress and toll-like receptor 4 signaling as a key pathway of acute lung injury. *Cell*. (2008) 133:235–49. doi: 10.1016/j.cell.2008.02.043
28. To KKW, Hung IFN, Li IWS, Lee K, Koo C, Yan W, et al. Delayed clearance of viral load and marked cytokine activation in severe cases of pandemic H1N1 2009 influenza virus infection. *Clin Infect Dis*. (2010) 50:850–9. doi: 10.1086/650581
29. Shahangian A, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY, et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest*. (2009) 119:1910–20. doi: 10.1172/JCI35412
30. Califano D, Furuya Y, Metzger DW. Effects of influenza on alveolar macrophage viability are dependent on mouse genetic strain. *J Immunol*. (2018) 201:134–44. doi: 10.4049/jimmunol.1701406
31. Bot A, Bot S, Bona CA. Protective role of gamma interferon during the recall response to influenza virus. *J Virol*. (1998) 72:6637–45. doi: 10.1128/JVI.72.8.6637-6645.1998
32. Karupiah G, Chen JH, Mahalingam S, Nathan CF, MacMicking JD. Rapid interferon γ-dependent clearance of influenza a virus and protection from consolidating pneumonitis in nitric oxide synthase 2- deficient mice. *J Exp Med*. (1998) 188:1541–6. doi: 10.1084/jem.188.8.1541
33. Weiss ID, Wald O, Wald H, Beider K, Abraham M, Galun E, et al. IFN-γ treatment at early stages of influenza virus infection protects mice from death in a NK cell-dependent manner. *J Interferon Cytokine Res*. (2010) 30:439–49. doi: 10.1089/jir.2009.0084
34. Couper KN, Blount DG, and Riley EM. IL-10: the master regulator of immunity to infection. *J Immunol*. (2008) 180:5771–7. doi: 10.4049/jimmunol.180.9.5771
35. Shouval DS, Ouahed J, Biswas A, Goettel JA, Horwitz BH, Klein C, et al. Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol*. (2014) 122:177–210. doi: 10.1016/B978-0-12-800267-4.00005-5
36. Donkor ES. Understanding the pneumococcus: transmission and evolution. *Front Cell Infect Microbiol*. (2013) 4:7. doi: 10.3389/fcimb.2013.00007
37. Vuononvirta J, Toivonen L, Gröndahl-Yli-Hannuksela K, Barkoff AM, Lindholm L, Mertsola J, et al. Nasopharyngeal bacterial colonization and gene polymorphisms of mannose-binding lectin and toll-like receptors 2 and 4 in infants. *PLoS ONE*. (2011) 6:e26198. doi: 10.1371/journal.pone.0026198
38. Yuan FF, Marks K, Wong M, Watson S, De Leon E, McIntyre PB, et al. Clinical relevance of TLR2, TLR4, CD14 and FcγRIIA gene polymorphisms in *Streptococcus pneumoniae* infection. *Immunol Cell Biol*. (2008) 86:268–70. doi: 10.1038/sj.icb.7100155
39. Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature*. (1995) 377:435–8. doi: 10.1038/377435a0

40. Rijneveld AW, Weijer S, Florquin S, Speelman P, Shimizu T, Ishii S, et al. Improved host defense against pneumococcal pneumonia in platelet-activating factor receptor–deficient mice. *J Infect Dis*. (2004) 189:711–6. doi: 10.1086/381392
41. Zhang JR, Mostov KE, Lamm ME, Nanno M, Shimida S, Ohwaki M, et al. The polymeric immunoglobulin receptor translocates pneumococci across nasopharyngeal human epithelial cells. *Cell*. (2000) 102:827–37. doi: 10.1016/S0092-8674(00)00071-4
42. Puchta A, Naidoo A, Verschoor CP, Loukov D, Thevaranjan N, Mandur TS, et al. TNF drives monocyte dysfunction with age and results in impaired anti-pneumococcal immunity. *PLoS Pathogens*. (2016) 12:1005368. doi: 10.1371/journal.ppat.1005368
43. LeMessurier KS, Häcker H, Chi L, Tuomanen E, and Redecke V. Type I interferon protects against pneumococcal invasive disease by inhibiting bacterial transmigration across the lung. *PLoS Pathog*. (2013) 9:e1003727. doi: 10.1371/journal.ppat.1003727
44. Parker D, Martin FJ, Soong G, Harfenist BS, Aguilar JL, Ratner AJ, et al. (2011). *Streptococcus pneumoniae* DNA initiates type I interferon signaling in the respiratory tract. *mBio*, 2. doi: 10.1128/mBio.00016-11
45. Rubins JB, Pomeroy C. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infect Immunity*. (1997) 65:2975–7. doi: 10.1128/IAI.65.7.2975-2977.1997
46. Sun K, Salmon SL, Lotz SA, and Metzger DW. Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory *Streptococcus pneumoniae* infection. *Infect Immunity*. (2007) 75:1196–202. doi: 10.1128/IAI.01403-06
47. Kerr AR, Kirkham LAS, Kadioglu A, Andrew PW, Garside P, Thompson H, et al. Identification of a detrimental role for NK cells in pneumococcal pneumonia and sepsis in immunocompromised hosts. *Microbes Infect*. (2005) 7:845–52. doi: 10.1016/j.micinf.2005.02.011
48. Lemire P, Galbas T, Thibodeau J, Segura M. Natural killer cell functions during the innate immune response to pathogenic streptococci. *Front Microbiol*. (2017) 8:1196. doi: 10.3389/fmicb.2017.01196
49. Baranek T, Morello E, Valayer A, Aimar RF, Bréa D, Henry C, et al. FHL2 regulates natural killer cell development and activation during *Streptococcus pneumoniae* infection. *Front Immunol*. (2017) 8:123. doi: 10.3389/fimmu.2017.00123
50. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, and Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc Natl Acad Sci USA*. (2005) 102:4848–53. doi: 10.1073/pnas.0501254102
51. Roche AM, Richard AL, Rahkola JT, Janoff EN, Weiser JN. Antibody blocks acquisition of bacterial colonization through agglutination. *Mucosal Immunol*. (2015) 8:176–85. doi: 10.1038/mi.2014.55
52. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest*. (2009) 119:1899–909. doi: 10.1172/JCI36731
53. Peñaloza HF, Nieto PA, Muñoz-Durango N, Salazar-Echegarai FJ, Torres J, Parga MJ, et al. Interleukin-10 plays a key role in the modulation of neutrophils recruitment and lung inflammation during infection by *Streptococcus pneumoniae*. *Immunology*. (2015) 146:100–12. doi: 10.1111/imm.12486
54. Van Der Poll T, Marchant A, Keogh CV, Goldman M, and Lowry SF. Interleukin-10 impairs host defense in murine. *J Infect Dis*. (1996) 174:994–1000. doi: 10.1093/infdis/174.5.994
55. Brundage JF, Shanks GD. Deaths from bacterial pneumonia during 1918 19 influenza pandemic. *Emerg Infect Dis*. (2008) 14:1193–9. doi: 10.3201/eid1408.071313
56. Morens DM, Taubenberger JK, Fauci AS. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis*. (2008) 198:962–70. doi: 10.1086/591708
57. Shieh WJ, Blau DM, Denison AM, DeLeon-Carnes M, Adem P, Bhatnagar J, et al. 2009 pandemic influenza A (H1N1): pathology and pathogenesis of 100 fatal cases in the United States. *Am J Pathol*. (2010) 177:166–75. doi: 10.2353/ajpath.2010.100115
58. Sharma-Chawla N, Sender V, Kershaw O, Gruber AD, Volckmar J, Henriques-Normark B, et al. Influenza A virus infection predisposes hosts to secondary infection with different *Streptococcus pneumoniae* serotypes with similar outcome but serotype-specific manifestation. *Infect Immun*. (2016) 84:3445–57. doi: 10.1128/IAI.00422-16
59. Eisenhut M. Influenza Virus Amplifies Interaction Of Polymeric Immunoglobulin Receptor With Pneumococcal Surface Protein A, which mediates invasion by pneumococcus. *J Infect Dis*. (2010) 201:1272–3. doi: 10.1086/651432
60. McCullers JA, Rehg JE. Lethal synergism between influenza virus and *Streptococcus pneumoniae*: characterization of a mouse model and the role of platelet-activating factor receptor. *J Infect Dis*. (2002) 186:341–50. doi: 10.1086/341462
61. Van Der Sluijs KF, Van Elden LJR, Nijhuis M, Schuurman R, Florquin S, Shimizu T, et al. Involvement of the platelet-activating factor receptor in host defense against *Streptococcus pneumoniae* during postinfluenza pneumonia. *Am J Physiol*. (2006) 172:7603–09. doi: 10.1152/ajplung.00050.2005
62. Rowe HM, Meliopoulos VA, Iverson A, Bomme P, Schultz-Cherry S, and Rosch JW. Direct interactions with influenza promote bacterial adherence during respiratory infections. *Nat Microbiol*. (2019) 4:1328–36. doi: 10.1038/s41564-019-0447-0
63. Goulding J, Godlee A, Vekaria S, Hilty M, Snelgrove R, and Hussell T. Lowering the threshold of lung innate immune cell activation alters susceptibility to secondary bacterial superinfection. *J Infect Dis*. (2011) 204:1086–94. doi: 10.1093/infdis/jir467
64. Hanada S, Pirzadeh M, Carver KY, and Deng JC. (2018). Respiratory viral infection-induced microbiome alterations and secondary bacterial pneumonia. *Front Immunol*. (2011) 9:2640. doi: 10.3389/fimmu.2018.02640
65. Lee KH, Gordon A, Shedden K, Kuan G, Ng S, Balmaseda A, et al. The respiratory microbiome and susceptibility to influenza virus infection. *PLoS ONE*. (2019) 14:e207898. doi: 10.1371/journal.pone.0207898
66. Yildiz S, Mazel-Sanchez B, Kandasamy M, Manicassamy B, and Schmolke M. Influenza A virus infection impacts systemic microbiota dynamics and causes quantitative enteric dysbiosis. *Microbiome*. (2018) 6:9. doi: 10.1186/s40168-017-0386-z
67. Ding T, Song T, Zhou B, Geber A, Ma Y, Zhang L, et al. Microbial composition of the human nasopharynx varies according to influenza virus type and vaccination status. *mBio*. (2019) 10:4. doi: 10.1128/mBio.01296-19
68. Ramos-Sevillano E, Wade WG, Mann A, Gilbert A, Lambkin-Williams R, Killingley B, et al. The effect of influenza virus on the human oropharyngeal microbiome. *Clin Infect Dis*. (2019) 68:1993–2002. doi: 10.1093/cid/ciy821
69. de Steenhuijsen Piters WAA, Jochems SP, Mitsi E, Rylance J, Pojar S, Nikolaou E, et al. Interaction between the nasal microbiota and S. pneumoniae in the context of live-attenuated influenza vaccine. *Nat Commun*. (2019) 10:2981. doi: 10.1038/s41467-019-10814-9
70. Rylance J, de Steenhuijsen Piters WAA, Mina MJ, Bogaert D, French N, and Ferreira DM. Two randomized trials of the effect of live attenuated influenza vaccine on pneumococcal colonization. *Am J Resp Crit Care Med*. (2019) 199:1160–3. doi: 10.1164/rccm.201811-2081LE
71. Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-γ during recovery from influenza infection. *Nat Med*. (2008) 14:558–64. doi: 10.1038/nm1765
72. Arredouani M, Yang Z, Ning YY, Qin G, Soininen R, Tryggvason K, et al. The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles. *J Exp Med*. (2004) 200:267–72. doi: 10.1084/jem.20040731
73. Crisler WJ, Lenz LL. Crosstalk between type I and II interferons in regulation of myeloid cell responses during bacterial infection. *Curr Opin Immunol*. (2018) 54:35–41. doi: 10.1016/j.coi.2018.05.014
74. Eshleman EM, Delgado C, Kearney SJ, Friedman RS, Lenz LL. Down regulation of macrophage IFNGR1 exacerbates systemic L. monocytogenes infection. *PLoS Pathog*. (2017) 13:e1006388. doi: 10.1371/journal.ppat.1006388
75. Martinez FO, Helming L, and Gordon S. Alternative activation of macrophages: an immunologic functional perspective. *Ann Rev Immunol*. (2009) 27:451–83. doi: 10.1146/annurev.immunol.021908.132532
76. van der Sluijs KF, van Elden LJR, Nijhuis M, Schuurman R, Pater JM, Florquin S, et al. IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *J Immunol*. (2004) 290:7603. doi: 10.4049/jimmunol.172.12.7603

77. Franceschi C, Garagnani P, Vitale G, Capri M, Salvioli S. Inflammaging and 'Garb-aging.' *Trends Endocrinol Metab*. (2017) 28:199–212. doi: 10.1016/j.tem.2016.09.005
78. Franceschi C, Campisi J. Chronic inflammation (Inflammaging) and its potential contribution to age-associated diseases. *J Gerontol.* (2014) 69:S4–9. doi: 10.1093/gerona/glu057
79. Pérez-Padilla R, Fernández R, García-Sancho C, Franco-Marina F, Aburto O, López-Gatell H, et al. Pandemic (H1N1) 2009 virus and down syndrome patients. *Emerg Infect Dis*. (2010) 16:1312–4. doi: 10.3201/eid1608.091931
80. Yang Q, Rasmussen SA, Friedman JM. Mortality associated with Down's syndrome in the USA from 1983 to 1997: a population-based study. *Lancet*. (2002) 359:1019–25. doi: 10.1016/S0140-6736(02)08092-3
81. Horvath S, Garagnani P, Bacalini MG, Pirazzini C, Salvioli S, Gentilini D, et al. Accelerated epigenetic aging in Down syndrome. *Aging Cell.* (2015) 14:491–5. doi: 10.1111/acel.12325
82. Broers CJM, Gemke RJBJ, Weijerman ME, Van Der Sluijs KF, Van Furth AM. Increased pro-inflammatory cytokine production in down syndrome children upon stimulation with live influenza a virus. *J Clin Immunol*. (2012) 32:323–9. doi: 10.1007/s10875-011-9625-4
83. Broers CJM, Gemke RJBJ, Morré SA, Weijerman ME, Van Furth AM. Increased production of interleukin-10 in children with Down syndrome upon *ex vivo* stimulation with *Streptococcus pneumoniae*. *Pediatr Res*. (2014) 75:109–13. doi: 10.1038/pr.2013.173
84. Torre D, Zeroli C, Ferrario G, Casalone R, Broggini M, Agrifoglio L, et al. Serum levels of gamma interferon in patients with Down's syndrome. *Infection*. (1995) 23:66–7. doi: 10.1007/BF01710065
85. Barroeta O, Nungaray L, Lopez-Osuna M, Armendares S, Salamanca F, Kretschmer RR. Defective monocyte chemotaxis in children with down's syndrome. *Pediatr Res*. (1983) 17:292–5. doi: 10.1203/00006450-198304000-00013
86. McDowell KM, Craven DI. Pulmonary complications of down syndrome during childhood. *J Pediatr*. (2011) 158:319–25. doi: 10.1016/j.jpeds.2010.07.023

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Inhibition of CXCR4 and CXCR7 Is Protective in Acute Peritoneal Inflammation

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Our previous studies revealed a pivotal role of the chemokine stromal cell-derived factor (SDF)-1 and its receptors CXCR4 and CXCR7 on migratory behavior of polymorphonuclear granulocytes (PMNs) in pulmonary inflammation. Thereby, the SDF-1-CXCR4/CXCR7-axis was linked with adenosine signaling. However, the role of the SDF-1 receptors CXCR4 and CXCR7 in acute inflammatory peritonitis and peritonitis-related sepsis still remained unknown. The presented study provides new insight on the mechanism of a selective inhibition of CXCR4 (AMD3100) and CXCR7 (CCX771) in two models of peritonitis and peritonitis-related sepsis by injection of zymosan and fecal solution. We observed an increased expression of SDF-1, CXCR4, and CXCR7 in peritoneal tissue and various organs during acute inflammatory peritonitis. Selective inhibition of CXCR4 and CXCR7 reduced PMN accumulation in the peritoneal fluid and infiltration of neutrophils in lung and liver tissue in both models. Both inhibitors had no anti-inflammatory effects in A_{2B} knockout animals (A_{2B}–/–). AMD3100 and CCX771 treatment reduced capillary leakage and increased formation of tight junctions as a marker for microvascular permeability in wild type animals. In contrast, both inhibitors failed to improve capillary leakage in A_{2B}–/– animals, highlighting the impact of the A_{2B}-receptor in SDF-1 mediated signaling. After inflammation, the CXCR4 and CXCR7 antagonist induced an enhanced expression of the protective A_{2B} adenosine receptor and an increased activation of cAMP (cyclic adenosine mono phosphate) response element-binding protein (CREB), as downstream signaling pathway of A_{2B}. The CXCR4- and CXCR7-inhibitor reduced the release of cytokines in wild type animals via decreased intracellular phosphorylation of ERK and NFκB p65. *In vitro*, CXCR4 and CXCR7 antagonism diminished the chemokine release of human cells and increased cellular integrity by enhancing the expression of tight junctions. These protective effects were linked with functional A_{2B}-receptor signaling, confirming our *in vivo* data. In conclusion, our study revealed new protective aspects of the pharmacological modulation of the SDF-1-CXCR4/CXCR7-axis during acute peritoneal inflammation in terms of the two hallmarks PMN migration and barrier integrity. Both anti-inflammatory effects were linked with functional adenosine A_{2B}-receptor signaling.

Keywords: PMN, neutrophil, SDF-1, stromal cell-derived factor, tight junction proteins, adenosine receptor A_{2B}

INTRODUCTION

Peritonitis and peritonitis-related sepsis are still associated with a high mortality for up to 40–60% (1). In the United States, sepsis is more common than myocardial infarction or colon cancer (2, 3). Despite decades of research, the underlying mechanisms are still not understood and therefore, there is still no functional treatment of sepsis possible (4–6). Sepsis is caused by an overshooting answer of the immune system on the infection, resulting in injuring its own organs. This acute pro-inflammatory response of the body is mainly driven by polymorphonuclear neutrophils (PMNs) as the first cells of the immune system to be recruited to the side of inflammation (7, 8). Accordingly, PMNs are considered as a prognostic marker for mortality in terms of sepsis (9) as they migrate from the circulatory system into the inflamed tissue.

Beside PMN migration, the second hallmark of sepsis is capillary leakage (10). Tight junction proteins (TJP) are intracellular adhesion complexes controlling paracellular permeability and are therefore involved in maintaining tissue homeostasis (11). More precisely, TJPs are located apically in polarized cells and regulate the passage of water, ions and molecules (12, 13) and are also involved in cellular signaling (14). Inflammation and hypoxia alter the integrity of the tissue and paracellular permeability (15–18), leading to the clinically observed tissue edema (19, 20).

In case of inflammation, the chemokine stromal cell-derived factor (SDF)-1 in the bone marrow decreases and PMNs are released into the vasculature to migrate to inflamed areas (21). SDF-1 has two receptors—CXCR4 and CXCR7—both widely expressed on hematopoietic and non-hematopoietic cells (22–24). Both SDF-1-receptors drive endothelial and epithelial transmigration of leukocytes during acute inflammation (23, 25, 26). Pharmacological inhibition of CXCR4 protects lung tissue and keeps tissue homeostasis during acute and chronic pulmonary inflammation by reducing infiltration of PMNs, respectively CXCR4-positive cells (25, 27). CXCR4 blockade improved stroke-related damage and reduced the blood-brain barrier disruption by reducing the release of inflammatory cytokines in the ischemic region (28). Also, CXCR7 regulated acute inflammatory and allergic-related edema formation by stabilizing the pulmonary epithelial barrier (29, 30).

Recent literature linked SDF-1 related signaling to a functional adenosine A_{2B}-receptor (25, 31). The nucleoside adenosine exerts its functions through four different adenosine receptors. The cell surface G protein-coupled adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 play a central role in various inflammatory diseases (15, 32–34). Activation of the A_{2B}-receptor plays a protective role in terms of tissue homeostasis and maintaining cellular barrier function during inflammation (15, 32, 35).

In contrast, patients on the intensive care unit (ICU) reveal altered expression of adenosine receptors and compromised ligand affinity (36, 37). Accordingly, if therapy of sepsis is linked to functional adenosine receptor signaling, the expression level and the functionality of the receptors should be evaluated to adapt and elaborate an individualized therapy.

Current literature demands the identification of subgroups of patients for a customized therapy (38, 39). In the presented study, we investigated the specific role of the SDF-1 receptors CXCR4 and CXCR7 during acute inflammatory peritonitis and peritonitis-related sepsis concerning the two hallmarks of acute inflammation, migration of PMNs and barrier permeability. Additional, we hypothesized that the protection through CXCR4 and CXCR7 antagonism depends on functional A_{2B}-receptors. To enlarge the impact of our study, we determined these aspects in a zymosan- and additionally in fecal-induced peritonitis.

MATERIALS AND METHODS

Animals

Mice were housed under pathogen-free conditions and on standard light-dark cycle. Mice were male and 8–12 weeks old (wild type: C57BL/6N; Charles River; Germany; A_{2B} knockout mice: $A_{2B}-/-$; kindly gift from Dr. Katya Ravid; Boston University; School of Medicine; Department of Biochemistry; USA). All animal protocols were approved by the Animal Care and Use Committee of the University of Tübingen.

Reagents

CCX771, the specific CXCR7 antagonist (10 mg/kg body weight [bw]; ChemoCentryx; USA), was injected subcutaneously and the specific CXCR4 antagonist AMD3100 (10 mg/kg bw; Sigma Aldrich; Germany) was administrated intraperitoneally (i.p.) 1 h before zymosan application (zymosan-A of Saccharomyces cerevisiae; 50 mg/kg bw; i.p. injection; Sigma-Aldrich; Germany).

Zymosan-Induced Peritonitis and Sepsis

Peritoneal inflammation was induced by zymosan application i.p. (1 mg per mouse; concentration: 1 mg/ml). Four hours after zymosan administration, 5 ml of PBS- were injected into the peritoneal cavity and 3 ml peritoneal fluid lavage were retrieved. After thoracotomy, blood samples were collected by right ventricle punctuation and the vascular system was flushed by 3 ml PBS- for blood-free organs. Peritoneal lavage and tissue (lung and liver) samples were removed for flow cytometry analysis and partly saved for subsequent experiments at −80°C.

Fecal-Induced Peritonitis and Sepsis

To prepare the fecal solution, we collected fecal dry pellets randomly from C57BL/6N male mice cages with same age and diet. Fecal material was pooled, diluted with normal saline to a concentration of 80 mg/ml, aliquoted and the same fecal stock solution used for this whole project. The fecal solution was injected intraperitoneally. After 4 h, peritoneal lavage, blood and organs were collected as described above.

RT-PCR

Total RNA was isolated from murine peritoneum, lungs and liver by using pegGOLD TriFast (Peqlab, Germany), and cDNA synthesis was performed by using a Bio-Rad iScript kit (Bio-Rad, Germany) according to the manufacturer's directions. We evaluated the gene expression of murine SDF-1, CXCR4, CXCR7,

A_1 adenosine receptor, A_{2A} adenosine receptor, A_3 adenosine receptor, A_{2B} adenosine receptor, CD73, TNFα, and IL6 by using RT-PCR and the following primers: SDF-1 (5′-GAG AGC CAC ATC GCC AGA G-3′ and 5′-TTT CGG GTC AAT GCA CAC TTG-3′), CXCR4 (5′-AGC ATG ACG GAC AAG TAC C-3′ and 5′-GAT GAT ATG GAC AGC CTT ACA C-3′), CXCR7 (5′-GGA GCC TGC AGC GCT CAC CG-3′ and 5′-CTT AGC CTG GAT ATT CAC CC-3′), A_1 (5′-ATT GTC ACT CAG CTC CCG C-3′ and 5′-TCA CCA GTA CAT TTC CGG GC-3′), A_{2A} (5′-TCA ACA GCA ACC TGC AGA AC-3′ and 5′-GGC TGA AGA TGG AAC TCT GC-3′), A_{2B} (5′-GCG TCC CGC TCA GGT ATA AA-3′ and 5′-CAG TGG AGG AAG GAC ACA CC-3′), A_3 (5′-GGG TTC CTG TAC TTC CTC TTG G-3′ and 5′-TCA ACC TCA GCC GCT TAT CC-3′), CD73 (5′-GTT CTC TCT GTT GGC GGT G-3′ and 5′-GGA TGC CAC CTC CGT TTA C-3′), TNFα (5′-GGA GCC TGC AGC GCT CAC CG-3′ and 5′-CTT AGC CTG GAT ATT CAC CC-3′), and IL6 (5′-GGA GCC TGC AGC GCT CAC CG-3′ and 5′-CTT AGC CTG GAT ATT CAC CC-3′). Gene levels of barrier integrity related proteins were evaluated by utilizing subsequent primers for murine occludin (OCLDN), tight junctions proteins 1–3 (TJP 1, 2, and 3), e-cadherin 1 (CDH1), and claudin (CLDN) 1, 3, and 5: OCLDN (5′- GTG GGA TAA GGA ACA CAT TT-3′ and 5′-GAC ACA TTT TTA ACC CAC TC-3′), TJP1 (5′-CCT TGG CCT AGC ATA CAC A-3′ and 5′-GAA ATC GTG CTG ATG TGC C- 3′), TJP2 (5′-CAG CAA GCA GAC CCT CAT C-3′ and 5′- TCC AGC TCA TTC CCG ATC C- 3′), TJP3 (5′-CGA CTA TGA GGA CAC CGA C-3′ and 5′-TGT CCC ATG ACC CAT CAG C- 3′), CDH1 (5′-CAG CTC CTT CCC TGA GTG-3′ and 5′-GCA CCC ACA CCA AGA TAC-3′), CLDN1 (5′- CCA CCA TTG GCA TGA AGT GC-3′ and 5′-AGA GGT TGT TTT CCG GGG AC-3′), CLDN3 (5′-CCT ACG ACC GCA AGG ACT AC-3′ and 5′-CTG GTA GTG GTG ACG GTA CG-3′), CLDN5 (5′- CCA CCA TTG GCA TGA AGT GC-3′ and 5′-AGA GGT TGT TTT CCG GGG AC-3′).

To reveal the gene expression of the human adenosine A_{2B}-receptor, IL6, and IL8, we used the following primers and performed RT-PCR: A_{2B} (5′-ATC TCC AGG TAT CTT CTC-3′ and 5′-GTT GGC ATA ATC CAC ACA G-3′), IL6 (5′-CCA CCA TCT ACT CCA TCA TCT TC-3′ and 5′-ACT TGT CCG TCA TGC TTC TC-3′), and IL8 (5′-AGC ACA GCC AGG AAG GCG AG-3′ and 5′-TCA TAG CCT GTG TTG GC-3′).

18S was used as house keeping gene (5′-GTA ACC CGT TGA ACC CCA TT-3′ and 5′-CCA TCC AAT CGG TAG TAG CG-3′).

Microvascular Permeability

Protein extravasation into the peritoneal lavage as a marker of capillary leakage was determined 4 h after zymosan, respectively 4 h after fecal-injection by using a BCA protein assay kit according to the standard protocol (Pierce; Thermo Fisher Scientific; Germany). Endothelial leakage was assessed by fluorescein isothiocyanate conjugated albumin (FITC-albumin; A9771; Sigma-Aldrich) extravasation in separate experiments. FITC-albumin (80 mg/kg BW) was injected into the tail vein 30 min before removal of peritoneal lavage. FITC-albumin concentration was measured in the lavage.

Cytokine Concentrations

The release of TNFα, IL6, CXCL1 (keratinocyte-derived chemokine), CXCL2/3 (macrophage inflammatory protein-2), and SDF-1α was determined in the peritoneal lavage of mice, 4 h after zymosan- and fecal-injection by ELISA kits (DY406; DY453; DY452; DY410, and DY460; R&D Systems; USA). Zymosan-induced release of IL6 and IL8 by human H441 cells was also detected by ELISA kits (DY206, respectively DY208; R&D Systems; USA).

In vivo PMN Extravasation

As previously described, lungs and liver samples were homogenized and prepared for flow cytometer staining procedure (25, 40). Peritoneal lavage (PL), lungs and liver samples were stained with a fluorescent antibody-mix, consisting of CD45 (clone 30-F11; 103132; BioLegend; USA) and Ly6G (clone 1A8; 127618; BioLegend; USA) to detect PMNs. The detailed description of the gating process is described in **Supplemental Figure 1A**. Samples were measured with a FACSCanto II flow cytometer (BD Biosciences; USA). The cytometer was calibrated routinely using the cytometer setup and tracking beads (BD Biosciences; USA) recommended by the manufacturer. BD FACSDiva software (Version 6; BD Biosciences; USA) was employed to control the flow cytometer settings, including the calibration procedures, and to acquire data. Detailed data analysis was performed using FlowJo software (version 7.8.2; Ashland; USA).

Western Blot Analysis

Mice were treated as described above and peritoneal tissue from wild type and A_{2B}−/− animals prepared for western blot analysis. Equivalent protein levels were determined by a protein assay kit (Pierce; Thermo Fisher Scientific; Germany) and loaded on SDS gels. After blotting on polyvinyldene difluoride membranes, the rabbit polyclonal anti–phospho NF-κB p65 (Ser536)(#3033; Cell Signaling Technology; Germany), the rabbit polyclonal anti–phospho ERK1/2 (Thr202/Tyr204) (#4370; Cell Signaling Technology; Germany) and the rabbit monoclonal anti-phospho CREB (Ser133) (#9198; Cell Signaling Technology; Germany) were used. For analyzing the impact of CXCR4 and CXCR7 on the formation of tight junctions 4 h after zymosan and autologous fecal administration, we used rabbit polyclonal anti–tight junction protein (TJP)-1 (1 mg/ml; Thermo Fisher Scientific; Germany) and mouse monoclonal anti-occludin (0.5 mg/ml; Thermo Fisher Scientific; Germany). The rabbit monoclonal anti-GAPDH served as housekeeping protein (G9545; Sigma-Aldrich; Germany).

Tissue Culture

In absence of a human peritoneal epithelial cell line, a human pulmonary epithelial cell line (H441; NCI-H441; ATCC® HTB-174™) and a human intestinal epithelial cell line (CaCo2; ATCC® HTB-37™) was used. H441 and CaCo2 cells were maintained in RPMI containing 10%FCS and 40 μg/ml gentamicin in a humidified atmosphere of 5% CO_2 at 37°C. H441 and CaCo2 were grown confluent and stimulated with NaCl

or zymosan 100 μg/ml for 4 h. Additional groups were treated with CCX771 (1 μM) or AMD3100 (1 μM) 1 h before zymosan administration. Supernatants were secured for protein analysis. Cells were removed and total RNA was isolated following the manufacturer's directions (pegGOLD TriFast; Peqlab; Germany and Bio-Rad iScript kit; Bio-Rad; Germany).

In additional experiments, we used siRNA to knock down the human adenosine receptor A_{2B} (sc-29642; Santa Cruz Biotechnology; USA) in H441 and CaCo2 cells. After the cell monolayer achieved 50% of confluence, medium was exchanged and cell layer transfected with jetPRIME® reagent (114-07; Polyplus transfection; France) and adenosine A_{2B} human siRNA added according to the manufacturer's instructions. 24 h after transfection, cells were harvested and total RNA was isolated for gene expression analysis. The success of siRNA transfection was evaluated by detection of gene levels of the adenosine receptor A_{2B} (**Supplemental Figure 1B**). Non-targeting siRNA (sc-37007; Santa Cruz Biotechnology; USA) was used as control.

Immunofluorescence Staining

Paraffin-embedded lung sections were fixed for 10 min in acetone and methanol. After washing and fixation, lung sections were permeabilized with 1% Triton X-100 and blocked with 5% BSA in PBS for 1 h. Sections were stained with rabbit polyclonal anti-A_{2B} adenosine receptor (sc-28996; Santa Cruz Biotechnology; USA), goat polyclonal anti-CXCR7 (sc-107515; Santa Cruz Biotechnology; USA), rabbit polyclonal anti-CXCR4 (sc-9046; Santa Cruz Biotechnology; USA), rabbit monoclonal anti-phospho CREB (Ser133) (#9198; Cell Signaling Technology) and goat polyclonal anti-cytokeratin 12 (sc-17101; Santa Cruz Biotechnology; USA). For visualization, the following secondary antibodies were employed: polyclonal donkey anti-goat IgG Alexa Fluor 488 (A11055; Thermo Fisher Scientific; Germany), polyclonal goat anti-rabbit IgG Alexa Fluor488 (A11008; Thermo Fisher Scientific; Germany), and polyclonal rabbit anti-goat IgG Alexa Fluor 546 (A21085; Thermo Fisher Scientific; Germany). For nuclei counterstaining, we used Roti-Mount FluorCare DAPI (HP20.1; Carl Roth; Germany). IgG controls are displayed in **Supplemental Figure 1C**.

For *in vitro* immunofluorescence experiments, H441 and CaCo2 cells were grown on chamber slides (Sarstedt Neumbrecht; Germany). After stimulation for 4 h with 100 ng/ml zymosan with CXCR4- (AMD3100; 1 μM) or CXCR7-treatment (CCX771; 1 μM), cells were fixed with 4% paraformaldehyde. After permeabilization with 1% Triton X-100, cells were blocked for 1 h with 5% BSA in PBS. Cells were stained by using rabbit polyclonal anti–tight junction protein (TJP)-1 (1 mg/ml; Thermo Fisher Scientific; Germany) and mono l anti-occludin (0.5 mg/ml; Thermo Fisher Scientific; Germany) followed by the secondary antibodies as described above. Rhodamin phalloidin was used to tackle β-actin (R415; Thermo Fisher Scientific; Germany). Images were analyzed by using ZEN software (Black edition 2011; Zeiss; Germany) and mean fluorescence intensities were measured by ImageJ (Version 1.49v; National Institute of Health; USA).

Immunohistochemical PMN Detection

PMN accumulation in peritoneal tissue, lung and liver sections was visualized via immunohistochemistry by using a Vectastain ABC kit (PK-4000; Vector Laboratories; Germany). Sections were blocked with Avidin solution (Vector Laboratories; Germany) for 1 h to avoid unspecific binding sites. PMNs were stained with rabbit anti-mouse Ly-6G (clone 1A8; Abcam; UK). Rabbit IgG was used as control (31235; Invitrogen; USA). Sections were incubated with biotinylated anti-rabbit IgG (BA-4000; Vector Laboratories; USA) for 1 h, followed by Vectastain ABC reagent (PK-4000; Vector Laboratories; USA) for 30 min and then incubated with DAB substrate. Nuclear fast red (H-3403; Linaris; Germany) was used for tissue counterstaining. Tissue slides were processed with a Leitz DM IRB microscope (Leica) and analyzed with AxioVision v4.8.2 (Carl Zeiss MicroImaging; Germany). Neutrophil counts were examined by enumerating the positive and therefore brown stained cells in a masked fashion. PMN numbers were scored from four random sections of four different tissue samples in each group by two independent observers (41).

String Analysis

STRING is a biological database and free web resource to identify known and predicted protein-protein networks. The STRING database includes the information of numerous experimental data, various computational predictions, and public text data. Furthermore, the STRING analysis identifies molecular partnerships and functional interactions from targets of interest by consolidating knowledge and providing context in biological systems (42). To generate reliable results, we set a medium confidence (>0.400) over our analysis.

Software and Statistical Analysis

Statistical analysis was performed by using Graph Pad Prism version 8.1 for Windows (Graph Pad Software; San Diego; USA). For comparison between two groups statistical analysis was done by an unpaired Student's *t*-test. Differences between the groups were evaluated by one-way ANOVA followed by Bonferroni's *post-hoc* test. Data are presented as mean ± SEM unless indicated otherwise.

RESULTS

Expression of CXCR4 and CXCR7 in Acute Inflammation *in vivo*

We evaluated gene expression of the chemokine SDF-1 and its receptors CXCR4 and CXCR7 in various organs and peritoneal tissue during zymosan- and fecal-induced peritonitis. We detected a significant mRNA increase of SDF-1 in the peritoneum and lung 4 h after zymosan- (**Figure 1A**) or fecal-administration (**Figure 1B**). Inflammation did not affect gene expression of SDF-1 in liver tissue. Gene expression of the two receptors CXCR4 and CXCR7 rose significantly after the onset of inflammation in the peritoneum, lung and liver tissue in both models. To verify these findings on protein level, we evaluated the surface expression of SDF-1, CXCR4, and CXCR7 in peritoneal tissue by immunofluorescence. According to the results of

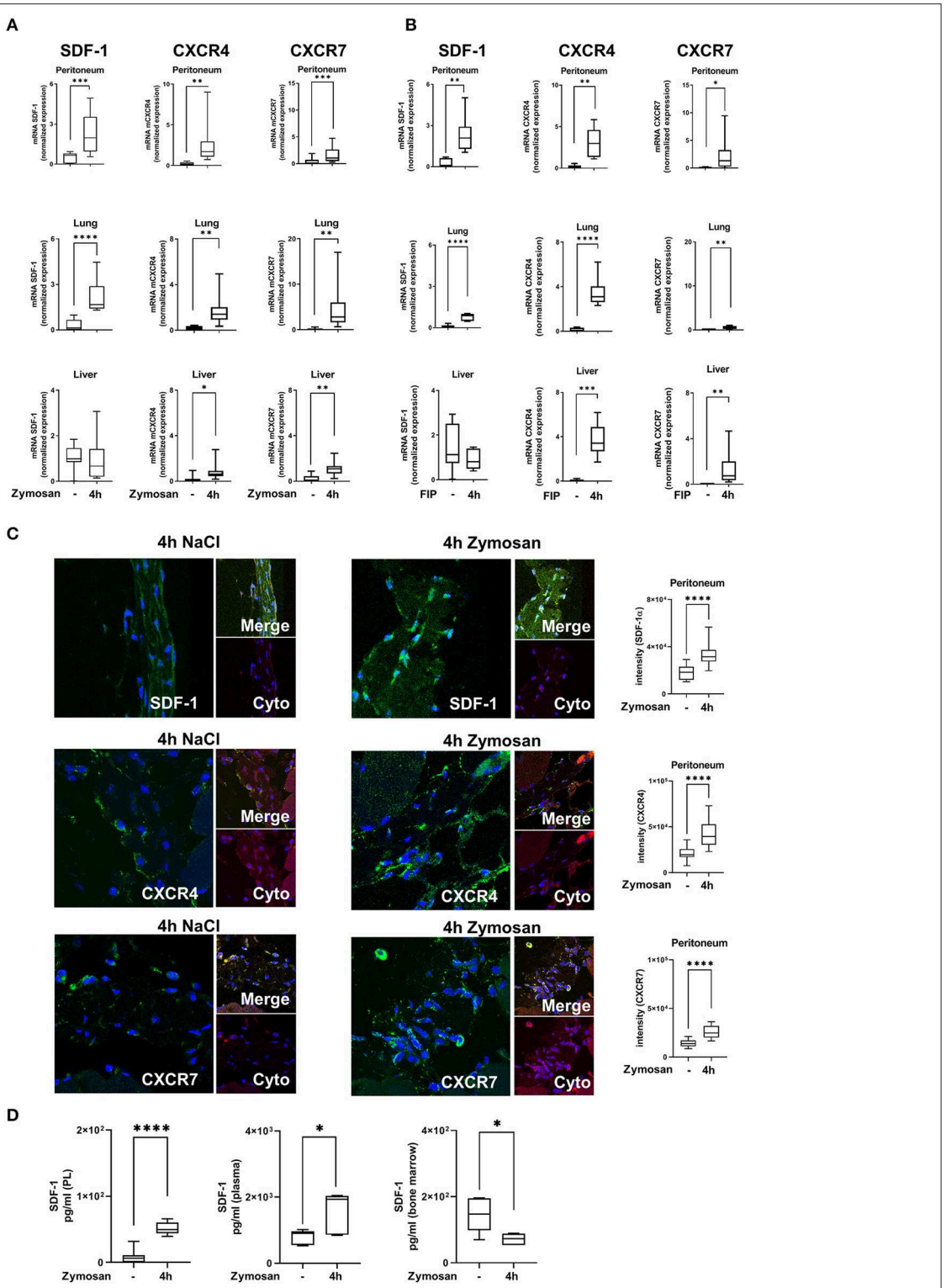

FIGURE 1 | Expression of stromal cell-derived factor-1 (SDF-1) and its receptors CXCR4 and CXCR7 in acute inflammation. **(A)** SDF-1, CXCR4, and CXCR7 mRNA levels were evaluated in peritoneal, lung and liver tissue 4 h after zymosan ($n = 8–12$) or **(B)** fecal administration ($n = 6–12$). **(C)** Immunofluorescence detection of the expression of SDF-1, CXCR4, and CXCR7 (all green) in peritoneal tissue (Cyto; cytokeratin; red) 4 h after zymosan (63x original magnification). Images are representatives of $n = 3$ experiments. Intensity was measured by ImageJ. **(D)** The release of SDF-1α in the peritoneal cavity, in the plasma, and in the bone marrow of wild type mice was detected by using ELISA ($n = 6–12$). Student's *t*-test was used for statistical analysis. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

gene expression, protein levels of SDF-1 and both receptors increased after zymosan (**Figure 1C**). The release of SDF-1 in the peritoneal lavage, plasma and bone marrow was assessed by ELISA (**Figure 1D**). A significant rise of SDF-1 was observed in the plasma, respectively in the peritoneal lavage of WT animals. SDF-1 levels in the bone marrow were reduced, allowing the zymosan-induced mobilization of PMNs into the circulation.

CXCR4- and CXCR7-Antagonism Controls PMN Migration in Acute Inflammation

Zymosan-induced PMN migration into peritoneum, lung and liver tissue was evaluated by using a flow cytometry-based method. In wild type mice, zymosan increased the PMN influx into the peritoneal lavage, lung and liver tissue (**Figure 2A**). The inhibition of both receptors, CXCR4 and CXCR7, significantly reduced the infiltration of PMNs into the peritoneal cavity, lung and liver. To visualize these findings and determine the PMN infiltration into the organs quantitatively, we stained PMNs immunohistochemical so that they appear brown and evaluated these tissue sections blinded (**Figure 2B**). Four hours after zymosan application, PMN infiltration increased in all tissues compared to untreated animals (**Figures 2C–E**). Specific CXCR4 and CXCR7 inhibition reduced PMN infiltration in all tissues, confirming our data from flow cytometry. These results highlight the impact of CXCR4- and CXCR7-inhibition on the migratory behavior of PMNs during acute inflammation.

The Anti-inflammatory Effects of CXCR4- and CXCR7-Inhibition Are Linked to a Functional A_{2B}-Receptor

Following our hypothesis that the protective effects of CXCR4- and CXCR7-antagonism in acute peritonitis are linked to A_{2B}-receptor signaling, we performed experiments with A_{2B}−/− animals. Zymosan induced a significant rise of PMNs in the peritoneal lavage, lung and liver tissue (**Figure 2F**). In these knockout animals, the inhibition of CXCR4 and CXCR7 did not show any protective effects concerning PMN migration, neither in all tissues nor in the peritoneal lavage. Blinded evaluation of immunohistochemical slides on PMN counts confirmed our flow cytometry data (**Figure 2G**). PMN infiltration is represented in histological sections of the peritoneal (**Figure 2H**), lung (**Figure 2I**) and liver tissue (**Figure 2J**).

CXCR4- and CXCR7-Inhibition Initiates Adenosine Receptor A_{2B} Signaling During Acute Inflammation

Our results demonstrated a link between the anti-inflammatory effects of the inhibition of both SDF-1 receptors and functional A_{2B}-receptor signaling. Now, we investigated if the inhibition of CXCR4 and CXCR7 influences the expression of the A_{2B}-receptor. After the application of zymosan, the expression of the A_{2B}-receptor was significantly reduced in wild type animals and the inhibition of both receptors increased the expression again to baseline levels without inflammation (**Figure 3A**). To further verify this link and to exclude other influences from adenosine signaling, we also determined the expression of the ecto-5′-nucleotidase CD73. CD73 is critically involved in the generation of extracellular adenosine, which plays a pivotal role itself in acute inflammation (43). Comparable to the expression of A_{2B}, CD73 was significantly reduced after the onset of inflammation and the inhibition of CXCR4 and CXCR7 increased the expression of the enzyme again to baseline levels. We also determined the expression in A_{2B}−/− animals and observed the same result as in wild type animals, indicating that the A_{2B}-receptor is the critical key in this setting (**Figure 3B**).

To confirm our data on protein level, we identified the surface expression of A_{2B} in the peritoneal tissue by immunofluorescence (**Figure 3C**). We detected a decrease of the expression of A_{2B} after zymosan, whereas CCX771 and AMD3100 elevated the surface expression of A_{2B} again.

The STRING analysis aims to collect, score and integrate all available knowledge of protein-protein interaction and to complement these with computational predictions of connections. By using STRING analysis, we searched for an association between the SDF-1-CXCR4/CXCR7-axis and the adenosine receptor A_{2B}. STRING analysis showed a link between the SDF-1-CXCR4/CXCR7-axis and intracellular signaling proteins like RELA, MAPK1, and CREB1 with TJP1. Also, the adenosine receptor A_{2B} is linked with TJP1 through CREB1 (**Figure 3D**). The aim of this analysis was to provide a critical assessment of interactions from targets of interest. To get strong results, we set a medium confidence (>0.400) over our analysis.

To further investigate the connection between the adenosine receptor A_{2B}, the phosphorylation of CREB and the SDF-1-CXCR4/CXCR7-axis in the setting of our study, we performed additional western blot experiments. We detected a significantly reduced activation of CREB in the peritoneal tissue after zymosan stimulation. Furthermore, the CXCR4 and CXCR7 inhibition augmented the activation of CREB (**Figure 3E**). Additionally, fluorescence studies confirmed our western blot results (**Figure 3F**). CREB is a downstream signaling pathway of A_{2B} and a cellular transcription factor. The activation of CREB initiates mainly anti-inflammatory effects, for example stabilization of tight junction proteins (44) and inhibiting NF-κB (45). The A_{2B} receptor is known to activate CREB (46). This increased phosphorylation of CREB through the adenosine receptor A_{2B} may explain the anti-inflammatory effects of the CXCR4, respectively CXCR7 inhibition on barrier integrity in the presented study.

To exclude any effects of the CXCR4 and CXCR7 inhibition on the adenosine receptors A_1, A_{2A}, and A_3, we performed additional RT-PCR experiments. Four hours after zymosan, we observed a significant decrease of A_{2A} and A_3 gene expression. A_1 adenosine receptor expression did not alter during zymosan-induced peritonitis. The pharmacologic inhibition of CXCR4 and CXCR7 showed no effects on the expression of the adenosine receptors A_1, A_{2A}, and A_3 (**Supplemental Figure 2**).

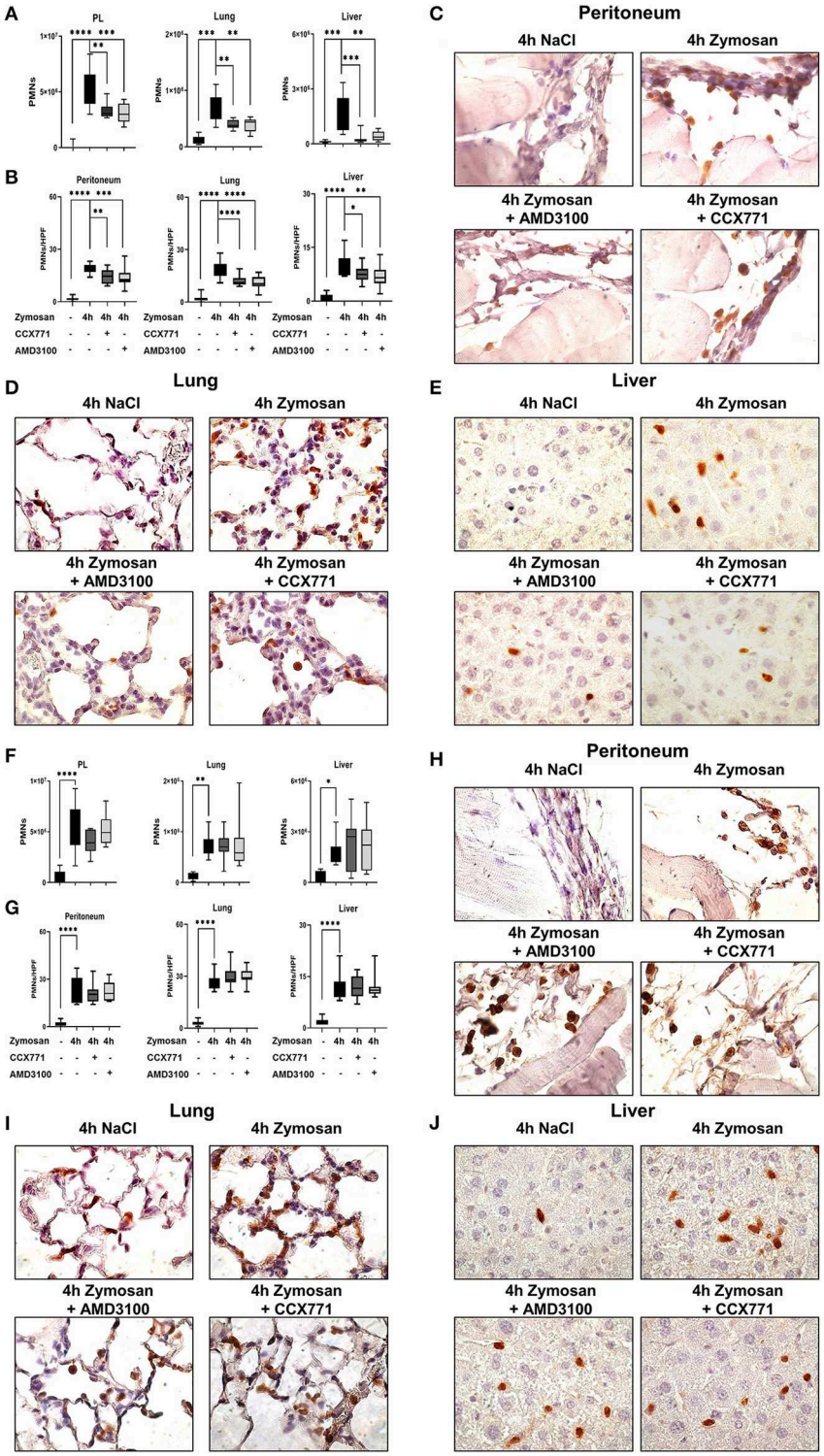

FIGURE 2 | CXCR4 and CXCR7 treatment dampens neutrophil migration into inflamed tissue through A_{2B} purinergic signaling. **(A)** We evaluated the influx of polymorphonuclear leukocytes (PMNs) into the peritoneal lavage, lung and liver tissue in wild type ($n = 8–12$) and A_{2B}–/– animals **(F)** ($n = 6–12$) with or without zymosan administration by flow cytometry. Animals were treated with AMD3100 (CXCR4-antagonist) or CCX771 (CXCR7-antagonist). PMN infiltration was also shown by immunohistochemistry, where PMNs were marked brown and counted per high power field **(B,G)** ($n = 16$). Representative histological examination of the **(C,H)** peritoneum, **(D,I)** lung and **(E,J)** liver tissue in wild type and A_{2B}–/– animals are shown. Images are representatives of $n = 3$ experiments. For statistical analysis, one-way ANOVA was used with Bonferroni *post-hoc* test. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{****}p < 0.0001$.

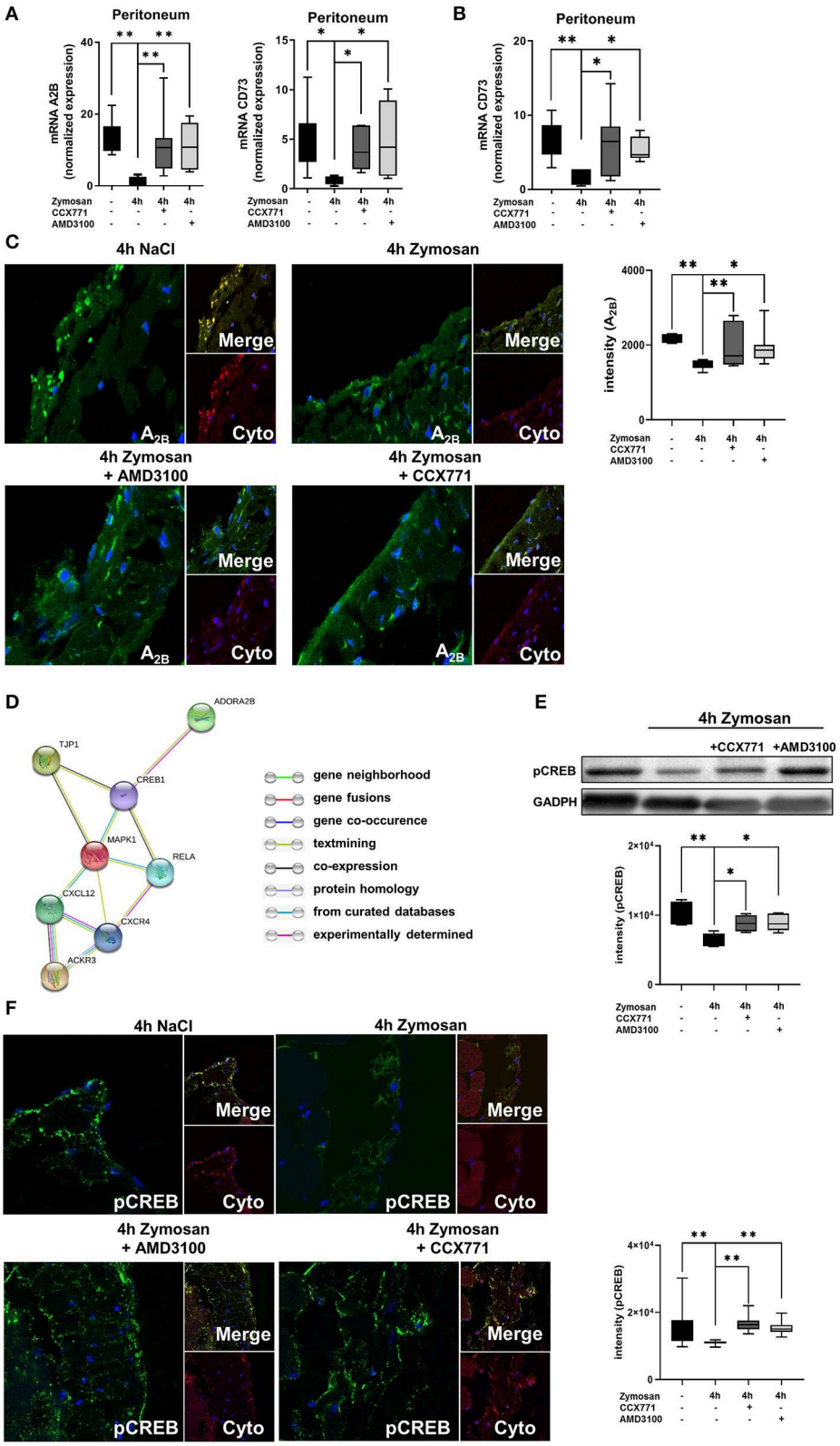

FIGURE 3 | Link between the SDF-1-CXCR4-CXCR7-axis and the adenosine receptor A_{2B}. **(A)** Expression levels of adenosine receptor A_{2B} and CD73 in peritoneal tissue 4 h after NaCl or zymosan with or without AMD3100 or CCX771 in wild type (WT) animals ($n = 7–8$). **(B)** Gene expression of CD73 in the peritoneum of A_{2B}–/–

(Continued)

FIGURE 3 | mice at indicated conditions ($n = 6–8$). **(C)** Immunofluorescence staining of the A_{2B}-expression (green) in peritoneal tissue (Cyto; cytokeratin red) (63x original magnification). Images are representatives of n=3 experiments. Adenosine receptor A_{2B} fluorescence intensity was assessed by using ImageJ. **(D)** Relationship between the SDF-1 (CXCL12)-CXCR4/CXCR7-axis, tight junction protein 1(TJP1), adenosine receptor A_{2B} (ADORA2B) and intracellular signaling proteins like NF-κB p65 (Rela), Mapk1 (ERK2), respectively cAMP response element-binding protein (CREB1) by String analysis. **(E)** Western blot analysis of the effects of CXCR4 and CXCR7 antagonism on the phosphorylation of intracellular CREB in peritoneal tissue of WT. Intensity of the blots were evaluated by ImageJ. **(F)** Detection of phosphoCREB (green) in peritoneal tissue of WT mice (Cyto; cytokeratin red) by using immunofluorescence staining (63x original magnification). Images are representatives of $n = 3$ experiments. Fluorescence intensity of phosphoCREB was assessed by using ImageJ. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $n = 6–12$; $^{*}p < 0.05$; $^{**}p < 0.01$.

Inhibition of CXCR4 and CXCR7 Stabilizes the Capillary Leakage

To evaluate the peritonitis-induced barrier destruction, we determined protein- and FITC-albumin extravasation into the peritoneal cavity. Zymosan-induced a significant increase of protein extravasation, respectively albumin, whereas the treatment of CCX771 and AMD3100 significantly reduced protein accumulation and therefore stabilized microvascular permeability (**Figure 4A**). For the endothelial and epithelial integrity, tight junction proteins are essential to control paracellular diffusion of water, ions and cells. Accordingly, we measured the expression of relevant tight junction proteins, like occludin (OCLDN), tight junction protein 1-3 (TJP1-3), e-cadherin (CDH1) and claudin1, 3, and 5 (CLDN1; CLDN3; CLDN5). We observed a significant depression of all analyzed tight junction proteins as inflammatory response in the peritoneum (**Figure 4B**). After the inhibition of CXCR4 and 7, we detected a significant elevation almost to baseline levels without inflammation of OCDLN, TJP1-3, CDH1, and CLDN3. The inhibition of CXCR7 resulted in a significant augmentation of CLDN1, whereas the inhibition of CXCR4 increased CLDN5. Western blot analysis from peritoneal tissue confirmed our above described findings for TJP1 and occludin (**Figure 4C**). Peritonitis reduced both tight junction proteins and the inhibition of CXCR4 and CXCR7 increased them again.

In A_{2B}−/− animals, zymosan significantly increased protein extravasation and FITC-albumin accumulation into the peritoneal cavity (**Figure 4D**). CXCR4 and CXCR7 inhibition were unable to prevent capillary leakage and FITC-albumin extravasation in these knockout animals, highlighting again the pivotal role of a functional adenosine A_{2B}-receptors in this setting. Accordingly, inhibition of CXCR4 and CXCR7 did not lead to any changes of gene (**Figure 4E**) and protein (**Figure 4F**) expression of the tight junction proteins in A_{2B}−/− animals.

Specific CXCR4- and CXCR7-Inhibition Dampens the Release of Inflammatory Cytokines by Controlling Intracellular Pathways

To further define the protective role of CXCR4 and CXCR7 antagonism during acute inflammatory peritonitis, we evaluated the expression and release of inflammatory cytokines in peritoneal tissue and peritoneal lavage. Zymosan significantly increased gene expression of TNFα and IL6 in wild type animals, and both inflammatory cytokines were reduced after the administration of CCX771 and AMD3100 (**Figure 5A**). Based on these results, we evaluated the release of TNFα, IL6, and additionally CXCL1 and CXCL2/3 as the main PMN chemoattractants, in the peritoneal cavity. Zymosan increased all inflammatory cytokines and the inhibition of both receptors reduced the release significantly (**Figure 5B**), confirming and explaining our results from the PMN migration assay.

To identify the mechanism behind these findings, we determined the phosphorylation and therefore activation of the intracellular signaling proteins ERK1/2 and NF-κB p65. ERK1/2 activates the transcription factor NF-κB, which regulates the replication of cytokines (47, 48). Zymosan induced the activation of ERK1/2 and NF-κB p65, whereas AMD3100 reduced mainly the activation of ERK1/2 and CCX771 mostly the phosphorylation of the NF-κB subunit 65 (**Figure 5C**).

In A_{2B}−/− animals, TNFα and IL6 gene expression increased after inflammation and the administration of AMD3100 and CCX771 did not lead to significant changes (**Figure 5D**). Correspondingly, AMD3100 and CCX771 failed to control the release of TNFα, IL6, CXCL1, and CXCL2/3 into the peritoneal cavity (**Figure 5E**) and did not influence the phosphorylation of ERK1/2 and NF-κB p65 in the peritoneal tissue of A_{2B}−/− mice (**Figure 5F**).

Functional Inhibition of CXCR4 and CXCR7 Dampens the Inflammatory Response During Polymicrobial Inflammation

To further verify the clinical impact of the inhibition of both SDF-1 receptors, we performed additional experiments with the injection of a fecal-solution to induce a polymicrobial inflammation. We determined PMN infiltration into the peritoneal lavage, lung and liver tissue by flow cytometry. In wild type mice, fecal solution led to increased PMN accumulation in the peritoneal cavity, lung and liver tissue (**Figure 6A**), whereas specific CXCR4 and CXCR7 antagonism significantly reduced PMN infiltration. Immunohistochemical blinded evaluation (**Figure 6B**) of the peritoneal tissue (**Figure 6C**), lung (**Figure 6D**), and liver (**Figure 6E**) confirmed our results.

We also investigated the second hallmark of acute inflammation in the polymicrobial model, the microvascular leakage. Four hours after fecal injection into the peritoneal cavity of wild type animals, protein extravasation increased significantly. CCX771 and AMD3100 administration dampened protein extravasation and protected the cellular integrity (**Figure 7A**). To verify the impact of CXCR4 and CXCR7 on capillary barrier, we evaluated the expression of tight junction proteins (**Figure 7B**). After polymicrobial peritonitis, all tight junction proteins were significantly decreased. The inhibition

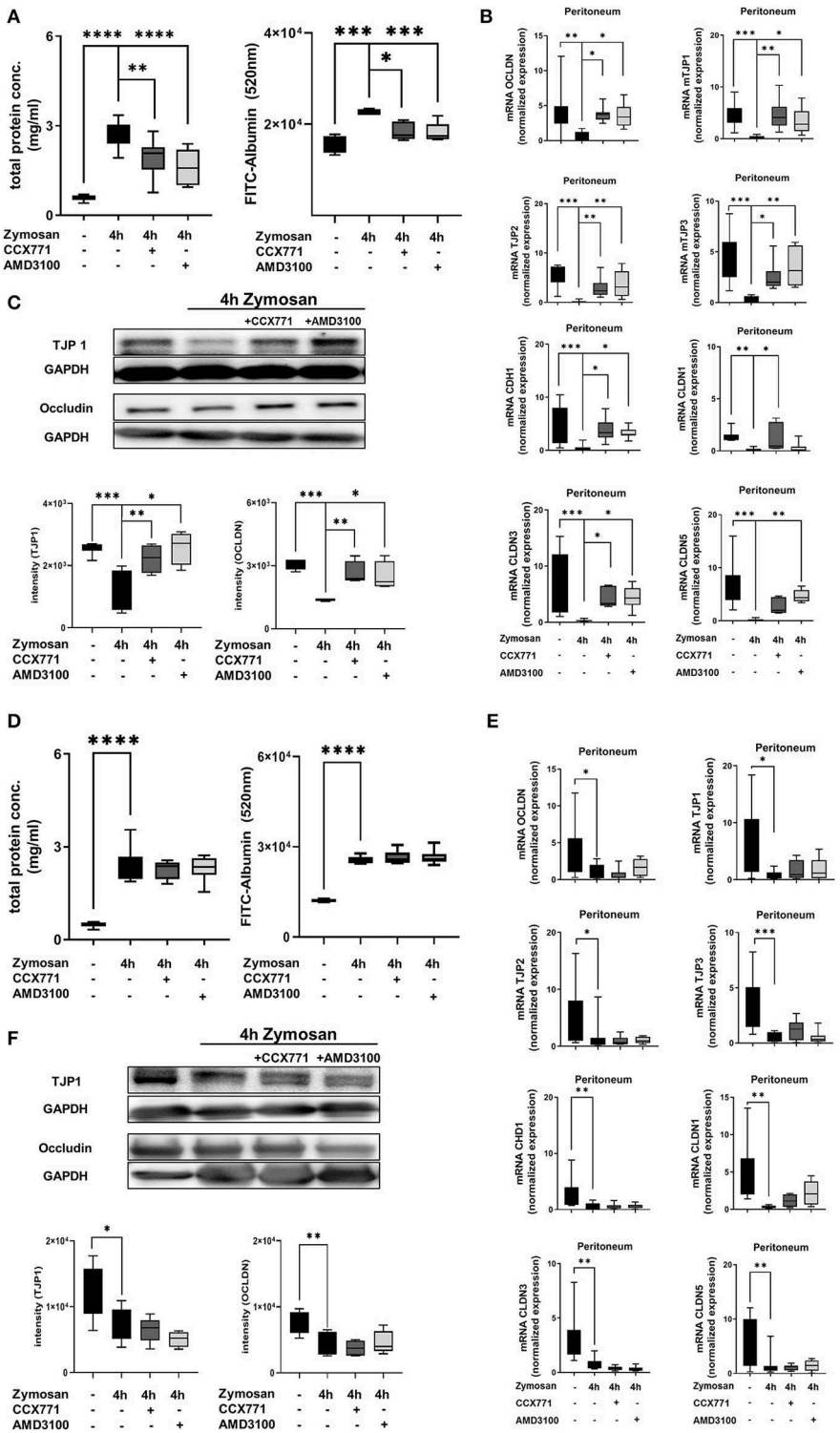

FIGURE 4 | Influence of CXCR4 and CXCR7 on microvascular permeability and cellular integrity. **(A)** Protein accumulation ($n = 6–12$) and FITC-Albumin extravasation ($n = 4–6$) were evaluated in the peritoneal cavity 4 h after NaCl or zymosan injection in wild type and **(D)** A_{2B}–/– animals. Effects of AMD3100 or CCX771 on capillary leakage were determined. **(B)** Gene levels of integrity-related tight junction proteins like occludin (OCLDN), tight junction protein 1, 2, and 3 (TJP1; TJP2; TJP3), e-cadherin 1 (CDH1), claudin 1, 3, and 5 (CLDN1; CLDN3; CLDN5) were measured in peritoneal tissue of wild type ($n = 8–12$) and **(E)** A_{2B}–/– animals ($n = 6–12$). **(C)** Representative western blots of TJP1 and occludin protein of wild type and **(F)** A_{2B}–/– peritoneal tissue are shown (representatives blots of $n = 3$ experiments). Intensity of the blots was evaluated by ImageJ. For statistical analysis, one-way ANOVA was used with Bonferroni *post-hoc* test. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

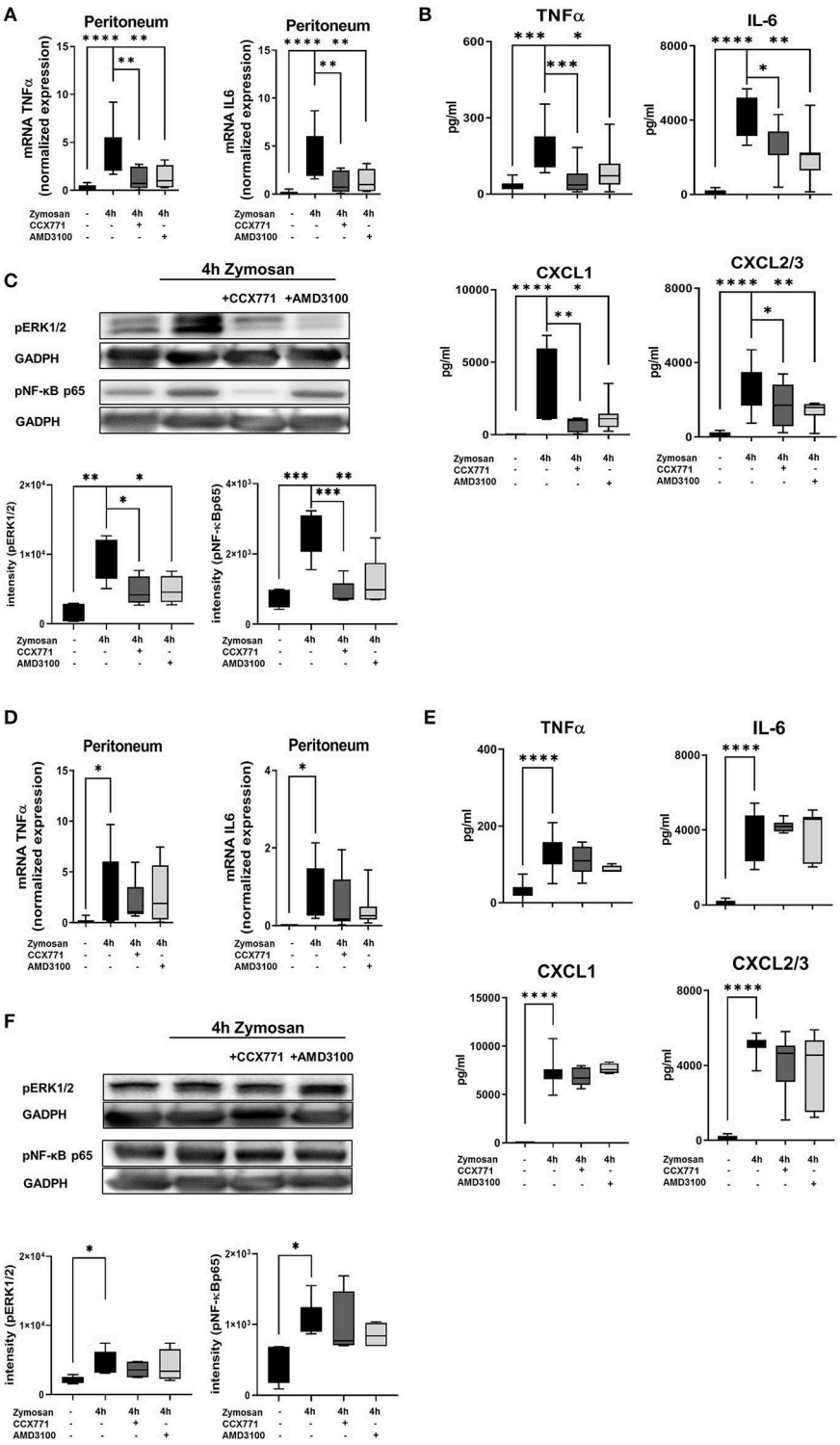

FIGURE 5 | Impact of the SDF-1 receptors CXCR4 and CXCR7 on the release of inflammatory cytokines and intracellular pathways. **(A)** After the induction of peritonitis by zymosan, effects of AMD3100 or CCX771 on the gene expression of TNFα and IL6 were evaluated in peritoneal tissue of wild type and **(D)** A_{2B}–/– mice. **(B)** The release of inflammatory cytokines (TNFα and IL6) and chemokines (CXCL1 and CXCL2/3) in the peritoneal cavity of wild type and **(E)** A_{2B}–/– animals was detected. **(C)** Effects of CXCR4 and CXCR7 inhibition on the phosphorylation of intracellular ERK1/2 and NF-κB subunit 65 in peritoneal tissue of wild type and **(F)** A_{2B}–/– animals 4 h after NaCl or zymosan with or without AMD3100- or CCX771 were determined. Blots are representatives of $n = 3$ experiments. Intensity of the blots was evaluated by ImageJ. For statistical analysis, one-way ANOVA was used with Bonferroni *post-hoc* test. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $n = 6$–12; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, and $^{****}p < 0.0001$.

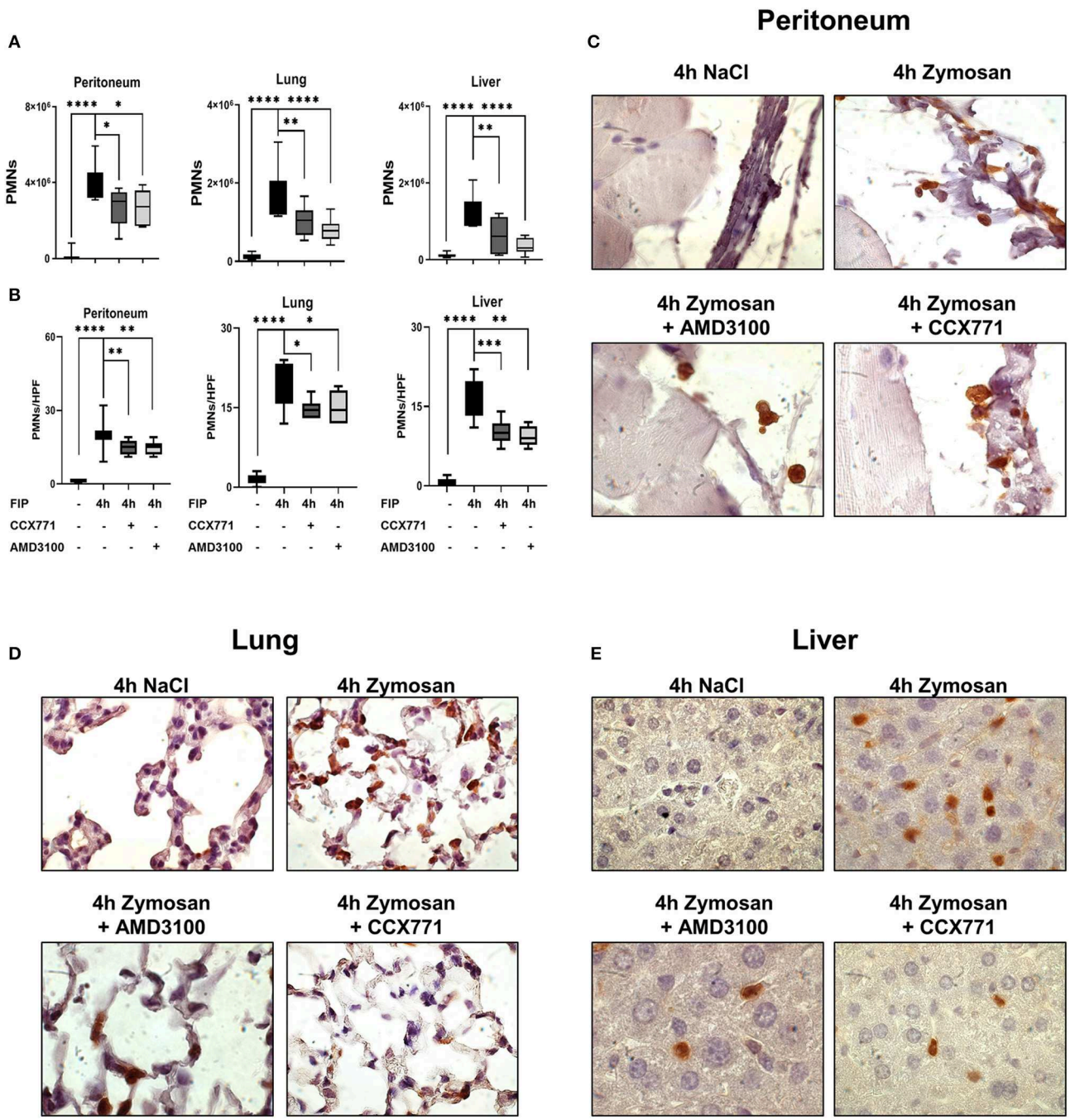

FIGURE 6 | Pharmacological modulation of CXCR7 and CXCR4 dampens PMN accumulation during polymicrobial peritonitis. **(A)** In wild type animals, PMN counts in peritoneal, lung and liver tissue were evaluated by flow cytometry after injection of autologous feces and the effects of AMD3100 and CCX771 determined. Immonofluorescence staining marked PMNs brown and neutrophil infiltration was assessed and counted per high power field **(B)**, **(C)** in the peritoneum, **(D)** lung and **(E)** liver tissue at indicated conditions. Images are representatives of $n = 3$ experiments. For statistical analysis, one-way ANOVA was used with Bonferroni *post-hoc* test. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $n = 6$–12; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, and $^{****}p < 0.0001$.

of CXCR7 elevated gene expression of occludin, TJP1, TJP3, claudin 1, and 5. The selective CXCR4 antagonist ameliorated gene levels of occludin, TJP1, TJP2, TJP3, e-cadherin 1, claudin 1, 3, and 5 in fecal peritonitis. Protein analyses confirmed the results from gene expression with a pivotal effect of AMD3100 and CCX711 administration on the protein expression of tight junctions (**Figure 7C**).

Further on, we evaluated the expression and release of inflammatory cytokines in the peritoneal lavage. The administration of CCX771 and AMD3100 significantly reduced

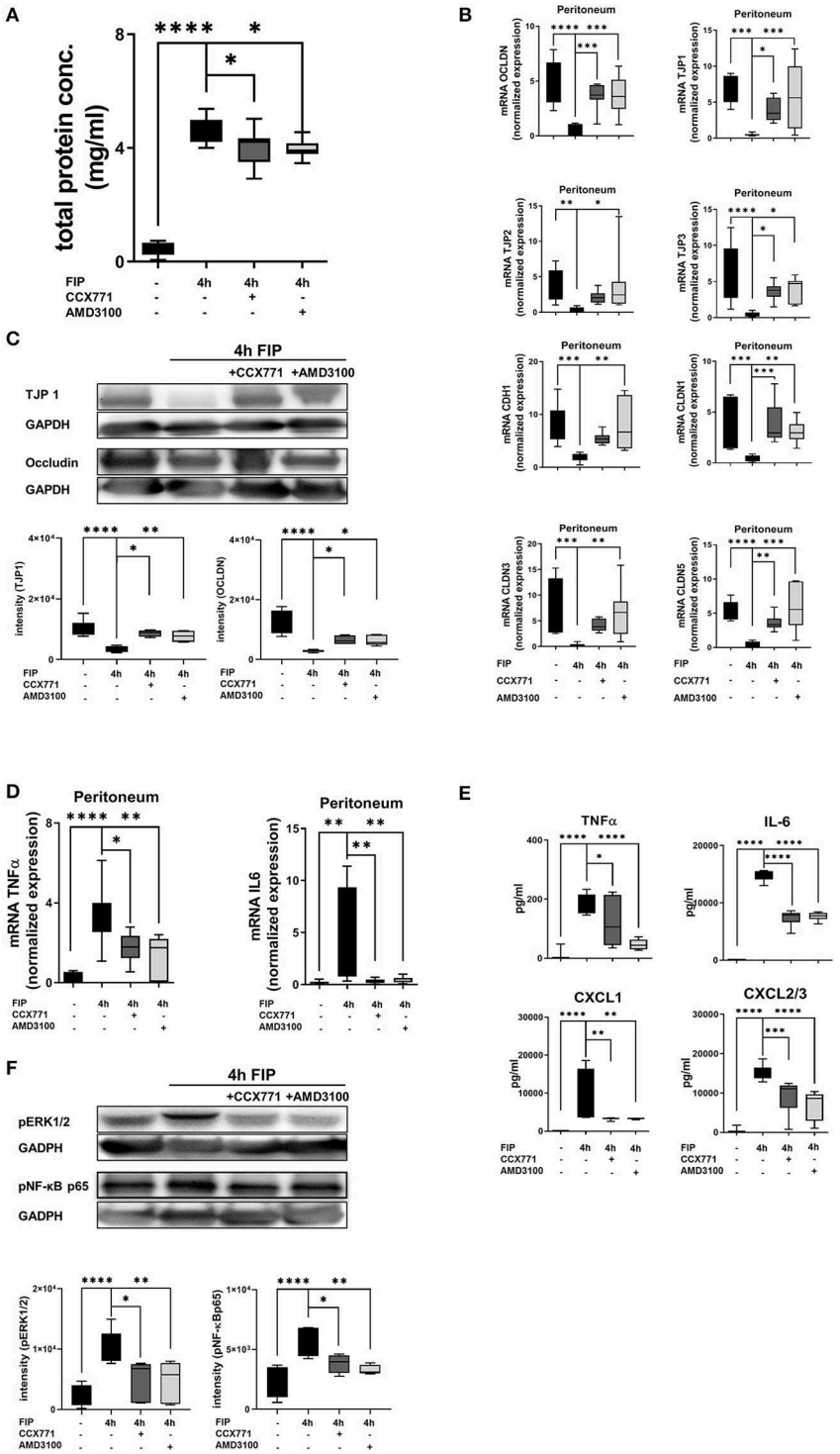

FIGURE 7 | Impact of CXCR4 and CXCR7 inhibition on microvascular permeability and cytokine release during polymicrobial peritonitis. **(A)** Protein extravasation was evaluated in the peritoneal cavity 4 h after fecal solution in wild type animals ($n = 8$–12). **(B)** mRNA of tight junction proteins was measured in peritoneal tissue of wild type animals ($n = 6$–12). **(C)** Protein levels of TJP1 and occludin were quantified in peritoneal tissue (representative blot of $n = 3$ experiments) and also **(D)** gene expression of inflammatory cytokines TNFα and IL6 ($n = 6$–8). **(E)** TNFα, IL6, CXCL1, and CXCL2/3 release was determined in the peritoneal cavity at indicated time points and conditions ($n = 8$). **(F)** Effects of CXCR4- and CXCR7 blockade on the phosphorylation of ERK1/2 and NF-κB subunit 65 in peritoneal tissue of wild type were identified (representative blots of $n = 3$ experiments). Intensity of the blots was evaluated by ImageJ. For statistical analysis, one-way ANOVA was used with Bonferroni *post-hoc* test. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, and $^{****}p < 0.0001$.

TNFα and IL6 gene expression in peritoneal tissue 4 h after the injection of autologous feces (**Figure 7D**). The detection of protein levels of TNFα and IL6 confirmed these findings. Additionally, the main PMN chemoattractants CXCL1 and CXCL2/3 were also significantly reduced after the inhibition of both receptors, supporting our results of PMN migration into the different organs (**Figure 7E**). To verify our findings, we evaluated the phosphorylation of ERK1/2 and NF-κB p65 in peritoneal tissue during fecal-induced peritonitis (**Figure 7F**), since ERK1/2 induces the activation of NF-κB p65, which controls the transcription of inflammatory cytokines (47, 48). Polymicrobial peritonitis activated ERK1/2 and NF-κB p65 signaling, whereas AMD3100 and CCX771 showed protective effects on the phosphorylation of both intracellular proteins.

AMD3100 and CCX771 Enhances Barrier Integrity and Controls Cytokine Release *in vitro*

To support our data, we added *in vitro* experiments and determined the impact of CXCR4 and CXCR7 inhibition on human epithelial cells. To visualize the effects of a pharmacolocic inhibition of CXCR4 and CXCR7, we evaluated the protein expression of occludin and TJP1 by immunofluorescence. Confirming our *in vivo* data, immunofluorescence slides revealed a very high expression of occludin and TJP1 on epithelial cells. Zymosan installation reduced the expression of both proteins and specific pharmacologic inhibition of CXCR4 and CXCR7 improved the surface presentation of occludin (**Figure 8A**) and TJP1 (**Figure 8B**). Visual aspects were verified by the detection of the mean fluorescence intensities (MFI) of occludin and TJP1 (**Figure 8C**). Furthermore, zymosan induced a strong reduction of TJP1 gene expression, which significantly increased again after the inhibition of CXCR4 and CXCR7, confirming our *in vivo* data (**Figure 8D**). A_{2B}-depletion by siRNA impeded the recovery of TJP1 expression after the treatment with both inhibitors. Additionally, we evaluated the release of IL6 and IL8 after zymosan stimulation. Zymosan increased the release of IL6 and IL8 by epithelial cells. These chemokine levels were significantly reduced after the administration of CCX771 and AMD3100 (**Figure 8E**). Depletion of A_{2B} abolished these protective effects on chemokine release (**Figure 8F**). These findings confirm our *in vivo* data and highlight the pivotal role of pharmacological inhibition of CXCR4 and CXCR7 in acute inflammation. The zymosan-induced reduction of the A_{2B} expression on H441 cells was significantly increased after the pharmacologic inhibition of CXCR4 and CXCR7 and confirmed our previous *in vivo* results (**Figure 8G**). To further investigate the impact of CXCR4 and CXCR7 inhibition on different cells, we performed additional experiments with the human intestinal epithelial cell line CaCo2. We evaluated the expression of occludin (**Supplemental Figure 3A**) and TJP1 (**Supplemental Figure 3B**) by immunofluorescence. Zymosan stimulation induced a significant reduction of both membrane proteins and AMD3100, respectively CCX771 significantly enhanced the expression of occludin and TJP1. The determination of the mean fluorescence intensities confirmed our *in vivo* and *in vitro* results (**Supplemental Figure 3C**). Additionally, gene levels of TJP1 were evaluated and the inhibition of both SDF-1 receptors induced a significant increase of TJP1 expression. Gene silencing of A_{2B} abolished the protective effects of CXCR4 and CXCR7 inhibition on TJP1 expression and confirmed our previous data with H441 cells and the A_{2B}-dependent anti-inflammatory effects of blocking the SDF-1 receptors (**Supplemental Figure 3D**). The release of IL8 in the supernatant of CaCo2 cells after zymosan application was evaluated. Like our data with H441 cells, the CXCR4 and CXCR7 blockade reduced significantly the zymosan-induced IL8 liberation from the CaCo2 cells (**Supplemental Figure 3E**). The A_{2B}-depletion by gene silencing abrogated the effects of AMD3100 and CCX771 on the release of IL8 and confirmed our previous *in vitro* data (**Supplemental Figure 3F**). To highlight the effects of CXCR4, respectively CXCR7 antagonism on the expression of A_{2B}, we performed immunofluorescence experiments with CaCo2 cells. Zymosan stimulation decreased the surface expression of A_{2B} on CaCo2 cells, while the blockade of CXCR4 or CXCR7 augmented the A_{2B} expression (**Supplemental Figure 3G**).

DISCUSSION

The stromal cell-derived factor-1 and it's both receptors, CXCR4 and CXCR7, are expressed in various hematopoietic cells and non-hematopoietic tissue (25, 49–51). It is well-known, that SDF-1 and the CXCR4 receptor orchestrate the hematopoietic niche and regulate neutrophil release from the bone marrow into the circulatory system during inflammation (21). In the presented project, we determined the detailed role of CXCR4 and CXCR7 in acute septic inflammation concerning PMN migration and capillary leakage. Furthermore, our study provided new insights about the link between the SDF-1-CXCR4/CXCR7-axis and the adenosine receptor A_{2B} during acute peritoneal inflammation.

In the presented study, both receptors and the chemokine SDF-1 underwent an inflammation-related elevation of their expression in the peritoneum, liver and lung during zymosan- and polymicrobial-induced peritonitis. In line with our data, current studies demonstrated an increase of CXCR4 and CXCR7 after LPS stimulation (29, 50). Additionally, CXCR4 and CXCR7 were shown to play a detrimental role in inflammatory conditions like atherosclerosis (23), chronic hypoxia-related pulmonary hypertension (52), and ischemic cardiac disease (53, 54).

In a model of polymicrobial sepsis, blocking CXCR4 decreased sepsis-induced mortality (55). Additionally, Gosh et al. demonstrated that inhibition of CXCR4 reduced migration of cells by regulating cytoskeletal remodeling (56) and CXCR4 is considered as biomarker for peritoneal sepsis (57). Nevertheless, data on CXCR4 and sepsis is still conflicting. Delano and colleagues detected an increased mortality after inhibiting SDF-1 in a model of polymicrobial sepsis (58). In the presented study, inhibition of CXCR4 played a pivotal role in terms of PMN influx and microvascular permeability. To our knowledge, we are the first, who described a pivotal role of the inhibition

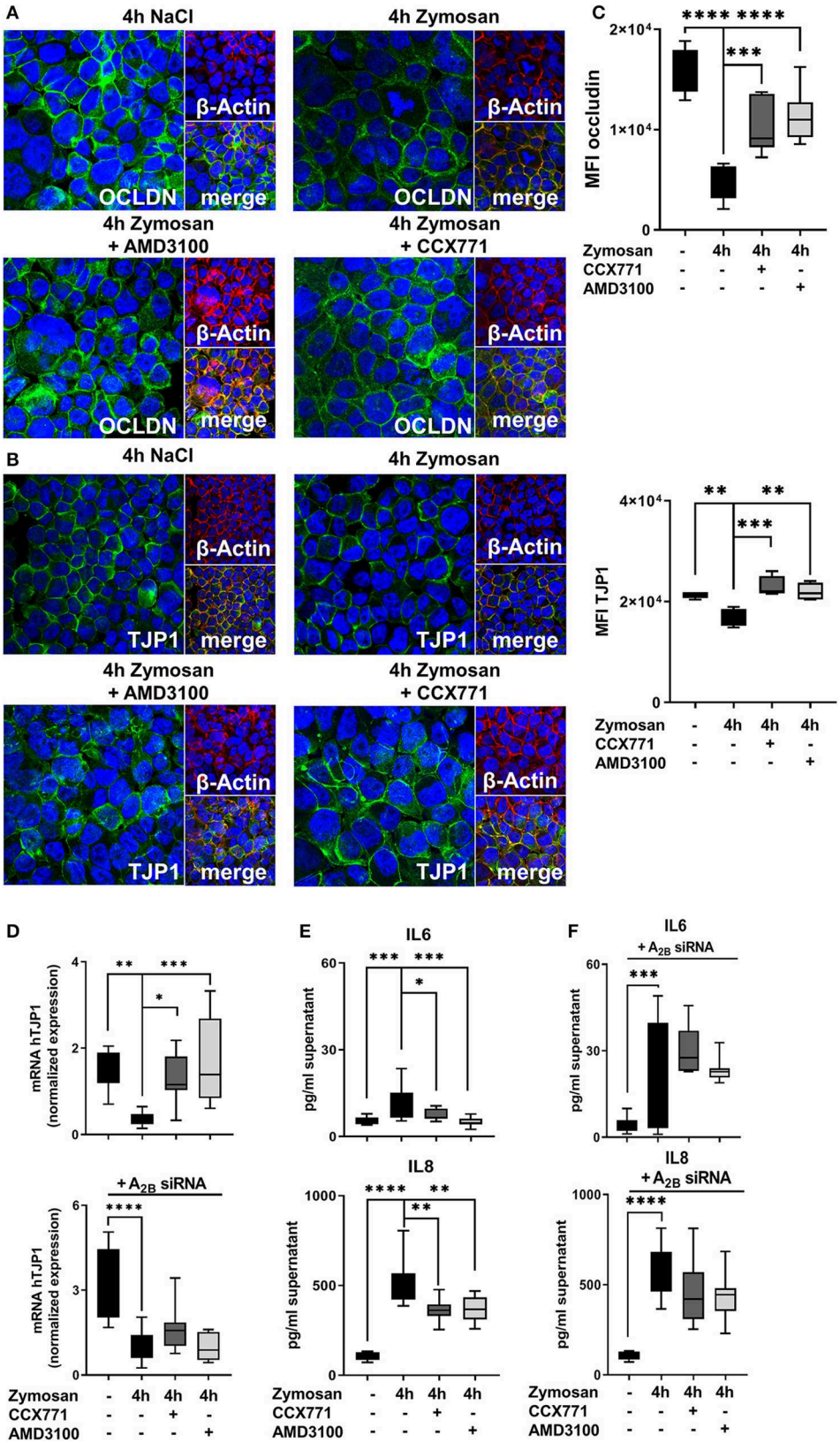

FIGURE 8 | Continued

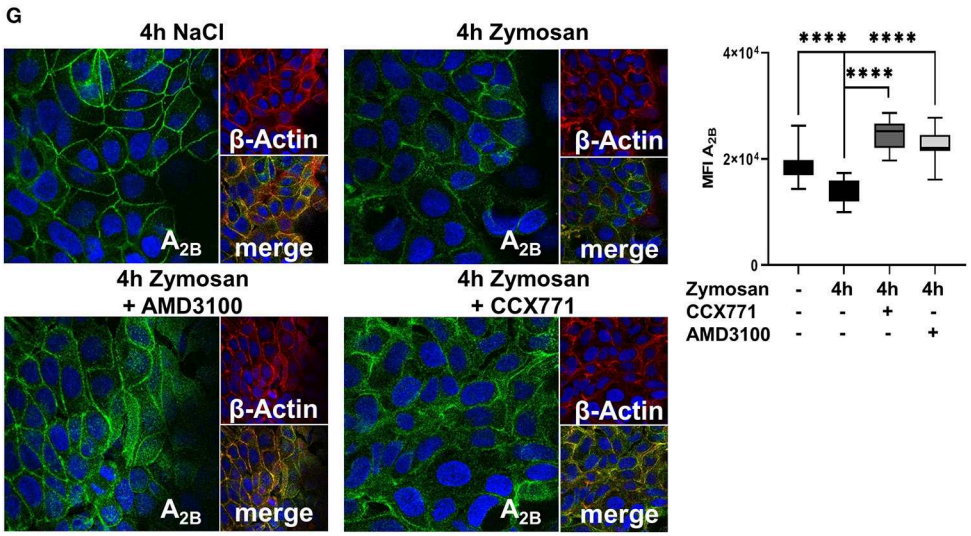

FIGURE 8 | Effects of the adenosine receptor A_{2B} blockade on permeability and cytokine release *in vitro*. **(A)** Expression of occludin (OCLDN; green) and **(B)** tight junction protein 1 (TJP1; green) in human epithelial cells (β-Actin; red) 4 h after NaCl or zymosan with or without AMD3100 or CCX771 (63x original magnification). Images are representatives of $n = 3$ experiments. **(C)** Fluorescence intensity of occludin and TJP1 was measured at indicated conditions by using ImageJ ($n = 8–12$). **(D)** TJP1 expression and the effects of CXCR4 and CXCR7 inhibition after adenosine receptor A_{2B} knock down was evaluated ($n = 8–10$). **(E)** The inflammation-related release of human IL6 and IL8 from epithelial cells and **(F)** the effects of A_{2B}-siRNA were determined in cell supernatants at indicated conditions ($n = 8–12$). **(G)** Protein levels of A_{2B} (green) in human H441 cells (β-Actin; red) at indicated conditions (63x original magnification). Image are representatives of $n = 3$ experiments. Fluorescence intensity was measured by ImageJ ($n = 8–12$). For statistical analysis, one-way ANOVA was used with Bonferroni *post-hoc* test. Data are presented as box and whisker graph with error bars indicating the range from minimum to maximum value; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

of CXCR7 on PMN migration into the peritoneal lavage and various organs and, additionally, on capillary leakage during acute peritonitis and peritonitis-related sepsis. These findings are in line with the results of our previous publications, where blocking CXCR4 and CXCR7 influenced PMN migration and microvascular permeability in acute pulmonary inflammation (25, 29).

The string analysis, reflects protein-protein interactions in direct (physical) and indirect (functional) associations based on the actual literature. For this project, it showed a connection between SDF-1 and CXCR4/7 with the tight junction proteins over the mitogen-activated protein kinases (MAPKs). ERK1/2 belongs to the extracellular signal-regulated kinases, which are part of the MAPKs. ERK1/2 activates the transcription factor NF-κB. In the presented study, ERK1/2 and NF-κB p65 were activated in both peritonitis models. NF-κB controls the transcription of various genes, that are related with the release of inflammatory cytokines and barrier integrity (59–61). Selective antagonism of CXCR4 and CXCR7 dampened the phosphorylation and therefore activation of ERK1/2 and NF-κB p65, explaining the protective effects of CXCR4 and CXCR7 antagonism on tight junction proteins.

The expression of tight junction proteins is dampened by peritonitis, affecting barrier integrity and leading to tissue edema (62). In the presented study, we show for the first time, that antagonism of CXCR4 and CXCR7 restored microvascular permeability and increased the expression of essential tight junctions like tight junction protein 1 and occludin in peritoneal tissue. Furthermore, the treatment with AMD3100 and CCX771 enhanced the expression of e-cadherin, claudin 1, claudin 3, and claudin 5 in peritoneal tissue. These findings are in line with our previous publications from the impact of CXCR4 and CXCR7 inhibition on pulmonary permeability (25, 29), where blocking CXCR4 and CXCR7 stabilized and improved tight junctions like TJP1- and occludin. Further verifying our findings about the pivotal role of both SDF-1 receptors on stabilizing cellular integrity, inhibition of CXCR4 increased TJP-1, occludin, and claudin 5 in the blood/brain barrier in terms of an ischemic stroke and brain-specific metastasis in lung cancer (28, 61). Also, the inactivation of CXCL12 stabilized endothelial tight junction expression like TJP-1 and occludin in breast cancer metastasis (63).

Phosphorylation of ERK1/2 and NF-κB during inflammation initiates the transcription of inflammatory cytokines and chemokines, which induce the migration of neutrophils. CXCR4 and CXCR7 inhibition reduced gene transcription of cytokines in the peritoneum and the release of TNFα, IL6, CXCL1, and CXCL2/3 in the peritoneal lavage. In accordance with the presented data, CXCR4 and CXCR7 blockade decreased the release of inflammatory chemokines in acute pulmonary inflammation and human alcoholic hepatitis (25, 64).

In addition, signaling via adenosine and adenosine receptors decreased the cytokine release in inflammation as well (34, 65, 66). Adenosine receptors influence leukocyte migration and protect tissue from inflammatory damage (32, 67, 68). Numerous studies highlight the anti-inflammatory potential of the adenosine receptor A_{2B} in terms of acute inflammation or ischemia-reperfusion injury. These studies show the impact

of the expression of the A_{2B} adenosine receptor in terms of myocardial infarction (69, 70), in acute pulmonary inflammation (71–73), and the expression of the receptor on the vascular endothelium (74, 75), and on different epithelial cell lines (32, 35, 76). Adenosine signaling stabilizes the cellular barrier and therefore microvascular permeability by inducing actin polymerization and changes in the cytoskeleton (77, 78). In the presented study, inhibiting CXCR4 and CXCR7 signaling enhanced the expression of the adenosine receptor A_{2B} and an increased phosphorylation of CREB. The A_{2B} adenosine receptor is known to activate CREB (46). CREB is a downstream signaling pathway of A_{2B} and a cellular transcription factor. The activation of CREB initiates mainly anti-inflammatory effects, for example stabilization of tight junction proteins (44) and inhibiting NF-κB (45). This increased phosphorylation of CREB through the adenosine receptor A_{2B} may explain the anti-inflammatory effects of the CXCR4, respectively CXCR7 inhibition on barrier integrity in the presented study. Lack of the adenosine receptor A_{2B} abrogated the protective effects of the pharmacological inhibition of CXCR4 and CXCR7. To our knowledge, we are the first detecting a link between the SDF-1/CXCR4/7 axis and functional A_{2B}-receptor signaling in acute peritonitis. This finding is crucial, since patients on the intensive care unit may have altered adenosine receptor distribution and ligand affinity (37). Accordingly, the expression of the A_{2B}-receptor should be investigated before the administration of the specific CXCR4 or CXCR7 antagonist.

CONCLUSION

Our study identified a previously uncharacterized role of the SDF-1 receptors CXCR4 and CXCR7 in peritonitis and peritonitis-related sepsis. The inhibition of both receptors demonstrated anti-inflammatory effects on PMN-migration and tissue integrity and therefore revealed a pivotal anti-inflammatory role of pharmacological modulation of CXCR4 and CXCR7 in acute septic inflammation. The anti-inflammatory effects of the specific CXCR4 and CXCR7 inhibition depend on functional A_{2B}-receptor signaling, enabling the identification of subgroups of patients, who would take advantage of this treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All animal protocols were approved by the Animal Care and Use Committee of the University of Tübingen.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to this article. K-CN, CJ, RP, KS, CE, DK, and JG-T mainly contributed by participation in the data acquisition, analysis and interpretation. K-CN and FK contributed to the conception and design of the study, as well as the analysis and interpretation of the data. K-CN and FK wrote 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.2020.00407/full#supplementary-material

REFERENCES

1. Nasa P, Juneja D, Singh O, Dang R, Arora V. Severe sepsis and its impact on outcome in elderly and very elderly patients admitted in intensive care unit. *J Intensive Care Med.* (2012) 27:179–83. doi: 10.1177/0885066610397116
2. Yang KM, Park IJ, Lee JL, Yoon YS, Kim CW, Lim SB, et al. Does the different locations of colon cancer affect the oncologic outcome? A propensity-score matched analysis. *Ann Coloproctol.* (2019) 35:15–23. doi: 10.3393/ac.2018.07.25
3. Yeh RW, Sidney S, Chandra M, Sorel M, Selby JV, Go AS. Population trends in the incidence and outcomes of acute myocardial infarction. *N Engl J Med.* (2010) 362:2155–65. doi: 10.1056/NEJMoa0908610
4. Coopersmith CM, De Backer D, Deutschman CS, Ferrer R, Lat I, Machado FR, et al. Surviving sepsis campaign: research priorities for sepsis and septic shock. *Intensive Care Med.* (2018) 44:1400–26. doi: 10.1007/s00134-018-5175-z
5. Kasugai D, Nishikimi M, Nishida K, Higashi M, Yamamoto T, Numaguchi A, et al. Timing of administration of epinephrine predicts the responsiveness to epinephrine in norepinephrine-refractory septic shock: a retrospective study. *J Intensive Care.* (2019) 7:20. doi: 10.1186/s40560-019-0377-1
6. Nunnally ME, Patel A. Sepsis—what's new in 2019? *Curr Opin Anaesthesiol.* (2019) 32:163–8. doi: 10.1097/ACO.0000000000000707
7. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol.* (2013) 13:159–75. doi: 10.1038/nri3399
8. Paudel S, Baral P, Ghimire L, Bergeron S, Jin L, DeCorte JA, et al. CXCL1 regulates neutrophil homeostasis in pneumonia-derived sepsis caused by streptococcus pneumoniae serotype 3. *Blood.* (2019) 133:1335–45. doi: 10.1182/blood-2018-10-878082
9. Ahn C, Kim W, Lim TH, Cho Y, Choi KS, Jang BH. The delta neutrophil index (DNI) as a prognostic marker for mortality in adults with sepsis: a systematic review and meta-analysis. *Sci Rep.* (2018) 8:6621. doi: 10.1038/s41598-018-24211-7

10. Deutschman CS, Tracey KJ. Sepsis: current dogma and new perspectives. *Immunity.* (2014) 40:463–75. doi: 10.1016/j.immuni.2014.04.001
11. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol.* (2016) 17:564–80. doi: 10.1038/nrm.2016.80
12. Boivin MA, Ye D, Kennedy JC, Al-Sadi R, Shepela C, Ma TY. Mechanism of glucocorticoid regulation of the intestinal tight junction barrier. *Am J Physiol Gastrointest Liver Physiol.* (2007) 292:G590–8. doi: 10.1152/ajpgi.00252.2006
13. Forster C. Tight junctions and the modulation of barrier function in disease. *Histochem Cell Biol.* (2008) 130:55–70. doi: 10.1007/s00418-008-0424-9
14. Balda MS, Matter K. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. *EMBO J.* (2000) 19:2024–33. doi: 10.1093/emboj/19.9.2024
15. Eckle T, Grenz A, Laucher S, Eltzschig HK. A2B adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice. *J Clin Invest.* (2008) 118:3301–15. doi: 10.1172/JCI34203
16. Eltzschig HK, Abdulla P, Hoffman E, Hamilton KE, Daniels D, Schonfeld C, et al. HIF-1-dependent repression of equilibrative nucleoside transporter (ENT) in hypoxia. *J Exp Med.* (2005) 202:1493–505. doi: 10.1084/jem.20050177
17. Morote-Garcia JC, Rosenberger P, Kuhlicke J, Eltzschig HK. HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. *Blood.* (2008) 111:5571–80. doi: 10.1182/blood-2007-11-126763
18. Konrad FM, Knausberg U, Hone R, Ngamsri KC, Reutershan J. Tissue heme oxygenase-1 exerts anti-inflammatory effects on LPS-induced pulmonary inflammation. *Mucosal Immunol.* (2016) 9:98–111. doi: 10.1038/mi.2015.39
19. Herrero R, Prados L, Ferruelo A, Puig F, Pandolfi R, Guillamat-Prats R, et al. Fas activation alters tight junction proteins in acute lung injury. *Thorax.* (2019) 74:69–82. doi: 10.1136/thoraxjnl-2018-211535
20. Santos TM, Franci D, Gontijo-Coutinho CM, Ozahata TM, de Araujo Guerra Grangeia T, Matos-Souza JR, et al. Inflammatory lung edema correlates with echocardiographic estimation of capillary wedge pressure in newly diagnosed septic patients. *J Crit Care.* (2018) 44:392–7. doi: 10.1016/j.jcrc.2017.11.036
21. Devi S, Wang Y, Chew WK, Lima R, A-González N, Mattar CN, et al. Neutrophil mobilization via plerixafor-mediated CXCR4 inhibition arises from lung demargination and blockade of neutrophil homing to the bone marrow. *J Exp Med.* (2013) 210:2321–36. doi: 10.1084/jem.20130056
22. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest.* (2010) 120:2423–31. doi: 10.1172/JCI41649
23. Chatterjee M, von Ungern-Sternberg SN, Seizer P, Schlegel F, Buttcher M, Sindhu NA et al. Platelet-derived CXCL12 regulates monocyte function, survival, differentiation into macrophages and foam cells through differential involvement of CXCR4-CXCR7. *Cell Death Dis.* (2015) 6:e1989. doi: 10.1038/cddis.2015.233
24. Suratt BT, Petty JM, Young SK, Malcolm KC, Lieber JG, Nick JA, et al. Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. *Blood.* (2004) 104:565–71. doi: 10.1182/blood-2003-10-3638
25. Konrad FM, Meichssner N, Bury A, Ngamsri KC, Reutershan J. Inhibition of SDF-1 receptors CXCR4 and CXCR7 attenuates acute pulmonary inflammation via the adenosine A2B-receptor on blood cells. *Cell Death Dis.* (2017) 8:e2832. doi: 10.1038/cddis.2016.482
26. Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, Allendorf DJ, et al. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. *J Mol Histol.* (2004) 35:233–45. doi: 10.1023/B:HIJO.0000032355.66152.b8
27. Chen Y, Yu X, He Y, Zhang L, Huang X, Xu X, et al. Activation of A2aR attenuates bleomycin-induced pulmonary fibrosis via the SDF-1/CXCR4 axis-related pathway. *Am J Transl Res.* (2017) 9:4125–36.
28. Huang J, Li Y, Tang Y, Tang G, Yang GY, Wang Y. CXCR4 antagonist AMD3100 protects blood-brain barrier integrity and reduces inflammatory response after focal ischemia in mice. *Stroke.* (2013) 44:190–7. doi: 10.1161/STROKEAHA.112.670299
29. Ngamsri KC, Muller A, Bosmuller H, Gamper-Tsigaras J, Reutershan J, Konrad FM. The pivotal role of CXCR7 in stabilization of the pulmonary epithelial barrier in acute pulmonary inflammation. *J Immunol.* (2017) 198:2403–13. doi: 10.4049/jimmunol.1601682
30. Chang HC, Huang PH, Syu FS, Hsieh CH, Chang SL, Lu J, et al. Critical involvement of atypical chemokine receptor CXCR7 in allergic airway inflammation. *Immunology.* (2018) 154:274–84. doi: 10.1111/imm.12881
31. Yang D, Koupenova M, McCrann DJ, Kopeikina KJ, Kagan HM, Schreiber BM, et al. The A2b adenosine receptor protects against vascular injury. *Proc Natl Acad Sci U S A.* (2008) 105:792–6. doi: 10.1073/pnas.0705563105
32. Hoegl S, Brodsky KS, Blackburn MR, Karmouty-Quintana H, Zwissler B, Eltzschig HK. Alveolar epithelial A2B adenosine receptors in pulmonary protection during acute lung injury. *J Immunol.* (2015) 195:1815–24. doi: 10.4049/jimmunol.1401957
33. Granja TF, Kohler D, Schad J, de Oliveira CB, Konrad F, Hoch-Gutbrod M, et al. Adenosine receptor adora2b plays a mechanistic role in the protective effect of the volatile anesthetic sevoflurane during liver ischemia/reperfusion. *Anesthesiology.* (2016) 125:547–60. doi: 10.1097/ALN.0000000000001234
34. Konrad FM, Witte E, Vollmer I, Stark S, Reutershan J. Adenosine receptor A2b on hematopoietic cells mediates LPS-induced migration of PMNs into the lung interstitium. *Am J Physiol Lung Cell Mol Physiol.* (2012) 303:L425–38. doi: 10.1152/ajplung.00387.2011
35. Aherne CM, Saeedi B, Collins CB, Masterson JC, McNamee EN, Perrenoud L, et al. Epithelial-specific A2B adenosine receptor signaling protects the colonic epithelial barrier during acute colitis. *Mucosal Immunol.* (2015) 8:1324–38. doi: 10.1038/mi.2015.22
36. Gaubert M, Marlinge M, Kerbaul F, Resseguier N, Laine M, Cautella J, et al. Adenosine plasma level and A2A receptor expression in patients with cardiogenic shock. *Crit Care Med.* (2018) 46:e874–80. doi: 10.1097/CCM.0000000000003252
37. Kreth S, Kaufmann I, Ledderose C, Luchting B, Thiel M. Reduced ligand affinity leads to an impaired function of the adenosine A2A receptor of human granulocytes in sepsis. *J Cell Mol Med.* (2009) 13:985–94. doi: 10.1111/j.1582-4934.2008.00530.x
38. Dushianthan A, Grocott MP, Postle AD, Cusack R. Acute respiratory distress syndrome and acute lung injury. *Postgrad Med J.* (2011) 87:612–22. doi: 10.1136/pgmj.2011.118398
39. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest.* (2012) 122:2731–40. doi: 10.1172/JCI60331
40. Konrad FM, Zwergel C, Ngamsri KC, Reutershan J. Anti-inflammatory effects of heme oxygenase-1 depend on adenosine A2A- and A2B-receptor signaling in acute pulmonary inflammation. *Front Immunol.* (2017) 8:1874. doi: 10.3389/fimmu.2017.01874
41. Nagata K, Browne KD, Suto Y, Kumasaka K, Cognetti J, Johnson VE, et al. Early heparin administration after traumatic brain injury: prolonged cognitive recovery associated with reduced cerebral edema and neutrophil sequestration. *J Trauma Acute Care Surg.* (2017) 83:406–12. doi: 10.1097/TA.0000000000001590
42. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* (2017) 45:D362–8. doi: 10.1093/nar/gkw937
43. Eckle T, Koeppen M, Eltzschig HK. Role of extracellular adenosine in acute lung injury. *Physiology.* (2009) 24:298–306. doi: 10.1152/physiol.00022.2009
44. Chen J, Xiao L, Rao JN, Zou T, Liu L, Bellavance E, et al. JunD represses transcription and translation of the tight junction protein zona occludens-1 modulating intestinal epithelial barrier function. *Mol Biol Cell.* (2008) 19:3701–12. doi: 10.1091/mbc.e08-02-0175
45. Wen AY, Sakamoto KM, Miller LS. The role of the transcription factor CREB in immune function. *J Immunol.* (2010) 185:6413–9. doi: 10.4049/jimmunol.1001829
46. Darashchonak N, Koepsell B, Bogdanova N, von Versen-Hoynck F. Adenosine A2B receptors induce proliferation, invasion and activation of cAMP response element binding protein (CREB) in trophoblast cells. *BMC Pregnancy Childbirth.* (2014) 14:2. doi: 10.1186/1471-2393-14-2
47. Vales LD, Friedl EM. Binding of C/EBP and RBP (CBF1) to overlapping sites regulates interleukin-6 gene expression. *J Biol Chem.* (2002) 277:42438–46. doi: 10.1074/jbc.M207363200
48. Minshawi F, White MRH, Muller W, Humphreys N, Jackson D, Campbell BJ, et al. Human TNF-Luc reporter mouse: a new model to quantify inflammatory responses. *Sci Rep.* (2019) 9:193. doi: 10.1038/s41598-018-36969-x

49. Chatterjee M, Rath D, Gawaz M. Role of chemokine receptors CXCR4 and CXCR7 for platelet function. *Biochem Soc Trans.* (2015) 43:720–6. doi: 10.1042/BST20150113
50. Feng YF, Guo H, Yuan F, Shen MQ. Lipopolysaccharide promotes choroidal neovascularization by up-regulation of CXCR4 and CXCR7 expression in choroid endothelial cell. *PLoS ONE.* (2015) 10:e0136175. doi: 10.1371/journal.pone.0136175
51. Guyon A. CXCL12 chemokine and its receptors as major players in the interactions between immune and nervous systems. *Front Cell Neurosci.* (2014) 8:65. doi: 10.3389/fncel.2014.00065
52. Sartina E, Suguihara C, Ramchandran S, Nwajei P, Rodriguez M, Torres E, et al. Antagonism of CXCR7 attenuates chronic hypoxia-induced pulmonary hypertension. *Pediatr Res.* (2012) 71:682–8. doi: 10.1038/pr.2012.30
53. Stellos K, Rahmann A, Kilias A, Ruf M, Sopova K, Stamatelopoulos K, et al. Expression of platelet-bound stromal cell-derived factor-1 in patients with non-valvular atrial fibrillation and ischemic heart disease. *J Thromb Haemost.* (2012) 10:49–55. doi: 10.1111/j.1538-7836.2011.04547.x
54. Stellos K, Langer H, Daub K, Schoenberger T, Gauss A, Geisler T, et al. Platelet-derived stromal cell-derived factor-1 regulates adhesion and promotes differentiation of human CD34+ cells to endothelial progenitor cells. *Circulation.* (2008) 117:206–15. doi: 10.1161/CIRCULATIONAHA.107.714691
55. Ramonell KM, Zhang W, Hadley A, Chen CW, Fay KT, Lyons JD, et al. CXCR4 blockade decreases $CD4^+$ T cell exhaustion and improves survival in a murine model of polymicrobial sepsis. *PLoS ONE.* (2017) 12:e0188882. doi: 10.1371/journal.pone.0188882
56. Ghosh MC, Makena PS, Gorantla V, Sinclair SE, Waters CM. CXCR4 regulates migration of lung alveolar epithelial cells through activation of Rac1 and matrix metalloproteinase-2. *Am J Physiol Lung Cell Mol Physiol.* (2012) 302:L846–56. doi: 10.1152/ajplung.00321.2011
57. Badr HS, El-Gendy FM, Helwa MA. Serum stromal-derived-factor-1 (CXCL12) and its alpha chemokine receptor (CXCR4) as biomarkers in neonatal sepsis. *J Matern Fetal Neonatal Med.* (2018) 31:2209–15. doi: 10.1080/14767058.2017.1336760
58. Delano MJ, Kelly-Scumpia KM, Thayer TC, Winfield RD, Scumpia PO, Cuenca AG, et al. Neutrophil mobilization from the bone marrow during polymicrobial sepsis is dependent on CXCL12 signaling. *J Immunol.* (2011) 187:911–8. doi: 10.4049/jimmunol.1100588
59. Yano T, Torisawa T, Oiwa K, Tsukita S. AMPK-dependent phosphorylation of cingulin reversibly regulates its binding to actin filaments and microtubules. *Sci Rep.* (2018) 8:15550. doi: 10.1038/s41598-018-33418-7
60. Li Y, Bai YJ, Jiang YR, Yu WZ, Shi X, Chen L, et al. Apelin-13 is an early promoter of cytoskeleton and tight junction in diabetic macular edema via PI-3K/Akt and MAPK/Erk signaling pathways. *Biomed Res Int.* (2018) 2018:3242574. doi: 10.1155/2018/3242574
61. Li H, Chen Y, Xu N, Yu M, Tu X, Chen Z, et al. AMD3100 inhibits brain-specific metastasis in lung cancer via suppressing the SDF-1/CXCR4 axis and protecting blood-brain barrier. *Am J Transl Res.* (2017) 9:5259–74.
62. Li Q, Zhang Q, Wang C, Liu X, Li N, Li J. Disruption of tight junctions during polymicrobial sepsis *in vivo*. *J Pathol.* (2009) 218:210–21. doi: 10.1002/path.2525
63. Ahirwar DK, Nasser MW, Ouseph MM, Elbaz M, Cuitino MC, Kladney RD, et al. Fibroblast-derived CXCL12 promotes breast cancer metastasis by facilitating tumor cell intravasation. *Oncogene.* (2018) 37:4428–42. doi: 10.1038/s41388-018-0263-7
64. Liu H, Li J, Tillman B, Morgan TR, French BA, French SW. TLR3/4 signaling is mediated via the NFkappaB-CXCR4/7 pathway in human alcoholic hepatitis and non-alcoholic steatohepatitis which formed mallory-denk bodies. *Exp Mol Pathol.* (2014) 97:234–40. doi: 10.1016/j.yexmp.2014.07.001
65. Reutershan J, Cagnina RE, Chang D, Linden J, Ley K. Therapeutic anti-inflammatory effects of myeloid cell adenosine receptor A2a stimulationin lipopolysaccharide-induced lung injury. *J Immunol.* (2007) 179:1254–63. doi: 10.4049/jimmunol.179.2.1254
66. Reutershan J, Vollmer I, Stark S, Wagner R, Ngamsri KC, Eltzschig HK. Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *FASEB J.* (2009) 23:473–82. doi: 10.1096/fj.08-119701
67. Rosenberger P, Schwab JM, Mirakaj V, Masekowsky E, Mager A, Morote-Garcia JC, et al. Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia. *Nat Immunol.* (2009) 10:195–202. doi: 10.1038/ni.1683
68. Eckle T, Hughes K, Ehrentraut H, Brodsky KS, Rosenberger P, Choi DS, et al. Crosstalk between the equilibrative nucleoside transporter ENT2 and alveolar Adora2b adenosine receptors dampens acute lung injury. *FASEB J.* (2013) 27:3078–89. doi: 10.1096/fj.13-228551
69. Eckle T, Hartmann K, Bonney S, Reithel S, Mittelbronn M, Walker LA, et al. Adora2b-elicited Per2 stabilization promotes a HIF-dependent metabolic switch crucial for myocardial adaptation to ischemia. *Nat Med.* (2012) 18:774–82. doi: 10.1038/nm.2728
70. Eltzschig HK, Bonney SK, Eckle T. Attenuating myocardial ischemia by targeting A2B adenosine receptors. *Trends Mol Med.* (2013) 19:345–54. doi: 10.1016/j.molmed.2013.02.005
71. Eckle T, Kewley EM, Brodsky KS, Tak E, Bonney S, Gobel M, et al. Identification of hypoxia-inducible factor HIF-1A as transcriptional regulator of the A2B adenosine receptor during acute lung injury. *J Immunol.* (2014) 192:1249–56. doi: 10.4049/jimmunol.1100593
72. Ehrentraut H, Westrich JA, Eltzschig HK, Clambey ET. Adora2b adenosine receptor engagement enhances regulatory T cell abundance during endotoxin-induced pulmonary inflammation. *PLoS ONE.* (2012) 7:e32416. doi: 10.1371/journal.pone.0032416
73. Ehrentraut H, Clambey ET, McNamee EN, Brodsky KS, Ehrentraut SF, Poth JM, et al. CD73+ regulatory T cells contribute to adenosine-mediated resolution of acute lung injury. *FASEB J.* (2013) 27:2207–19. doi: 10.1096/fj.12-225201
74. Eltzschig HK, Weissmuller T, Mager A, Eckle T. Nucleotide metabolism and cell-cell interactions. *Methods Mol Biol.* (2006) 341:73–87. doi: 10.1385/1-59745-113-4:73
75. Idzko M, Ferrari D, Riegel AK, Eltzschig HK. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. *Blood.* (2014) 124:1029–37. doi: 10.1182/blood-2013-09-402560
76. Frick JS, MacManus CF, Scully M, Glover LE, Eltzschig HK, Colgan SP. Contribution of adenosine A2B receptors to inflammatory parameters of experimental colitis. *J Immunol.* (2009) 182:4957–64. doi: 10.4049/jimmunol.0801324
77. Schmit MA, Mirakaj V, Stangassinger M, Konig K, Kohler D, Rosenberger P. Vasodilator phosphostimulated protein (VASP) protects endothelial barrier function during hypoxia. *Inflammation.* (2012) 35:566–73. doi: 10.1007/s10753-011-9347-z
78. Lawrence DW, Comerford KM, Colgan SP. Role of VASP in reestablishment of epithelial tight junction assembly after Ca2+ switch. *Am J Physiol Cell Physiol.* (2002) 282:C1235–45. doi: 10.1152/ajpcell.00288.2001

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PERSPECTIVE
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IL-22 Plays a Critical Role in Maintaining Epithelial Integrity During Pulmonary Infection

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Pulmonary infection is a leading cause of hospitalization in world. Lung damage due to infection and host mediated pathology can have life threatening consequences. Factors that limit lung injury and/or promote epithelial barrier function and repair are highly desirable as immunomodulatory therapeutics. Over the last decade, interleukin−22 has been shown to have pulmonary epithelial protective functions at the mucosal immune interface with bacterial and viral pathogens. This article summarizes recent findings in this area and provides perspective regarding the role of IL-22 in mucosal host defense.

Keywords: lung, interleukin, mucosa, influenza, bacteria

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INTRODUCTION

Protective immune mechanisms of lung injury during infection are an important area of research that could provide new immunomodulatory therapeutic options. Maintenance of lung epithelial structural integrity during bacterial or viral pneumonia is of critical importance to limit lung edema and pathogen dissemination. The function of the cytokine interleukin (IL)−22 in the lung has only recently been elucidated and much is still to be discovered. Work over the last decade has shown that IL-22 likely plays a role in epithelial biology during infectious diseases (1–4). IL-22 is a member of the IL-10 cytokine family and is produced mainly by innate and adaptive T cells in response to lung injury during infection, allergy, and fibrosis (5). IL-22 signals through a heterodimeric receptor of IL-22Ra1 and IL-10R2, which is expressed on non-hematopoietic cells. Ligand binding leads to phosphorylation of the transcription factor STAT3 and nuclear translocation to regulate transcriptomic program in cells. IL-22 signaling is opposed by a soluble decoy receptor IL-22Ra2, or IL-22 binding protein (IL-22BP), which is coordinately regulated with IL-22 production to regulate IL-22 signaling. In this perspective article, the role of IL-22 in regulating the epithelial barrier in the lung during bacterial, viral, and super-infection will be discussed.

BACTERIAL INFECTION

Bacterial infections of the lung with Gram (−) and (+) bacteria are common causes of both community and hospital acquired pneumonia. The first description of a host defense role for IL-22 in the lung was demonstrated using the *Klebsiella pneumoniae* model of pneumonia (6). In that model, neutralization of IL-22 led to failed control of bacterial infection compared with control mice. Anti-IL-22 treatment resulted in increased bacterial burden in the lung and dissemination to the spleen. Induction of IL-22 in response to *K. pneumoniae* was dependent on IL-23 production. IL-23 is a canonical Type 17 immunity promoting cytokine. IL-22 promoted proliferation of human bronchial epithelial cells *in vitro* and improved transepithelial electrical resistance, a measure of barrier function, following scratch wounding. These data demonstrated an epithelial barrier

protective effect of IL-22. Shortly thereafter, therapeutic delivery of IL-22 to rat lungs was shown to improve epithelial barrier function in a model of ventilator induced lung injury (7). IL-22 treatment reduced lung edema and increased survival in this model providing further evidence for IL-22 acting and an epithelial protective cytokine.

Using the *Pseudomonas aeruginosa* model of lung infection, antibody blockade of IL-22 was shown to increase infection, lung damage, and neutrophil accumulation (8). Consistent with this finding, exogenous IL-22 or neutralization of IL-22BP reduced neutrophil recruitment to the lung and pathology. More recently, IL-22–/– mice were tested in a model of *P. aeruginosa* infection (9). IL-22–/– mice had worsened lung injury when compared with wild-type (WT) control mice. These authors also showed that the inflammatory environment in the lung during *P. aeruginosa* infection results in proteolytic degradation of IL-22 via the neutrophil serine protease 3. Further, the *P. aeruginosa* protease IV has also been shown to degrade IL-22 as a potential virulence factor (10). These data suggest a critical role for IL-22 in Gram (−) host defense and identify targeting of IL-22 by pathogens as an immune evasion mechanism.

The role of the IL-22/IL-22BP axis in Gram (+) host defense has also been shown. Deletion or antibody neutralization of IL-22 resulted in increased lung bacterial burden and dissemination compared with controls during *Staphylococcus aureus* infection (11). These data are consistent with a barrier protective role for IL-22 in the lung. The role of IL-22 has also been studied in the context of *Streptococcus pneumoniae* infection (12, 13). IL-22–/– mice were shown to have increased bacterial burden in the lung compared to WT mice. Exogenous IL-22 was able to decrease *S. pneumoniae* burden in the lung in WT mice. In a second study, IL-22BP–/– mice, lacking the inhibitory receptor of IL-22, were protected from lung injury and mortality compared with WT mice. Further, IL-22BP–/– mouse lungs had altered transcriptomes with a lack of oxidative phosphorylation gene expression, which was IL-22 dependent. Macrophages from IL-22BP–/– mice had increased glycolytic activity, suggesting that IL-22 may regulate macrophage metabolism. While much about the mechanisms by which IL-22/IL-22BP regulate anti-bacterial host defense remains to be discovered, it is clear that IL-22 is of importance in these settings.

A potential relationship between IL-22 and interferon lambda (IFNλ) has recently emerged. IFNλ is an antiviral cytokine family that has been shown to be protective during viral infections without pro-inflammatory effects, an important consideration in the lung. In the *K. pneumoniae* model, IFNλR–/– mice exhibited better bacterial control and improved epithelial barrier function (14). These IFNλR–/–mice had acute elevations in IL-22 production suggesting that IFNλ negatively regulates IL-22 in the lung. This regulation may be bi-directional as IL-22–/– mice had decreased IFNλ expression in the later phases of infection. Using IL-22–/– mice, IL-22 was shown to be critical for epithelial barrier stability in the *K. pneumoniae* model, confirming aforementioned work using IL-22 neutralization. Consistent with the findings regarding IL-22 and IFNλ cross-talk, IL-22 treatment increased IFNλ production and anti-IL-22 decreased IFNλ levels in a model of *P. aeruginosa* infection and in an *in vitro* alveolar epithelial cell line (15). This interaction between IL-22 and IFNλ has also been suggested in models of bacterial pneumonia exacerbated by preceding influenza virus infection. IFNλR–/– mice were shown to produce increased levels of IL-22 following influenza, *S. aureus* super-infection (16). However, others did not observe an increase in IL-22 following exogenous IFNλ delivery in a similar super-infection model (17). These data suggest a potentially complex role for IL-22 in modulating antiviral immunity in the lung.

INFLUENZA INFECTION

Influenza virus is the cause of world-wide, annual epidemics and can result in severe lung pathogenesis in people of all ages. Initial study into the role of IL-22 during influenza infection produced limited results. Antibody neutralization of IL-22 was not effective at reducing influenza mediated morbidity or mortality in mice, although a reductive effect on viral load was shown (18). Shortly thereafter, influenza infection was shown to induce IL-22 production by invariant natural killer T (iNKT) cells in the lung (19). The pro-Type 17 cytokines IL-1β and IL-23 were shown to induce iNKT cell production of IL-22 and lead to protection from lung epithelial damage both *in vitro* and *in vivo*. In this study, IL-22 production was manipulated by deletion of iNKT cells and no effect on viral load was observed. These data suggest an epithelial protective role for IL-22 during viral infections.

These studies were then followed by a trio of studies of influenza infection in IL-22–/– mice. Conventional natural killer cells were shown to be a primary source of IL-22 following influenza challenge in mice (20). IL-22–/– mice had decreased epithelial regeneration compared to WT mice after influenza infection. In addition, IL-22 stimulated epithelial proliferation *in vitro*. Another study found similar effects on lung epithelial repair following influenza infection (21). In that study, IL-22–/– mice had increased lung injury, decreased lung function, and increased pulmonary fibrosis compared with WT mice 3 weeks following influenza infection. A third study provided additional supportive data showing increased lung injury and decreased epithelial integrity after influenza challenge (22). However, the primary cellular source of IL-22 was γδT and innate lymphoid cells in that study. All three studies failed to observe an impact on viral burden, but were able to show an important role for IL-22 in preservation of the epithelial barrier in viral pneumonia.

Regulation of IL-22 signaling during influenza infection has also been studied. Two studies showed that IL-22Ra1 is highly expressed on airway epithelial cells prior to influenza infection (20, 21). Following infection, expression of IL-22Ra1 is highly increased in injured areas of the distal lung. Tlr3 activation by viral pathogen-associated molecular pattern molecules in epithelial cells results in increased expression of IL-22Ra1 via IFNβ dependent STAT1 signaling (23). Additional pathways have been shown to regulate IL-22Ra1 protein stability. Glycogen synthase kinase 3β (GSK3β) was shown to phosphorylate IL-22Ra1 and increase its protein stability (24). Further, IL-22 was shown to inactivate GSK3β, perhaps as a feedback mechanism to terminate IL-22Ra1 signaling. IL-22Ra1 was also shown to be

regulated via ubiquitination by the E3 ligase FBXW12 (25). This interaction destabilizes IL-22Ra1. During infections, neutrophil serine proteases also can degrade IL-22Ra1 providing another mechanism of control of IL-22 signaling (26). Regulation of IL-22Ra1 is complex and it is likely that we only have a limited understanding at this time.

Finally, deletion of IL-22BP negative regulation of IL-22 was shown to decrease lung inflammation and injury compared with WT mice after influenza infection (27). Increased tight junction protein expression was observed *in vivo*. Treatment of human bronchial epithelial cells with IL-22 resulted in increased expression of tight junction proteins and claudins, suggesting a direct role for IL-22 in mediating epithelial barrier integrity. Delivery of IL-22 to mice decreased influenza mediated inflammation and lung leak. IL-22 was also shown to inhibit viral induced expression of programmed death-ligand 1 (PD-L1) on epithelial cells (28). PD-L1 expression is a mechanism by which viruses evade T cell activation and clearance. In this manner, IL-22 may also promote antiviral immunity distinct from its barrier protective role. These data suggest that the IL-22/IL-22BP axis may be of critical importance in prevention of infectious lung injury.

INFLUENZA, BACTERIAL SUPER-INFECTION

An important clinical exacerbation of influenza infection is secondary bacterial pneumonia. In both epidemic and pandemic seasons, bacterial super-infection is a severe complication that leads to a need for intensive care and in some cases, death. *S. pneumoniae* has classically been the most common cause of influenza related super-infections, however over the last decade, *S. aureus* has become more predominant. IL-22–/– mice were shown to have increased bacterial burden and mortality compared with WT mice in a model of influenza, *S. pneumoniae* super-infection (22). In this model, exogenous IL-22 delivered to WT mice decreased bacterial dissemination, but did not affect lung bacterial load (29). IL-22 therapy increased epithelial barrier function and decreased lung leak. In support of these findings, deletion of IL-22BP in mice improved outcomes of influenza super-infection with both *S. aureus* and *S. pneumoniae* (30). Severe lung pathology induced by influenza, *S. pneumoniae* infection was significantly attenuated in IL-22BP–/– mice. Less lung leak and increased expression of claudin proteins were observed in IL-22BP–/– mice compared with WT mice. Finally, IL-22 treatment of air liquid interface cultures of human bronchial epithelial cells resulted in preserved tight junction function following injury with *S. aureus*. These data demonstrate and important role for IL-22/IL-22BP in the context of polymicrobial infection.

CORONAVIRUS INFECTION

The current COVID-19 pandemic has raised interest in immunomodulatory therapeutics. There are no published reports regarding lung IL-22 in coronavirus infections caused by severe acute respiratory syndrome viruses (SARS-CoV1 or SARS-CoV2) or middle east respiratory syndrome virus (MERS). A recent report suggests that IL-22 may be systemically elevated in the acute phase of SARS-CoV2 infection and may play a role in cardiovascular injury (31). Severe SARS-CoV2 infection is associated with development of acute respiratory distress syndrome (ARDS) with lung hyper-inflammation and edema (32, 33). The degree of similarity between SARS-CoV2 and influenza pathogenesis is currently unclear; however, it is likely that IL-22 plays a role in epithelial barrier integrity. Data is needed to assess the potential for targeting the IL-22 pathway in this context.

SUMMARY

The highlighted work and those of others have shown an emerging role for IL-22 in promoting epithelial integrity and repair following infectious pathogen challenge in the lung. Precise mechanisms of IL-22 action have been more elusive, as IL-22 often has limited biologic effects on uninjured cells *in vitro*. While data exist regarding the effects of IL-22 on epithelial cells *in vitro*, there remains much to be discovered. It will be important to assess the effects of IL-22 on epithelial cells in inflammatory settings, perhaps in combination with pathogen associated molecular patterns (PAMPs) or toxins. Evidence for direct impact of IL-22 on immune cells is less abundant and controversial. More work in this area is needed to determine if IL-22 affects lymphocyte function in the absence of signaling through lung stromal cells. Mechanisms by which IL-22 mediates pathogen clearance, in the case of bacterial infection, are mostly unknown. Several studies have shown that lung bacterial burden is altered when IL-22 is manipulated, indicating that IL-22's role is not solely focused on preventing dissemination from the lung. It is likely that IL-22, signaling through the epithelium, impacts host defense in several undiscovered ways. IL-22 has many described functions outside of the lung in the gastrointestinal tract, liver, and thymus. It is possible that IL-22 regulation of immunity in the lung is not restricted to direct effects in lung tissue. Focused use of floxed IL-22Ra1 mice should enable high resolution study of tissue and cellular compartments where IL-22 signaling is required during infection. Regardless, pre-clinical animal models suggest that IL-22 has significant therapeutic potential in the context of infectious diseases. Additional study is required to confirm the current reports in the literature and expand the field beyond the few pathogens mentioned herein. Undoubtedly, studies of IL-22 in SARS-CoV2 infection would be of great interest. Beyond pre-clinical animal models, translational studies are now needed to determine if IL-22 can limit lung damage and promote repair in humans. Less is known with regard to human IL-22 production and signaling in human infectious diseases. This area will be critical to evaluate in human pneumonia.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JA conceived and wrote the manuscript.

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REFERENCES

1. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. *Nat Immunol*. (2011) 12:383–90. doi: 10.1038/ni.2025
2. Manni ML, Robinson KM, Alcorn JF. A tale of two cytokines: IL-17 and IL-22 in asthma and infection. *Expert Rev Respir Med*. (2014) 8:25–42. doi: 10.1586/17476348.2014.854167
3. Mcaleer JP, Kolls JK. Directing traffic: IL-17 and IL-22 coordinate pulmonary immune defense. *Immunol Rev*. (2014) 260:129–44. doi: 10.1111/imr.12183
4. Eyerich K, Dimartino V, Cavani A. IL-17 and IL-22 in immunity: driving protection and pathology. *Eur J Immunol*. (2017) 47:607–14. doi: 10.1002/eji.201646723
5. Aujla SJ, Alcorn JF. T(H)17 cells in asthma and inflammation. *Biochimica et biophysica acta*. (2011) 1810:1066–79. doi: 10.1016/j.bbagen.2011.02.002
6. Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med*. (2008) 14:275–81. doi: 10.1038/nm1710
7. Hoegl S, Bachmann M, Scheiermann P, Goren I, Hofstetter C, Pfeilschifter J, et al. Protective properties of inhaled IL-22 in a model of ventilator-induced lung injury. *Am J Respir Cell Mol Biol*. (2010) 44:369–76. doi: 10.1165/rcmb.2009-0440OC
8. Broquet A, Jacqueline C, Davieau M, Besbes A, Roquilly A, Martin J, et al. Interleukin-22 level is negatively correlated with neutrophil recruitment in the lungs in a *Pseudomonas aeruginosa* pneumonia model. *Sci Rep*. (2017) 7:11010. doi: 10.1038/s41598-017-11518-0
9. Guillon A, Brea D, Luczka E, Herve V, Hasanat S, Thorey C, et al. Inactivation of the interleukin-22 pathway in the airways of cystic fibrosis patients. *Cytokine*. (2019) 113:470–4. doi: 10.1016/j.cyto.2018.10.015
10. Guillon A, Brea D, Morello E, Tang A, Jouan Y, Ramphal R, et al. *Pseudomonas aeruginosa* proteolytically alters the interleukin 22-dependent lung mucosal defense. *Virulence*. (2017) 8:810–20. doi: 10.1080/21505594.2016.1253658
11. Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, et al. Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol*. (2011) 186:1666–74. doi: 10.4049/jimmunol.1002194
12. Trevejo-Nunez G, Elsegeiny W, Conboy P, Chen K, Kolls JK. Critical role of IL-22/IL22-RA1 signaling in pneumococcal pneumonia. *J Immunol*. (2016) 197:1877–83. doi: 10.4049/jimmunol.1600528
13. Trevejo-Nunez G, Elsegeiny W, Aggor FEY, Tweedle JL, Kaplan Z, Gandhi P, et al. Interleukin-22 (IL-22) binding protein constrains IL-22 activity, host defense, and oxidative phosphorylation genes during pneumococcal pneumonia. *Infect Immun*. (2019) 87:e00550–19. doi: 10.1128/IAI.00550-19
14. Ahn D, Wickersham M, Riquelme S, Prince A. The Effects of IFN-lambda on epithelial barrier function contribute to *Klebsiella pneumoniae* ST258 Pneumonia. *Am J Respir Cell Mol Biol*. (2019) 60:158–66. doi: 10.1165/rcmb.2018-0021OC
15. Broquet A, Besbes A, Martin J, Jacqueline C, Vourc'h M, Roquilly A, et al. Interleukin-22 regulates interferon lambda expression in a mice model of *Pseudomonas aeruginosa* pneumonia. *Mol Immunol*. (2020) 118:52–9. doi: 10.1016/j.molimm.2019.12.003
16. Planet PJ, Parker D, Cohen TS, Smith H, Leon JD, Ryan C, et al. Lambda interferon restructures the nasal microbiome and increases susceptibility to staphylococcus aureus superinfection. *MBio*. (2016) 7:e01939–15. doi: 10.1128/mBio.01939-15
17. Rich HE, Mccourt CC, Zheng WQ, Mchugh KJ, Robinson KM, Wang J, et al. Interferon lambda inhibits bacterial uptake during influenza superinfection. *Infect Immun*. (2019) 87:e00114–19. doi: 10.1128/IAI.00114-19
18. Guo H, Topham DJ. Interleukin-22 (IL-22) production by pulmonary Natural Killer cells and the potential role of IL-22 during primary influenza virus infection. *J Virol*. (2010) 84:7750–9. doi: 10.1128/JVI.00187-10
19. Paget C, Ivanov S, Fontaine J, Renneson J, Blanc F, Pichavant M, et al. Interleukin-22 is produced by invariant natural killer T lymphocytes during influenza A virus infection: potential role in protection against lung epithelial damages. *J Biol Chem*. (2012) 287:8816–29. doi: 10.1074/jbc.M111.304758
20. Kumar P, Thakar MS, Ouyang W, Malarkannan S. IL-22 from conventional NK cells is epithelial regenerative and inflammation protective during influenza infection. *Mucosal Immunol*. (2013) 6:69–82. doi: 10.1038/mi.2012.49
21. Pociask DA, Scheller EV, Mandalapu S, Mchugh KJ, Enelow RI, Fattman CL, et al. IL-22 Is Essential for Lung Epithelial Repair following Influenza Infection. *Am J Pathol*. (2013) 182:1286–96. doi: 10.1016/j.ajpath.2012.12.007
22. Ivanov S, Renneson J, Fontaine J, Barthelemy A, Paget C, Fernandez EM, et al. Interleukin-22 reduces lung inflammation during influenza A virus infection and protects against secondary bacterial infection. *J Virol*. (2013) 87:6911–24. doi: 10.1128/JVI.02943-12
23. Hebert KD, Mclaughlin N, Zhang Z, Cipriani A, Alcorn JF, Pociask DA. IL-22Ra1 is induced during influenza infection by direct and indirect TLR3 induction of STAT1. *Respir Res*. (2019) 20:184. doi: 10.1186/s12931-019-1153-4
24. Weathington NM, Snavely CA, Chen BB, Zhao J, Zhao Y, Mallampalli RK. Glycogen synthase kinase-3beta stabilizes the interleukin (IL)-22 receptor from proteasomal degradation in murine lung epithelia. *J Biol Chem*. (2014) 289:17610–9. doi: 10.1074/jbc.M114.551747
25. Franz J, Jerome J, Lear T, Gong Q, Weathington NM. The human IL-22 receptor is regulated through the action of the novel E3 ligase subunit FBXW12, which functions as an epithelial growth suppressor. *J Immunol Res*. (2015) 2015:912713. doi: 10.1155/2015/912713
26. Guillon A, Jouan Y, Brea D, Gueugnon F, Dalloneau E, Baranek T, et al. Neutrophil proteases alter the interleukin-22-receptor-dependent lung antimicrobial defence. *Eur Respir J*. (2015) 46:771–82. doi: 10.1183/09031936.00215114
27. Hebert KD, Mclaughlin N, Galeas-Pena M, Zhang Z, Eddens T, Govero A, et al. Targeting the IL-22/IL-22BP axis enhances tight junctions and reduces inflammation during influenza infection. *Mucosal Immunol*. (2020) 13:64–74. doi: 10.1038/s41385-019-0206-9
28. Seki N, Kan OK, Matsumoto K, Fukuyama S, Hamano S, Tonai K, et al. Interleukin-22 attenuates double-stranded RNA-induced upregulation of PD-L1 in airway epithelial cells via a STAT3-dependent mechanism. *Biochem Biophys Res Commun*. (2017) 494:242–8. doi: 10.1016/j.bbrc.2017.10.045
29. Barthelemy A, Sencio V, Soulard D, Deruyter L, Faveeuw C, Le Goffic R, et al. Interleukin-22 immunotherapy during severe influenza enhances lung tissue integrity and reduces secondary bacterial systemic invasion. *Infect Immun*. (2018) 86:e00706–17. doi: 10.1128/IAI.00706-17
30. Abood RN, Mchugh KJ, Rich HE, Ortiz MA, Tobin JM, Ramanan K, et al. IL-22-binding protein exacerbates influenza, bacterial super-infection. *Mucosal Immunol*. (2019) 12:1231–43. doi: 10.1038/s41385-019-0188-7

31. Guzik TJ, Mohiddin SA, Dimarco A, Patel V, Savvatis K, Marelli-Berg FM, et al. COVID-19 and the cardiovascular system: implications for risk assessment, diagnosis, and treatment options. *Cardiovasc Res*. (2020) 30:cvaa106. doi: 10.1093/cvr/cvaa106
32. Felsenstein S, Herbert JA, Mcnamara PS, Hedrich CM. COVID-19: immunology and treatment options. *Clin Immunol*. (2020) 215:108448. doi: 10.1016/j.clim.2020.108448
33. Kakodkar P, Kaka N, Baig MN. A comprehensive literature review on the clinical presentation, and management of the pandemic Coronavirus Disease 2019 (COVID-19). *Cureus*. (2020) 12:e7560. doi: 10.7759/cureus.7560

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Pulmonary Innate Immune Response Determines the Outcome of Inflammation During Pneumonia and Sepsis-Associated Acute Lung Injury

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The lung is a primary organ for gas exchange in mammals that represents the largest epithelial surface in direct contact with the external environment. It also serves as a crucial immune organ, which harbors both innate and adaptive immune cells to induce a potent immune response. Due to its direct contact with the outer environment, the lung serves as a primary target organ for many airborne pathogens, toxicants (aerosols), and allergens causing pneumonia, acute respiratory distress syndrome (ARDS), and acute lung injury or inflammation (ALI). The current review describes the immunological mechanisms responsible for bacterial pneumonia and sepsis-induced ALI. It highlights the immunological differences for the severity of bacterial sepsis-induced ALI as compared to the pneumonia-associated ALI. The immune-based differences between the Gram-positive and Gram-negative bacteria-induced pneumonia show different mechanisms to induce ALI. The role of pulmonary epithelial cells (PECs), alveolar macrophages (AMs), innate lymphoid cells (ILCs), and different pattern-recognition receptors (PRRs, including Toll-like receptors (TLRs) and inflammasome proteins) in neutrophil infiltration and ALI induction have been described during pneumonia and sepsis-induced ALI. Also, the resolution of inflammation is frequently observed during ALI associated with pneumonia, whereas sepsis-associated ALI lacks it. Hence, the review mainly describes the different immune mechanisms responsible for pneumonia and sepsis-induced ALI. The differences in immune response depending on the causal pathogen (Gram-positive or Gram-negative bacteria) associated pneumonia or sepsis-induced ALI should be taken in mind specific immune-based therapeutics.

Keywords: pneumonia, sepsis, ALI, ILCs, neutrophils, macrophages

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INTRODUCTION

Lungs serve as vital organs for the gaseous exchange in the vertebrates. They have evolved from their very primitive stage (air sacs found in the very primitive and well-armored fossil placoderm fish Bothriolepis) to the most advanced form present in mammals depending on their habitat and the oxygen demand. Thus, due to continuous gaseous exchange function, lungs serve as a very easy target organ for the airborne pathogens, allergens, and other toxicants to cause pulmonary

infections or inflammation. However, pulmonary damage may be acute, or chronic depending on the intensity and the duration of the exposure. For example, chronic obstructive pulmonary disease (COPD) and allergic asthma cause chronic inflammatory changes in the lungs. Whereas, acute microbial (bacterial or viral) infections responsible for pneumonia or sepsis cause severe inflammatory damage to the lungs, leading to the development of acute lung injury/inflammation (ALI) or acute respiratory distress syndrome (ARDS) in critically ill patients (1).

ALI in response to the severe pulmonary microbial infections occurs as a result of the immunological recognition of the pathogen responsible for inducing a pro-inflammatory immune response. The ALI causes severe tissue damage, and in severe cases, irreversible pulmonary damage may lead to death. For example, the protein-rich hydrostatic pulmonary edema characterizing ALI causes refractory hypoxemia, stiffening of the lungs, and difficulty to respire. Rene Laennec (invented the stethoscope in 1861) in 1821 first described the ARDS as an "idiopathic pulmonary edema" occurring without heart failure, which was further modified into "wet lung or shock lung" (2, 3). However, Ashbaugh et al. for the first time, coined the term ARDS to describe the rapid onset of tachypnoea, hypoxemia, and the loss of compliance after a variety of stimuli (4). Sepsis is a leading (6–42%) cause of the ALI (5). Depending on the ALI/ARDS cause, age, and sex of the host, the pulmonary innate immune system plays a very significant role in the ALI pathogenesis (6).

The innate immune system serves as the first line of defense against foreign pathogens via recognizing their pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). Also, innate immune cells recognize the damage or danger-associated molecular patterns (DAMPs) generated during the pro-inflammatory conditions disturbing immune homeostasis (7). The recognition of PAMPs or MAMPs and DAMPs involves several pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and multiple germ line encoded receptors [NOD-like receptors (NLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs) and multiple intracellular DNA sensors expressed (cGAS-STING signaling pathway, Aim 2 like receptors (ALRs)] (8–11). This induces the pro-inflammatory immune response generating different cytokines, chemokines, interferons (IFNs), and other molecules, including reactive oxygen or nitrogen species (ROS or RNS) for clearing the infection to maintain the immune homeostasis. However, the innate immune response dysregulation during infection may increase its severity via increasing the pathogen load due to the inefficient pathogen clearance or by causing increased and irreversible organ damage in patients succumbed to sepsis (12, 13). Hence, a regulated innate immune response during both acute and chronic infections is essential for clearing the infection.

The organ-specific innate immune response determines infection severity. For example, the potent innate immune response generation in the lungs during localized pulmonary infections (pneumonia) or its dysregulation as seen in the non-pulmonary sepsis (sepsis originating from other sources, including the peritoneum, urinary tract, various soft tissues, and skin)-associated acute ALI or ARDS plays a crucial role in the disease outcome (12). Thus, the major aim of the present article is to describe the pulmonary innate immune response responsible for the ALI observed during bacterial pneumonia and sepsis, as evidenced by both animal and human findings.

LUNG AS AN INNATE IMMUNE ORGAN

Lungs are the vital organs designed not only for the gaseous exchange but also serve as a major immune organ to protect the host from diseases caused by the pathogen inhalation during respiration along with allergens and xenobiotics (allergic asthma, pneumonia, sepsis-associated ALI) (12, 14). In the early 1960s, Askonas and Humphrey showed upon intravenous injection of pneumococcal antigens lungs potentially contribute to developing more specific antibodies in comparison to the rest of the lymphoid organs (15). Later on, another study in rabbits showed that the local intranasal instillation of pneumococcal antigen-induced the specific immunity and pulmonary resistance to the infection without generating the antibody (Ab)-mediated systemic immunity (16). Furthermore, the pulmonary DNA vaccine-based immunization also induces the local $CD8^{+}$T cell-based protective anti-viral (vaccinia and influenza virus) immunity without recruiting peripheral T cells (17). This pulmonary immune response during vaccination can further be enhanced by the nasal administration of the adjuvants (18). However, the pulmonary challenge with recombinant vaccines has the potential to generate local (lung) as well as systemic immunity against pathogens (19). Thus, lung can induce protective immunity against respiratory pathogens without the involvement or activation of peripheral or systemic immunity by working as a potent immune organ.

Lungs can be categorized into two components both from a physiological and immunological point of view, (1) Upper respiratory tract serving as mucosal (IgA serves as predominating class of antibody) and glandular component, and (2) peripheral airways without any mucosal tissue (dominated by IgG antibody). Furthermore, the peripheral airways on the luminal side constantly remain in contact with the Broncho-alveolar cells (BACs, 90% of which under normal homeostasis comprise of alveolar macrophages), and 10% of which is comprised of lymphocytes (**Figure 1**). Thus, the pulmonary immune system is separable into different compartments, which have the potential to interact (20). Similar to the epithelial lymphocyte compartment of the gut, a compartment of lymphocytes residing in the respiratory tract epithelium over the epithelial membrane and between the epithelial cells also exists. Thus, protecting the host from invasive pulmonary infections. The other compartment of the respiratory lymphoid cells (RLCs) comprises of the organized lymphoid tissues lying within the bronchial walls. This RLC compartment comprises of either solitary lymphoid follicles (SLFs) or their aggregates resembling the Peyer's Patches (PPs) of the intestine (21, 22). Thus, this bronchus-associated lymphoid tissue (BALT) is morphologically and functionally analogous to the gut-associated lymphoid tissue (GALT) of the intestine (23, 24). For example, receptor activator

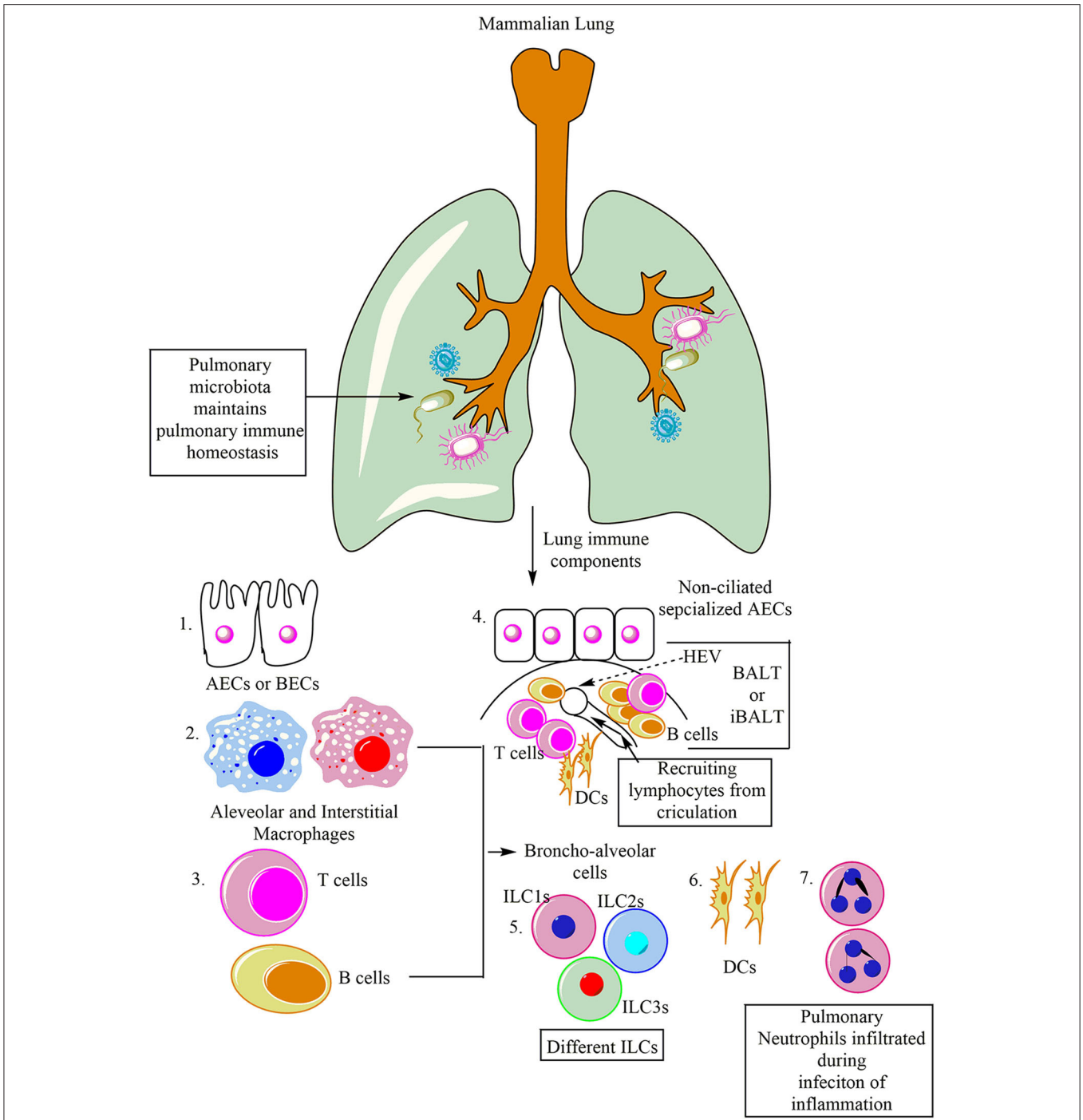

FIGURE 1 | Major immune cells in the mammalian lung. Lungs are potent immune organs and contain macrophages, which may be divided into alveolar macrophages (AM) and interstitial macrophages (IMs), alveolar and bronchial epithelial cells (AECs and BECs), DCs, NK cells along with other ILCs (ILC1s, ILC2s, and ILC3s), and adaptive immune cells (different T and B cells). Neutrophils also migrate to the lungs in response to the infection or inflammatory insult. Additionally, like Peyer's patches (PPs) of gut-associated lymphoid tissue (GALT), lungs also have BALT. BALT contains T cells (T cell Zone), B cells (B cell zone), and DCs. The BALT induced in response to the infection is called iBALT.

of nuclear factor-κB and its ligand (RANKL) is a common inducer of M cells in the lungs and gastrointestinal tracts (GITs) (25). M cells play a crucial role in respiratory diseases (26).

The BALT is covered by a lymphoepithelium, and its follicle-associated epithelium selectively samples both soluble and particulate matter from the respiratory tract lumen (**Figure 1**)

(27, 28). Of note, in humans, BALT is present only in the lungs of kids and adolescents, and adults show BALT only during chronic inflammatory diseases, where it is called inducible BALT (iBALT) (**Figure 1**) (29). On the other hand, BALT may present in the fetal and neonate's lungs, depending on the antigenic stimulation (30, 31). However, these RLCs comprising the lymphoid follicles of the BALT in humans expand or proliferate considerably in a group of patients suffering from recurrent respiratory tract infections (RTIs) of unknown etiology due to the occlusion of the bronchiolar or bronchial lumen (32). B cells are the major immune cell population of the BALT responsible to generate IgA (**Figure 1**) (20, 33). T cells comprising T cell zone are also present in BALT. T cell zones also have DCs. BALTs also have high endothelial venule (HEV), which serves to transport lymphocytes and antigens to and from the circulation (**Figure 1**). The IgA produced may bind to the lymphocytes to increase their Ab-dependent cytotoxic action. The secreted IgA also protects against viral and bacterial infections along with the allergy. The other compartment comprises of BACs, which can be obtained through broncho-alveolar lavage fluid (BALF) from the peripheral airways. BALF may contain alveolar macrophages (AMs), innate lymphoid cells (ILCs), and dendritic cells (DCs), providing protection against pathogens, toxicants, and allergens inhaled. These pulmonary innate immune cells serve as antigen-presenting cells (APCs) and secrete several cytokines and chemokines to regulate both the pulmonary innate and adaptive immunity. Under normal healthy conditions, BACs in BALF mainly comprises of AM (90%) and rest (10%) are lymphocytes (14). These lymphocytes, via lymph, circulate through the lung and patrol for potential antigen inhaled or entered into the lung through circulation.

The pulmonary immune system matures in the postnatal environment depending on the richness and the type of antigen exposure to the host (34). However, during *in utero* embryonic development lungs remain sterile, but during vaginal delivery, they acquire maternal microbiota (35, 36). The pulmonary microbiota helps in the pulmonary immune system development, tolerance induction, and its homeostasis (**Figure 1**) (37, 38). The pulmonary residential epithelial cells, ILCs, and AMs along with other pulmonary immune cells, are essential to maintain the steady-state in the lungs. However, their ability to recognize different airway pathogens and allergens also induces inflammatory changes in the lungs. Under some situations, these pulmonary inflammatory changes are mild and resolve by itself, but the ALI observed during bacterial pneumonia and sepsis may prove harmful to the host depending on the severity of the infection and the inflammatory innate immune response.

PULMONARY INNATE IMMUNE RESPONSE DURING BACTERIAL PNEUMONIA

According to the National Center for Health Statistics, bacterial pneumonia and influenza comprised eighth causes of mortality in the United States in 2014–2018 (39, 40). However, in children, among infectious diseases, pneumonia is the single most cause of death all over the world (41). Thus, pneumonia is a serious life-threatening infection among the children and older population. Pneumonia pathogenesis is a very complex process involving the microbial invasion of the lower respiratory tract through community or hospital spread. It may occur through inhalation of the causal pathogen. For example, *S. pneumoniae* is the most common pathogen responsible for community-acquired pneumonia (CAP). In addition to the *S. pneumoniae*, *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, and *Coxiella burnetii* are several other common pathogens responsible for CAP (42, 43). Most hospital-acquired pneumonia are caused by Gram-negative pathogens (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, etc.). The details of CAP and hospital-acquired pneumonia (HAP) are described somewhere else (41). The pulmonary innate immune response during pneumonia initiates with the activation of residential innate immune cells (AECs, AMs, etc.) inducing the neutrophil infiltration into the lungs. Toll-like receptor 4 (TLR4) activation induced immune response protects the experimental animals infected with Gram-positive (*Streptococcus pneumoniae*) or Gram-negative bacteria (*Klebsiella pneumoniae*) induced pneumonia (44).

Neutrophil Infiltration in Lungs During Pneumonia-Associated ALI

The mechanism of neutrophil infiltration in the lungs varies from the process involved in other organs and has been described in detail somewhere else (45, 46). The mechanism of neutrophil infiltration in the lungs during ALI varies during Gram-negative and -positive bacterial pneumonia (47). For example, during Gram-negative bacterial (*E. coli* or *P. aeruginosa*) pneumonia, alveolar neutrophil infiltration is mediated by CD18 or β2 integrin, whereas in Gram-positive bacterial (*S. pneumoniae*) pneumonia, it is mediated by the CD29 or β1 integrin (48). Additionally, patients with ALI show an elevated chemokine (CXCL8 or IL-8, CXCL1, or keratinocyte-cell derived chemokine (KC), CXCL5, or epithelial cell-derived neutrophil-activating peptide-78 (ENA-78), and CCL-2) levels in their BALF, which further regulate neutrophil infiltration into the lungs (49, 50).

The CXCR2 (a chemokine receptor) binding to different chemokines [CXCL1, CXCL8 (in humans), CXCL5, CXCL2, CXCL3, CXCL6, and CXCL7 (in humans)] regulates the neutrophil infiltration in the lungs (**Figure 2A**) (51). The lung epithelial cells (LECs) produce the CXCL5 during bacterial (*E. coli*) pneumonia that induces neutrophil infiltration in the lungs, whereas in naïve murine blood, platelets are a crucial source of CXCL5 (51). However, CXCL5 deficiency during *E. coli* pneumonia increases neutrophil influx in the lungs, accelerates the pathogen clearance, improves pulmonary edema, and protects the mice from severe pneumonia and, thus, the ALI (52). $CXCL5^{-/-}$ mice do not show much decrease in CXCR2 expression on bone marrow and blood neutrophils as compared to the wild type (WT) mice upon *E. coli*-induced pneumonia, but the CXCR2 expression on neutrophils remains unchanged during intranasal lipopolysaccharide (LPS)

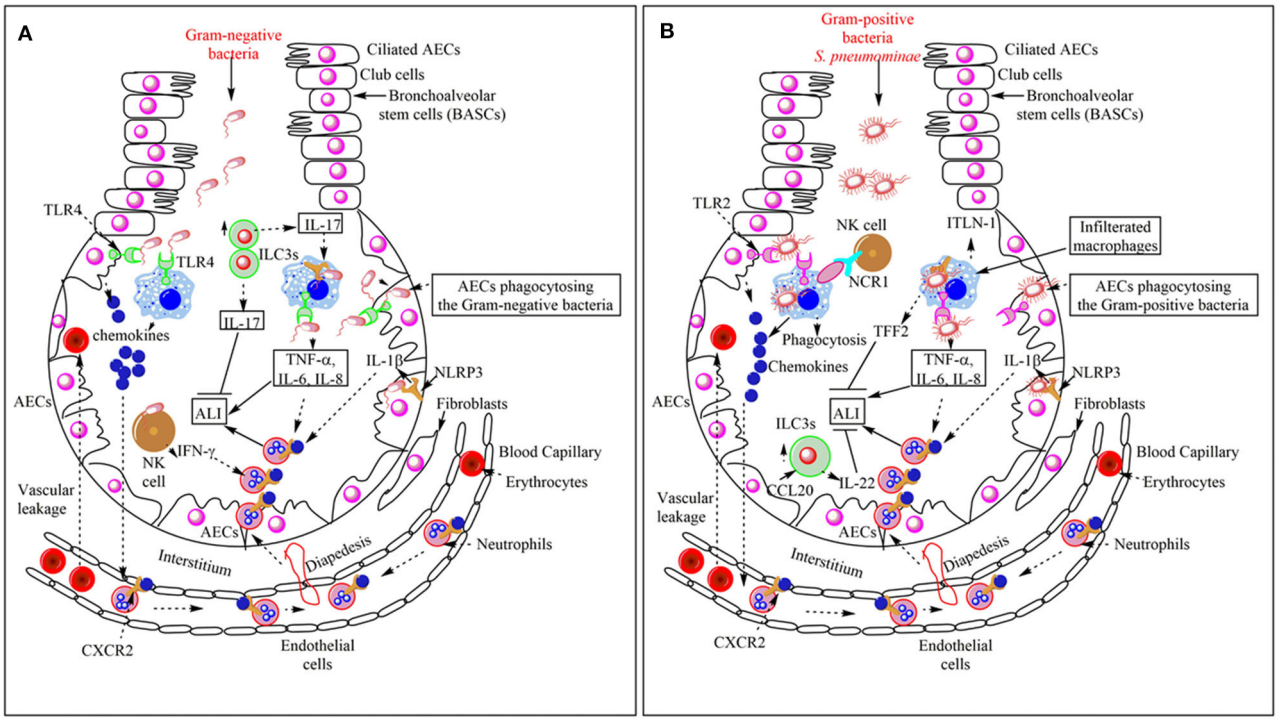

FIGURE 2 | Overview of the bacterial pneumonia-associated innate immune response responsible for ALI. **(A)** Gram-negative bacterial pneumonia and ALI. The PRRs [TLRs (TLR4) and Inflammasome proteins (NLRP3)] expressed on the pulmonary innate immune cells (AECs, BECs, AMs, DCs, and NK cells) recognize Gram-negative bacteria in the lungs. This recognition induces pro-inflammatory cytokine (TNF-α, IL-6, IL-8, and IL-1β) and chemokine release. Chemokines and pro-inflammatory cytokines induce the neutrophil infiltration in the alveoli from the pulmonary blood capillaries through their vascular endothelium via diapedesis. The infiltrated neutrophils help in the pathogen clearance but also cause bystander inflammatory pulmonary tissue damage via damaging PECs or AECs. In the severe pneumonia-associated ALI, the vascular leakage of proteins and erythrocytes also occurs in the lungs. The pulmonary NK cells also release IFN-γ, which further enhances neutrophil infiltration and ALI. However, the increase in ILC3s at later stages increases the IL-17 level. This cytokine helps in the increased phagocytosis of the pathogen and the resolution of the Gram-negative bacterial pneumonia-induced ALI. **(B)** Gram-positive bacterial pneumonia-associated ALI. The recognition of the Gram-positive bacteria (*S. aureus*) induces the increased the production of chemokines and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8), which via binding to the corresponding receptors (CXCR2) on the neutrophils induce their diapedesis to the lung alveoli from the pulmonary vascular endothelium. Neutrophils, along with bacterial clearance, also cause ALI. Pulmonary vascular endothelium damage also causes vascular leakage. The Pulmonary NK cells via their NCR1 interact with AMs to further increase the pro-inflammatory cytokine release, which further aggravates the neutrophil infiltration, pathogen clearance, and ALI also. The NLRP3 also works independently of inflammasome activation via inducing the release of TFF2 and ITLN-1. TFF2 inhibits ALI and helps in its resolution, whereas ITLN-1 clears the infection via increasing the pathogen phagocytosis. The CCL20 released from AECs increases the pulmonary ILC3 numbers, which release IL-22 that inhibits ALI and helps in its resolution.

challenge (52). In the absence of CXCL5, CXCL1, and CXCL2 bind to the Duffy Antigen Receptor for Chemokines (DARC) to increase the neutrophil infiltration in the lungs, which enhances bacterial clearance, and protects the animal from severe pneumonia (52). The levels of these chemokines (CXCL8) affect the severity of ALI and the mortality among patients regulating neutrophil infiltration (53). The CXCL1 regulates neutrophil infiltration and the bacterial clearance during *K. pneumoniae*-induced pneumonia via regulating the CXCL2/MIP-2 and CXCL5, and NF-κB and MAPKs activation in the lungs (54). Thus, the pulmonary neutrophil infiltration is crucial in the ALI induction and its resolution (55, 56). The pulmonary residential innate immune cells [Airway epithelial cells (AECs), macrophages, dendritic cells (DCs), and innate lymphoid cells (ILCs)] are crucial in the pathogenesis of bacterial pneumonia and associated ALI and its outcome.

Airway Epithelial Cells (AECs) and PRRs (TLRs and Inflammasomes) During Pneumonia

AECs comprising of bronchial epithelial cells (BECs) and alveolar epithelial cells [categorized into (1) type I AECs, which are primarily involved in facilitating gaseous exchange and may also recognize pathogens, and (2) type II AECs, also called type II pneumocytes and serve as innate immune cells] serve as a protective mechanical barrier against inhaled pathogens responsible for pneumonia (57, 58). Type II pneumocytes also secrete surfactant proteins on their apical side. These surfactant proteins serve as mucins. The type II AECs secrete repair enzymes (fibrinogen or FBG) basolaterally, which respond to the change in osmotic pressure of the cell very quickly and sense pore-forming toxins secreted by pathogenic bacteria (59). The released FBG helps in the cellular response in response to the inflammatory cell damage (60). AECs also serve as

potent regulators of the generation of the primary immune response against invading pathogens via releasing various immune mediators [antimicrobial peptides (AMPs), cytokines] and interacting directly with other immune cells (macrophages, neutrophils, and DCs) (61). The prolonged activation of AECs may prove harmful to the host due to the release of large quantities of pro-inflammatory cytokines, chemokines, and their increased cell death (necrosis or necroptosis).

During pneumonia, AECs recognize various pathogens due to the expression of different PRRs, including TLRs [TLR1, TLR2, TLR4, TLR5, TLR6 (Extracellular TLRs), and TLR3, TLR7, TLR8, and TLR9 (Intracellular TLRs)] and NLRs comprising inflammasome (58, 62–64). The recognition of pathogens by these PRRs serves as the first line of defense and helps in the pathogen clearance. The downstream adaptor molecules [myeloid differentiation primary response protein 88 (MyD88) and Toll/IL-1R domain-containing adaptor-inducing IFN-β (TRIF)] of TLR signaling play crucial roles in bacterial pneumonia. For example, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice develop severe pneumonia due to the profound bacterial growth during Gram-negative pneumonia (*K. pneumoniae*, *P. aeruginosa*, and *E. coli*) in response to the impaired immune response, including the reduced generation of Th1 immune response (TNF-α, IL-6, and IL-8) and almost no neutrophil influx and regulated upon activation normal T Cells expressed and presumably Secreted (RANTES or CCL5) production (65–68).

The TRIF signaling in response to the TLR activation during *K. pneumoniae*-induced pneumonia also exerts antibacterial defense via inducing the interferon (IFN)-x03B3 in the lungs (69). However, Toll/IL-1R Domain-Containing Adaptor Protein (TIRAP) plays a critical role during *K. pneumoniae*-induced pneumonia but not during *P. aeruginosa*-induced pneumonia due to the attenuation of neutrophil sequestration, and MIP-2, TNF-α, IL-6, and LIX (lipopolysaccharide-induced CXC chemokine) production (70). The LIX production, neutrophil infiltration, and bacterial clearance during *P. aeruginosa*-induced pneumonia do not require TIRAP (70). The TLR2-induced MyD88 activation is not required for the *S. aureus* clearance during pneumonia and only exerts the potent inflammatory immune response, but it plays a crucial role in *P. aeruginosa* clearance (71). Thus, TLR2 activation does not have a significant role in the pathogen clearance and survival of the mice but is only required for the inflammatory immune response during Gram-positive bacterial (*S. pneumoniae*) pneumonia (**Figure 2B**) (72). The TLR2 signaling activation during Gram-positive bacterial (*S. pneumoniae*) pneumonia increases the non-small cell lung cancer cell (NSCLC) metastasis (73). Thus, Gram-positive bacterial pneumonia may increase the metastasis of cancer cells in cancer patients.

Inflammasomes and their component proteins also play a crucial role in pathogen detection and clearance during pneumonia. For example, NLRP1 (NLR Family Pyrin Domain Containing 1) enhances the host's resistance to pneumonia via detecting their virulence factors [*Bacillus anthracis* lethal factor (LF) protease] (74). The LF protease induces the proteasome-mediated degradation of amino-terminal domains of NLRPB1 to liberate the carboxyl-terminal fragment, a potent caspase-1 (CASP1) activator (75). Also, the NLR Family Pyrin Domain Containing 3 (NLRP3) activation in BECs during various pneumonia-causing bacterial (*K. pneumoniae, S. pneumoniae, S. aureus, C. pneumoniae,* and *L. pneumophila*) infections protects the host from infections (**Figures 2A,B**) (63, 76, 77). The human BECs also express NLRP3 inflammasome (78). The NLRP3-mediated control of *K. pneumoniae*-induced pneumonia involves the increased neutrophil infiltration, macrophage necrosis, and the release of high-mobility group box-1 protein (HMGB-1) (**Figure 2A**) (79).

The apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) or pyrin domain (PYD) (ASC, also known as PYCARD) is an inflammasome adapter protein required for the formation of the absent in melanoma 2 (AIM2) and NLRP3 inflammasomes. Inflammasome activation causes the ASC speck formation, which forms a platform to activate caspase-1 (CASP-1). However, NLRP3 and ASC maintain pulmonary innate immune homeostasis during *S. pneumoniae*-induced pneumonia through an inflammasome independent manner without activating the CASP1 and CASP11 (80). During this process, they (NLRP3 and ASC) stimulate the optimal expression of several mucosal innate immune proteins, including trefoil factor 2 (TFF2) and intelectin-1 (ITLN-1, a secretory galactofuranose-binding lectin) via expressing the SAM pointed domain-containing Ets transcription factor (SPDEF), which facilitates the mucosal defense factor genes (**Figure 2B**) (80). SPDEF activation involves STAT6 activation. TFF2 protects from increased inflammatory damage via inducing decreased neutrophil recruitment through inhibiting the endothelial vascular cell adhesion molecule 1 (VCAM1) expression and nitric oxide ($NO^{\cdot}$) release from macrophages via inhibiting inducible nitric oxide synthase (iNOS) (81, 82). TFF2 also antagonizes IL-12 (a cytokine required for inducing IFN-γ production and activating Th1 immune cells) secretion from dendritic cells (DCs) and macrophages (83). TFF2 also serves as mucosal healers via protecting mucosal damage, promoting cell motility, and alveolar type 2 cell proliferation, and restores pulmonary gas exchange after infection (84, 85). TFF2 also induces IL-25 and IL-33 after infection to induce type 2 immunity and repair (86). Pulmonary macrophages also utilize the TFF2/Wnt axis to induce pulmonary epithelial cell proliferation to repair the damage following ALI (87). ITLN-1 provides protection via directly binding to the *S. pneumoniae* and representing them to phagocytes for phagocytosis (**Figure 2B**) (80, 87). The BECs express ITLN-1 and may also clear the *S. pneumoniae* via phagocytosis (88–90).

Aged mice exhibit a reduced NLRP3 expression and function, which increases their susceptibility to developing pneumonia, ALI, and mortality (91). The lower expression and function of NLRP3 in aged immune cells (macrophages, epithelial cells, and DCs) attribute to the increased unfolded protein responses (UPRs), which causes a decreased inflammasome assembly and function increasing the severity of pneumonia caused by *S. pneumoniae* (92). The aging also increases the susceptibility of the host to secondary *S. pneumoniae*-induced pneumonia due to the decreased NLRP3 expression and function in the aged

lung (93). The treatment of these aged mice with inflammasome activators [Nigericin, which promotes potassium (K^+) efflux increases the synthesis and release of inflammasome activation-dependent cytokines (IL-1β and IL-18)] increase their survival and decreases their susceptibility toward pneumonia and ALI. Furthermore, the pre-treatment of aged mice with endoplasmic reticulum (ER) chaperone and the stress-reducing agent tauroursodeoxycholic acid (TUDCA) decreases the pneumonia-associated mortality among the aged mice due to the activation of the NLRP3 inflammasome, which increases the pathogen clearance, and lowers the infection-associated pneumonitis (92). The aged mice also express lower levels of TLR1, TLR6, and TLR9 in the lungs, which also increases their susceptibility to pneumonia (93).

Of note, during lethal pneumonia caused by a low dose of serotype 3 *S. pneumoniae,* NLRP3 increases the incidence of ALI and mortality due to the bacterial dissemination and the development of the sepsis (94). Also, during *S. aureus*-induced pneumonia, NLRP3 deficiency prevents the onset of severe necrotic pneumonia via promoting bacterial clearance (95). The NLRP3 activation by α-hemolysin during *S. aureus* pneumonia induces necrotic pulmonary injury or necrotizing pneumonia independent of IL-1β signaling (95, 96). The NLRP3 activation by α-hemolysin in innate immune cells depends on A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) expression and activity (97). ADAM10 binding with α-hemolysin increases NLRP3 activation and cell death due to the availability of ADAM10 on the cell surface. However, ADAM10 protease activity does not play a significant role in NLRP3 activation. Thus, the profound NLRP3 inflammasome activation depending on the severity of the infection proves harmful to the host. In addition to the NLRP3 inflammasome, *S. aureus* pneumonia also activates NLRC4 inflammasome to induce necroptosis through inhibiting the IL-17A-induced neutrophil accumulation in the lungs and IL-18 production (98). The deficiency of NLRC4 increases the pulmonary neutrophil infiltration, decreases the necroptosis, increases the pathogen clearance, and improves the host survival. Thus, the loss of NLRC4 in both hematopoietic and non-hematopoietic cells protects the host against *S. aureus* pneumonia (98).

The murine AECs also express NLRP6 inflammasome (63). NLRP6 activation during *S. aureus* pneumonia also increases the pyroptosis and necroptosis, causing necrotizing pneumonia, increases bacterial burden in the lungs, and decreases the pulmonary neutrophil infiltration (99). The neutrophils isolated from NLRP6 knockout (KO) animals exhibit an increased NADPH-dependent reactive oxygen species (ROS) production and increased bacterial killing. Thus, therapeutic targeting of NLRP3, NLRC4, and NLRP4 inflammasome during Gram-positive bacteria-induced severe pneumonia responsible for ALI may prove beneficial to the host. An experimental study has shown the beneficial effects (inhibition of ALI, decrease in pro-inflammatory cytokines levels, and decrease in the mortality) of resveratrol during *K. pneumoniae*-induced pneumonia through the NLRP3 inflammasome inhibition (100). NLRC4 activation during Gram-negative bacterial (*K. pneumoniae*, *P. aeruginosa*) pneumonia proves beneficial to the host via producing IL-1β, IL-17A, and neutrophil chemoattractants (keratinocyte cell-derived chemokines, MIP-2, and LPS-induced CXC chemokines) in the lungs (101). However, during *P. aeruginosa* pneumonia, NLRC4 activation-induces inflammatory lung damage, increases pulmonary bacterial burden, and necroptosis (102). Hence the inhibition of NLRC4 inflammasome activation during Gram-negative pneumonia remains a tricky scenario, and further studies will prove helpful in the direction.

The TLR2, TLR4, and MyD88 deficiency did not alter the host response during lethal pneumonia. Any abnormality in the AEC function may lead to the predisposition of the host toward pulmonary infections, including bacterial pneumonia due to the enhanced microbial colonization. For example, patients with allergic asthma are more prone to develop bacterial pneumonia due to the increased pathogenic bacterial colonization, including *Staphylococcus aureus* (*S. aureus*). It may be explained as the higher Th2 cytokines (IL-4 and IL-13) decrease the antimicrobial action of AECs via suppressing the synthesis of human β-defensins 2. However, during streptococcal or pneumococcal pneumonia, AECs also express secreted and transmembrane (Sectm) 1, Sectm1a, and Sectm1b genes due to the type 1 IFN signaling induction in AECs via signal transducer and activator of transcription 1 (STAT-1) activation (103). The Sectm1a binds to the neutrophils only in the presence of the infection and increases the CXCL2 expression. Thus, Sectm1 synthesis and release by AECs during pneumonia increase the neutrophil infiltration into the lungs and helps to clear the infection. However, its dysregulated synthesis may lead to the development of ALI or ARDS.

PECs protect from *K. pneumoniae*-induced pneumonia via ingesting and controlling their number through phagocytosing them via producing the complement component C3, which opsonizes them for phagocytosis (**Figure 2A**) (104). CD46 recognizes the C3 opsonized *K. pneumoniae* for the AEC-mediated phagocytosis or internalization (105). However, the complement resistant strains of *K. pneumoniae* have been emerged and are posing a potential threat to the host (106). The type 1 AECs also highly express epithelial membrane protein 2 (EMP2), a tetraspan protein, which promotes recruitment of different integrins (α6β1, αVβ3) and adhesion molecules (ICAM-1) to the lipid rafts (107). Both rodent and human type II AECs and AMs do not express EMP2 (108). The EMP2 expression of type 1 AECs plays a crucial role in the transepithelial neutrophil migration into the alveoli by regulating the expression of integrins and adhesion molecules (ICAM-1) and suppression of caveolins during bacterial pneumonia (109). Mice lacking EMP2 show a decreased neutrophil infiltration in the alveoli and lung injury during pneumonia. Thus, the activation of the residential PECs as innate immune cells during bacterial pneumonia plays a crucial role in the pathogenesis of pneumonia-associated ALI and its outcome depending on the pathogens (Gram-positive or Gram-negative bacteria) causing pneumonia and the associated immune response.

Pulmonary Innate Lymphoid Cells (ILCs) During Pneumonia and Associated ALI

ILCs serve as immunoregulatory innate immune cells at mucosal surfaces and play a crucial role in the pathogenesis of inflammation and inflammatory diseases (110, 111). The details of their development, classification, regulatory transcription factor (TFs), and function are described somewhere else (112–114). ILCs are divided into three major categories depending on their effector functions and transcriptional requirements: (1) Group 1 ILCs include type 1 ILCs, and Natural Killer (NK) cells, (2) Group 2 ILCs or ILC2s, and (3) Group 3 ILCs or ILC3s (114). Group 1 ILCs, including NK cells, are a rapid source of interferon-γ (IFN-γ), and mice deficient in IFN-γ develop more severe *K. pneumoniae* or *L. pneumophila*-induced pneumonia upon intratracheal inoculation of the pathogen due to impaired IL-1 and IL-6 production, and the defective clearance of the bacteria (115, 116).

NK cells in the lungs are present in its parenchyma in humans and comprise 10–20% of total lung lymphocytes, and in mice, they account for 10% of total lung lymphocytes (117, 118). Human lung NK cells are mostly $CD16^{+}CD56^{low}$, and $KIR^{+}CD57^{+}NKG2A^{-}$ highly differentiated NK cells are also found in the lungs (117, 119). However, the pulmonary resident NK cells express CD69, CD49a, and CD103, and most of them are $CD56^{high}CD16^{-}$ and display a lesser mature form (120). In mice, pulmonary NK cells protect against *K. pneumoniae*-induced pneumonia via secreting IFN-γ and IL-22, which launch the bacterial growth-controlling interactions between alveolar macrophages and NK cells (**Figure 2A**) (121, 122). IFN-γ plays a crucial role in the bactericidal action of alveolar macrophages and the release of NK cell amplifying IL-12 and CXCL10 (122). The NCR1 (natural cytotoxicity receptor 1) on pulmonary NK cells controls their activation and the IFN-γ release during the early stages of *S. pneumoniae*-induced pneumonia without mediating the pathogen recognition (123). However, NCR1 ligands are expressed by pulmonary macrophages and DCs, which directly interact with NK cells during the early stages of *S. pneumoniae*-induced pneumonia (**Figure 2B**). This interaction increases their phagocytic activity required to clear the infection and mounting the effective immune response (**Figure 2B**).

Group 2 ILCs secrete Th2 cytokines [IL-4, IL-5, IL-6, IL-9, IL-13, and Amphiregulin (Arg)] and group 3 ILCs depending on the cytokine released, can be categorized into IL-17 secreting and IL-22 secreting ILC3s. In addition to these cytokines, ILC3s also secrete IL-26 (in humans), GM-CSF, and TNF-α (124). Lymphoid tissue inducer (LTi) cells also belong to group 3 ILCs and secrete IL-22 and IL-17. However, LTi cells have not been seen in the lungs in homeostasis and acute inflammation (125). In human lungs, they have also not been identified due to the lack of known human LTi markers (126). For example, CCR6 is a marker for mice LTi cells, but in humans, all ILC3s express CCR6, and therefore CCR6 does not serve as a marker for human LTi cells (127). Even tertiary lymphoid organs or follicles (TLOs or TLFs), such as iBALT form in the lung tissues of $Rorc^{-/-}$ and $Id2^{-/-}$ mice, which lack LTi cells, following influenza virus infection and inflammation (128). However, iBALT development depends on IL-17 secreted by Th17 cells, which triggers lymphotoxin-independent expression of CXCL13 and CCL19. Thus, LTi cells are dispensable for the aspect of lung immunity.

Lungs are the crucial sites for all the three groups of ILCs (125). Haemophilus influenza pulmonary infection increases the number of IFN-γ producing ILC1-like ($Lin^{-}IL\text{-}7R\alpha^{+}IL\text{-}12R\beta2^{+}IL\text{-}18R\alpha^{+}Tbet^{+}$) cells and increases the pulmonary inflammatory immune response due to the plasticity among pulmonary ILC2s (129). AECs or PECs or pulmonary macrophages during pneumonia secrete IL-1β that governs the ILC2s plasticity (130). IL-1β impacts ILC2 plasticity via inducing the low expression of T-bet (TF) and inducing the IL-12Rβ2 expression, which converts these cells into ILC1s in the presence of IL-12 (131). The treatment with IL-12 during pneumonia exerts a protective action via increasing the infiltration of inflammatory cells (ILC1s, NK cells, and neutrophils) and inflammatory cytokines (IFN-γ) (132, 133). The transforming growth factor-β1 (TGF-β1) secreted by AECs or PECs primes pulmonary ILC2s (134). Pulmonary ILC2s express TGF-βRII. The $CD127^{+}CD90^{+}CCR6^{+}ROR\gamma t^{+}$ group 3 ILCs have been identified in the lung mucosa (135). Pulmonary ILC2s are unable to migrate efficiently within the lung tissue in the absence of TGF-β (134). IL-33 protects against pneumonia via enhancing bacterial clearance and improving the mortality via increasing the neutrophil infiltration and pulmonary ILC2s number (136). The ILC2s convert into ILC1s, which clear the pathogens. Also, these ILC2s are crucial for IL-13-dependent differentiation of pulmonary M2 macrophages, required for the resolution phase of inflammation and infection (137).

The *S. pneumoniae* pneumonia frequently induces the group 3ILCs accumulation in the lungs, which produce IL-22 to protect against severe pneumonia (**Figure 2B**). Furthermore, the administration of TLR5 agonist (flagellin) enhances the IL-22 production from group 3 ILCs during *S. pneumoniae*-induced pneumonia (**Figure 2B**). Studies have also shown earlier, the protective action of mucosal (including sublingual root) flagellin administration to mice infected with *S. pneumoniae*-induced pneumonia without the activation of NLRC4 inflammasome (138, 139). Also, the TLR5 agonist (flagellin) administration increases the efficacy of antibiotic treatment during pneumonia (140). The group 3 ILCs activation to produce IL-22 during *S. pneumoniae*-induced pneumonia also involves the activation of pulmonary dendritic cells (DC). Thus, the activation of pulmonary mucosal group 3 ILCs may prove beneficial to contain the pulmonary infection or pneumonia associated with severe lung inflammation and ALI. The number of group 3 ILCs producing IL-17 also increases during *K. pneumoniae*-induced pneumonia, which helps in the resolution of pulmonary inflammation at later stages to prevent the development of ALI during pneumonia (**Figure 2A**) (141). The release of TNF-α increases the pulmonary ILC3s number and also acts on AECs or PECs to synthesize CCL20. CCL20 chemoattracts ILC3s at the site of infection and inflammation (**Figure 2B**). Also, ILC3 produce IL-17A, which enhances the phagocytic uptake and killing of the bacteria by pulmonary macrophages to clear pneumonia (**Figure 2A**). Thus, ILC3s secrete IL-17A to clear

infection during early stages and help in the resolution of the inflammation to prevent ALI during pneumonia.

The recruitment of IL-22 producing ILC3s into the lungs of neonate mice on exposure to commensal bacteria protects them from neonatal pneumonia (142, 143). This protection involves the intestinal mucosal DCs mediated sensing of commensal bacteria. Furthermore, the murine gut microbiota comprising segmented filamentous bacteria (Sfb) controls the resistance to the *S. aureus* pneumonia via enhancing the number of IL-22 and IL-17 producing innate immune cells (144). Hence pulmonary ILC3s protect the host from pneumonia and associated ALI during early childhood and later in adult life. Thus, pulmonary ILCs serve as crucial pulmonary innate immune cells to protect against pneumonia-induced ALI and in the resolution of the lung inflammation during pneumonia.

Pulmonary Macrophages During Bacterial Pneumonia and Associated ALI

Pulmonary macrophages account for 90–95% of lung immune cells at homeostasis (145). They are of two types: (1) Interstitial macrophages or IMs (reside in lung parenchyma and highly express CD11b but lower levels of CD11c), and (2) Alveolar macrophages or AMs (located in airway space, express high levels of CD11c and low levels of CD11b at their quiescent stage) in the healthy lung (146). Both AMs and IMs express the macrophage-specific markers [CD64 or Fc-gamma receptor 1 (FcγRI) and MER Proto-Oncogene or Tyro-Axl-MerTK (TAM) family of receptor Tyrosine Kinase (Mertk) is involved in efferocytosis] (147, 148). AMs are crucial for maintaining pulmonary immune homeostasis and host defense due to their unique location at the interface between the pulmonary mucosa and the external environment, and are inherently suppressive, whereas IMs exhibit the regulatory function in the lung (149). IMs produce high levels of IL-10 as compared to the AMs, which mainly produce non-specific antimicrobial molecules, including $NO^{\cdot}$, TNF-α, and IFN-γ (146). The steady-state AMs express CD206 (a mannose receptor, which is a C-type lectin and serves as a PRR) and β-glucan specific receptor (Dectin-1), which are also expressed by alternatively-activated macrophages (AAMs) or M2 as their definitive markers (150, 151). The serum CD206 (sCD206) levels increases in the patients of community-acquired pneumonia (CAP) with the increase in its severity [pneumonia severity index (PSI)], which can be used for CAP prognosis (152). Also, the infiltration of $CD206^{+}$ macrophages increases in the lungs of patients with fatal pneumonia.

AMs play a crucial role in the pathogenesis of bacterial pneumonia and associated ALI. For example, AMs during Gram-negative bacterial pneumonia produce TNF-α, which induces granulocyte-macrophage colony-stimulating factor (GM-CSF) in AECs that elicits proliferative signaling in AECs via autocrine stimulation contributing to the alveolar epithelial barrier restoration (153). However, during *S. pneumoniae*-induced pneumonia infiltrating peripheral macrophages replace the resident AMs and IMs. Also, the AM-mediated clearance of apoptotic cells decreases their potential to phagocytose the bacterial pathogens, which increases the bacterial burden in the lungs (**Figure 2B**) (154, 155). The efferocytosis induces the release of prostaglandin E2 (PGE2), which binds to the prostanoid receptors EP2-EP4 activating inhibitory cAMP and PKA pathway, which impairs the neutrophil infiltration and induces the IL-10 release to impair the pathogen clearance (155, 156). PGE2 also impairs the *S. pneumoniae* intracellular killing (ICK) by AMs via inhibiting the hydrogen peroxide (H_2O_2) production (157). IL-18 produced by AMs protects against pneumonia and ALI associated with *S. pneumoniae* infection via enhancing the bacterial clearance (158). However, IL-18 proves detrimental to *P. aeruginosa*-induced pneumonia and enhances its invasiveness to cause sepsis and ALI (159). Thus, type (Gram-positive or Gram-negative) of bacterial pathogens also determines the macrophage-mediated immune response, including the protective or detrimental action of IL-18 released.

The transition of M1 to M2 macrophages during the late stages of pneumonia mediates the inflammation resolution via producing IL-4 and IL-13, which promote STAT6 activation (160). Also, pulmonary macrophages secrete TNF-α stimulated gene-6 (TSG-6), which helps in the ALI resolution via promoting the M1 to M2 macrophage transition However, the efferocytosis of neutrophils by AMs during later stages of pneumonia helps in the resolution of lung inflammation due to expression of growth arrest-specific 6 (Gas6), a member of vitamin K-dependent family of proteins, which binds to its receptors Tyro3, Axl and Mer (TAM), or Mertk (148, 160). The Mertk activation causes ERK-mediated sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2) expression to decrease the cytosolic $Ca2^{+}$ levels, which suppresses the calcium//calmodulin-dependent protein kinase II (148). This process decreases the mitogen activating protein kinase (MAPK) and MK2 kinase activity to increase the abundance of non-phosphorylated cytosolic lipoxygenase (LOX), called 5-LOX, to enhance the production of specialized pro-resolving mediators (SPMs) mediating inflammation resolution (148). Thus, TSG-6 activates STAT6 to induce Gas6 expression in AMs for the ALI resolution during pneumonia.

Lipoxin A4 release by pulmonary endothelial cells, immigrated neutrophils, and pulmonary macrophages at later stages of pneumonia, inhibits neutrophil infiltration, promotes the efferocytosis of dead neutrophils by serving as a proapoptotic signal through downregulating Mac-1 (a β2 integrin) expression, to induce the pulmonary inflammation resolution (161–163). The lipoxin A4-induced neutrophil apoptosis involves the myeloperoxidase (MPO)-induced extracellular signal-regulated kinase (Erk) and Akt-mediated Bcl2-associated agonist of cell death (Bad) phosphorylation along with reducing the antiapoptotic protein myeloid cell leukemia-1 (Mcl-1) expression, which aggravates the mitochondrial dysfunction. This is because Mcl-1 promotes neutrophil survival through heterodimerization and neutralization of Bcl-2 interacting protein (Bim) or Bcl-2 homologous antagonist/killer (Bak) in the mitochondrial outer membrane (162, 164, 165). Lipoxin A4 also enhances the pathogen (*E. coli*) clearance by pulmonary macrophages through inducing the AMP expression (161). The Mac-1 binding to its ligands (ICAM-1, FBG, and MPO) suppresses the apoptosis (163). However, the

Mac-1-dependent phagocytosis of complement-opsonized pathogens triggers rapid neutrophil apoptosis that depends on NADPH oxidase-generated reactive oxygen species (ROS) and caspase (CASP) activation (166). Lipoxin A_4 also inhibits the CXCL8 or IL-8 release from pulmonary macrophages (167). Furthermore, Lipoxin A4 agonist, BML-111 induces autophagy in pulmonary macrophages through suppressing MAPK 1 and 8 signaling. The autophagy of pulmonary macrophages protects against ALI during Gram-negative bacterial pneumonia (168). Lipoxin A4-dependent autophagy among alveolar macrophages during pneumonia occurs independently of mTOR signaling. Hence pulmonary AMs play a crucial role in the induction of protective inflammatory immune during pneumonia and later on in the resolution of the inflammation.

This resolution process occurs at the expense of local pulmonary innate immunity comprising AMs (suppressing phagocytosis) to predisposes the recovering host to severe secondary pneumonia (169). This defective phagocytic function of AMs from pneumonia recovering animals stays for at least 28 days. Even the AMs transplanted intratracheally from normal mice to pneumonia recovered mice become paralyzed AMs, indicating the presence of long term inflammatory innate immune response suppression to make sure the complete resolution of the pulmonary inflammation (169). However, regulatory T cells (T_{regs}), cytokines (TGF-β1 and TNF-α), and DAMPs (HMGB1) do not play a significant role in the induction of paralyzed AMs during resolution of pulmonary inflammation following pneumonia. Also, these paralyzed AMs are not metabolically exhausted as they produce more lactate as compared to the control AMs and produce the same amount of TNF-α upon LPS challenge. Of note, the process of macrophage renewal in mice recovering from pneumonia is similar to normal mice. Thus, AMs of mice recovering from pneumonia are defective in phagocytosis and are unable to clear bacterial pathogens efficiently, increasing their susceptibility to secondary pneumonia. However, these defective or paralyzed AMs are derived from the local pulmonary macrophages in response to the increased expression of signal regulatory protein α (SIRPα), a regulator of tyrosine kinase-coupled signaling processes (phagocytosis) (170–172).

SIRPα increases during the resolution phase in response to the increased surfactant protein-D (SP-D) level. SP-D is an agonist for SIRPα and induces the immunosuppressive environment to produce trained but paralyzed AMs, which stay for weeks after infection to make sure the complete resolution of the inflammation. The increased Sirpα expression upregulates the Mir142 (a micro RNA regulating gene expression in mononuclear phagocytes) expression but down-regulates Setdb2 gene (encoding a histone methyltransferase, which controls the chemokine response during viral pneumonia) (173, 174). The Sedtb2 down-regulation may prevent neutrophil infiltration during the resolution phase to dampen the pneumonia-induced ALI, as indicated previously (173). Also, the Sedtb2 down-regulation alter the pro-inflammatory phenotype of macrophages to a reparative phenotype (175). Mir142 is also shown to regulate immunometabolic reprogramming and favors glycolysis via regulating fatty acid oxidation (FAO) through directly targeting carnitine palmitoyltransferase −1a (CPT1a), a key regulator of the FA pathway (176). Thus, pulmonary macrophages play a crucial role in the resolution of ALI associated with pneumonia.

The Interaction Between PECs and AMs During Pneumonia and Associated ALI

The AMs highly express CD200R (an OX2 glycoprotein of the superfamily of immunoglobulins) on their surface and its levels are maintained by epithelial expression of IL-10 and TGF-β (177). The PECs express ligand for CD200R called, CD200 on their apical side. The CD200R-CD200 interaction on AMs inhibits their pro-inflammatory action during pneumonia that prevents the induction of ALI (177). The CD200-CD200R interaction increases AAMs or M2a phenotype via cAMP-response element-binding protein-C/EBP-beta signaling and upregulates TGF-β expression (178). Also, M2a (anti-inflammatory or regulatory) macrophages generated in the presence of IL-4 and IL-13 also express CD200R in humans (179, 180). The CD200-CD200R interaction inhibits the downstream signaling pathway comprising of the ERK1/2 signaling pathway required for macrophage activation downstream of IFN-γ signaling through Janus-associated kinase (JAK)/STAT-1 activation (181). The $CD200^{-/-}$ mice develop ALI during pneumonia due to the increased pro-inflammatory function of macrophages and a decrease in the resolution of inflammation (177). Also, the AMs attached to the alveolar wall form connexin 43 (Cx43)-containing gap junction channels with the airway epithelium during bacterial pneumonia and intercommunicate through synchronized Ca^{2+} waves, through utilizing the epithelium as the conducting pathway (182, 183). This interaction further supports the anti-inflammatory role of PEC-AM interaction. As mice with AM-specific knockout of Connexin-43 show an increased neutrophil infiltration into the pulmonary alveoli and increased pro-inflammatory cytokine levels in BALF during Gram-negative bacterial (*P. aeruginosa*) pneumonia (182, 183). Thus, the interaction between PECs and AMs controls the inflammatory outcome of the pulmonary infections, including pneumonia leading to the development of ALI and its resolution.

PULMONARY INNATE IMMUNE RESPONSE DURING BACTERIAL SEPSIS

Sepsis leads to the pulmonary inflammation that does not resolve and leads to the development of ALI or ARDS, causing irreversible damage to the lungs (12). Earlier studies have shown that the sepsis is responsible for more than 210,000 cases of ALI/ARDS in the US alone/annually, causing over 74,500 deaths (184, 185). The sepsis-associated ALI//ARDS has a higher mortality rate as compared to the ALI occurring due to other causes (186). The sepsis-associated ALI/ARDS may initiate on any side, including direct lung injury due to the pulmonary epithelial damage or indirect damage comprising the endothelial cell damage (187, 188). The neutrophil infiltration plays a crucial event in this outcome, and the recruitment of neutrophils into the lungs depends on the expression of E-selectin [CD62E or endothelial-leukocyte adhesion molecule 1 (ELAM-1), or

leukocyte-endothelial cell adhesion molecule-2 (LECMA-2)]. E-selectin does not express on unstimulated endothelium, but its expression increases on pulmonary vascular endothelium due to the impact of pro-inflammatory cytokines and induces neutrophil infiltration in sepsis-induced ALI (**Figure 3**) (189).

The immunohistochemical analysis has shown an increased expression of CD62E or E-selectin in the pulmonary microvasculature in sepsis-associated fatalities. The pulmonary intravascular, interstitial and intra-alveolar leukocytes strongly express very late antigen-4 or VLA-4 (CD49d/CD29) or α4β1integrin in sepsis-associated casualties. The ICAM-1 (CD54) is highly expressed on the pulmonary endothelial cells, pulmonary macrophages, and lymphocytes in sepsis-associated fatalities (**Figure 3**). The pulmonary epithelial damage during ALI/ARDS seen in patients with pneumonia-associated sepsis may be indicated by an elevation of surfactant protein-D (SP-D). However, but these patients have low levels of von Willebrand factor (vWF) and IL-6, and IL-8, which are the markers of endothelial damage (**Figure 3**) (190). The pulmonary epithelial damage seen during direct sepsis-associated ALI is more severe as compared to the indirect non-pneumonia-mediated sepsis (190). However, the damage to the endothelium during direct sepsis-associated ALI is less severe. The pulmonary B1a cells exert a protective role in cecal-ligation and puncture (CLP)-induced sepsis via inhibiting neutrophil infiltration and MPO production in the lungs (**Figure 3**) (191). The $CXCR2^{-/-}$ mice exposed to peritoneal sepsis show a decreased pulmonary damage due to the low neutrophil infiltration in the lungs, and the increased CXCL10 expression in the peritoneum (192). CCL-3 or macrophage inflammatory protein-1α (MIP-1α) also mediates sepsis-induced ALI via promoting neutrophil infiltration, pulmonary vascular leakage, and early mortality (**Figure 3**) (193). The following sections highlight the pulmonary innate immune response during sepsis-induced ALI//ARDS.

PECs During Sepsis and Associated ALI

The generation of pro-inflammatory molecules (cytokines, ROS, and RNS) and hypoxia damage the pulmonary epithelial barrier during sepsis-induced ALI (**Figure 2**) (194, 195). This damage to the pulmonary epithelium alters its barrier function and induces the fluid and protein leakage into the alveolar space. The injury to both type I and II AECs during sepsis can easily be assessed in both plasma and pulmonary lavage fluid by the presence of several biomarkers as described previously (194, 196). The pulmonary epithelial damage and increase in its permeability during sepsis involve the change in actin organization (197). The PECs damage due to actin reorganization during sepsis does not include MAPK signaling or the alterations in the tight junction (TJ) proteins. The PECs (BECs and AECs) of the septic lung show an increased αvβ3 integrin, but its inhibition during sepsis-associated ALI needs to study as it may increase the endothelial permeability and thus the sepsis-associated ALI (198, 199). The later (proliferative or fibroproliferative stage observed during the second week of sepsis) stages of ALI during sepsis involves the transformation of the damaged epithelial cells to fibroblast-like cells (epithelial-mesenchymal transition), which requires mitochondrial ROS and hypoxia-inducible factor-1α (HIF-1α) (200).

The PECs express a higher Fas level during sepsis-associated ALI, and the increased infiltration of FasL expressing inflammatory immune cells in the lungs occurs (201). The apoptotic death of PECs during non-pulmonary sepsis (sepsis originating outside the lungs or in the absence of pulmonary infection) involves Fas-FasL interaction, and the Fas inhibition protects their apoptotic cell death via diminishing lung tissue TNF-α, IL-6, IL-10, IFN-γ, IL-12, and CASP-3 activity (202). The BECs show an increased expression of both C3aR and C5aR during sepsis (203). An increased intrapulmonary or intra alveolar C5a level during sepsis may cause severe ALI via binding to the C5aR1 or C5aR, which induces an increased neutrophil infiltration into the septic lung and cytokine/chemokine storm (**Figure 3**) (204, 205). The infiltrated neutrophils in the lungs during ALI/ARDS have a distinctive phenotype and are resistant to apoptosis, and exhibit an enhanced phosphoinositide 3-kinase-dependent (PI3K)-dependent respiratory burst (206). A human study has also indicated the infiltration of less apoptotic neutrophils in the lungs of Sepsis-associated ALI/ARDS patients (207). Hence neutrophils migrated to the lungs during sepsis-associated ALI exert more damaging effects to the lungs as compared to bacterial pneumonia (**Figure 3**).

The apoptosis of neutrophils enhances the resolution of the inflammation that is lost in the sepsis-associated ALI. However, the cyclin-dependent kinase (CDK) inhibitor, called AT7519 enhances the apoptosis of infiltrated neutrophils during sepsis-associated ALI or ARDS, can be used as a mediator of initiating the resolution phase of inflammation during sepsis-associated ALI (208). Of note, mechanisms causing ALI and resolution of inflammation occur in parallel during sepsis-associated ALI/ARDS. The first resolution step involves the reestablishment of the alveolar-capillary barrier and the migration of AT-II epithelial cells to replace injured AT I epithelial cells, following the proliferation of tissue-resident progenitor cells (187). However, the uncontrolled inflammatory process causing severe ALI overpowers the resolution process, which causes irreversible damage during Gram-negative bacteria (*Klebsiella pneumoniae*)-induced sepsis as indicated by accumulation of the lungs with apoptosis-resistant neutrophils and elevation of pro-inflammatory cytokines (IL-1α, TNF-α) in BALF (12). Furthermore, keratinocyte growth factor (KGF) treatment induced the resolution in PECs *in vitro* and *in vivo* in mice, but it failed in phase II clinical trial and aggravated the ALI (209–211). Thus, due to severe PEC damage during sepsis-induced ALI, it is more damaging and irreversible as compared to the ALI observed during bacterial pneumonia only without the development of sepsis.

ILCs During ALI/ARDS Observed During Sepsis

There is a doubt regarding the presence of ILC1s in naïve lungs or during homeostasis. However, their number increases during Haemophilus influenzea infection. It occurs due to the phenotypic change in lung-resident ILC2s in response to the

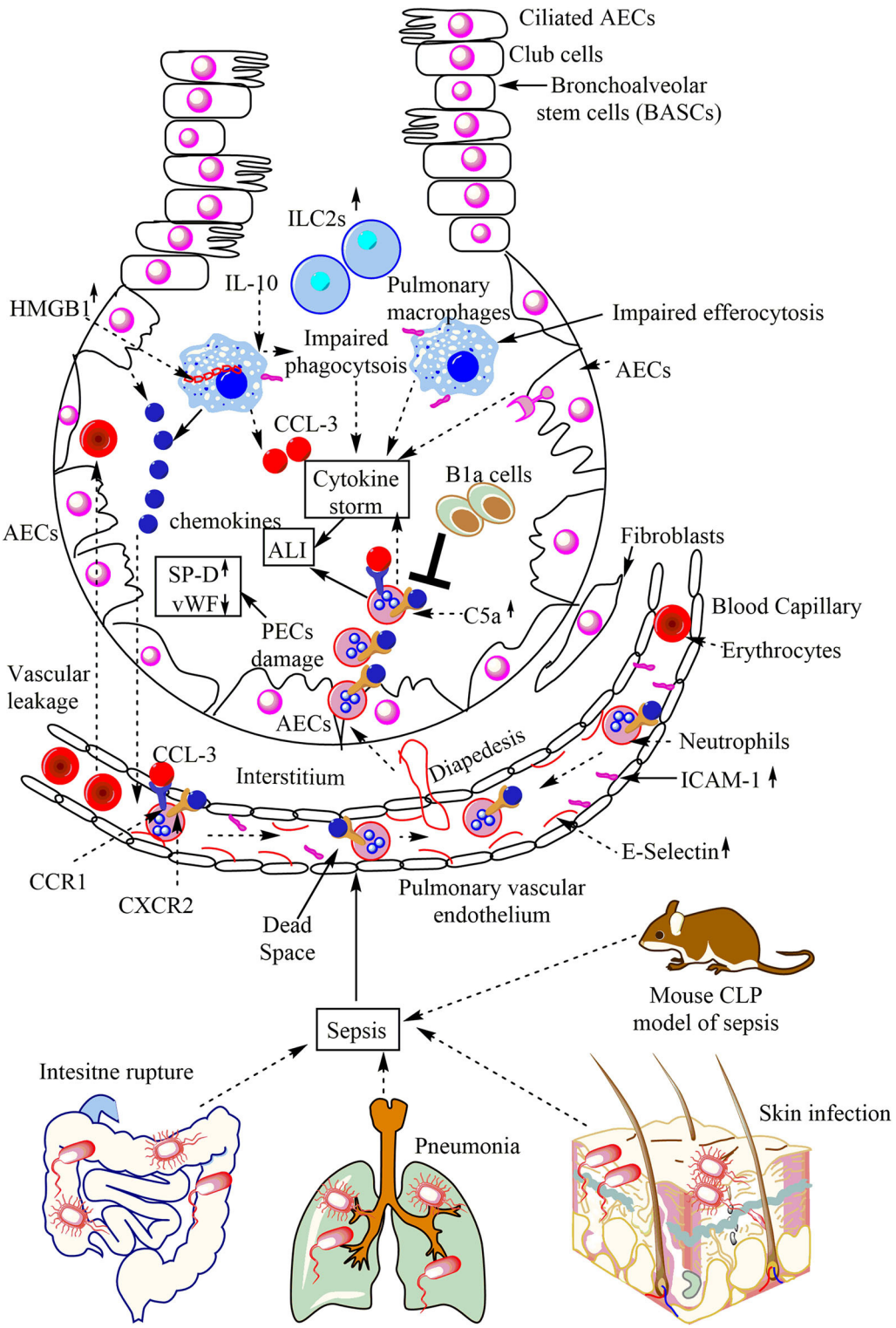

FIGURE 3 | Overview of sepsis-induced ALI. Local infections of the skin (*S. aureus*), lungs (pneumonia), and intestinal commensal bacteria leak into the blood may lead to sepsis development. Sepsis leads to the neutrophil and monocyte infiltration in the lung alveoli via pulmonary transendothelial migration due to the profound release of the pro-inflammatory mediators (cytokine storm) damaging endothelial monolayer and inducing endothelial vascular leakage. These neutrophils and monocytes reach into the lung alveoli through crossing the pulmonary epithelial layer due to damage of PECs (AECs and BECs). These PECs express C5aR and C3aR

(Continued)

FIGURE 3 | receptors. The profound release of complement component C5a during sepsis induces the inflammatory damage, and the death of PECs during sepsis causes sepsis-associated ALI. The PECs death/damage increases the SP-D, but Vwf levels decrease. The increase in the IL-10 levels in the lungs at later stages of sepsis impairs the bactericidal action of AMs along with inducing a defective efferocytosis. The defective efferocytosis among AMs further increases the ALI. The necrotic death of AMs (indicated by the cytosolic HMG-B1) at later stages of sepsis further aggravates the ALI. The neutrophils infiltrated into the lung alveoli during sepsis are apoptosis-resistant and aggravate the ALI due to their increased pro-inflammatory action on lung tissues. B1a cells inhibit neutrophil infiltration and, thus, the sepsis-induced ALI. The increase in pulmonary ILC2s also occurs.

downregulation of T1/ST2, GATA3, IL-5, and IL-13 expression (125). ILC2s represent the majority of ILCs in both mouse and human (30% of all ILCs in adult human lungs) lungs (212, 213). Although Lungs have a low number of ILC2s in the steady-state, and it increases only during pulmonary allergic diseases. Lungs have both NCR^+ and NCR^- ILC3s during a steady state. Lin-$CD127^+ROR\gamma t^+$ILC3s comprise 30% of ILCs in mice, and the majority of them also co-express CCR6. These can be activated with IL-23 and IL-1β *in vitro* to produce IL-22 and IL-17A (135). The human lungs also have ILC3s ($Lin^-CD127^+CRTh2^-CD117^+$) expressing RORγt. During *S. pneumoniae* lung infection, the depletion of ILC3s protects the host from ALI due to inhibition of IL-22 and IL-17A production (135).

The systemic levels of ILCs (ILC1s and ILC3s) significantly decrease in patients with sepsis in comparison to the control group due to their increased apoptotic death (214). The HLA-DR expression increases in the ILCs of the septic patients without any effect on their capacity to produce TNF-α in response to the TLR agonists. The apoptotic cell death among ILCs (ILC1s, ILC2s, and ILC3s) occurs due to the increase in CASP3 level and activity within <24 h of sepsis diagnosis (214). However, no significant decrease in systemic ILC2s occurs during the early stages of sepsis despite the increase in CASP3 activity. It may be attributed to the sphingosine-1-phosphate (S1P)-dependent migration of ILC2s to distant organs, including lungs (215). The plasma S1P-1 level decreases with the severity of the sepsis (216). The ILC2 migration to the lungs in response to S1P occurs due to increased expression of S1P receptors (S1PRs, S1P1-SIP5) (217). A study in CLP-induced sepsis has shown the increase in ILC2s in the peritoneum and small intestine along with the increased IL-13 and IL-33 levels in the peritoneal lavage fluid (PLF) within 24 h post sepsis development (218).

Patients with sepsis show increased plasma IL-33 levels (218). Many investigators have suggested different roles of pulmonary ILC2s during sepsis, depending on the experimental model. For example, increased IL-33 levels (released by epithelial cells of the lungs) in CLP-induced sepsis in mice cause sepsis-induced ALI, and IL-33 inhibition causes a decrease in sepsis-associated ALI due to the decreased neutrophil and monocyte infiltration into the lungs (219). This IL-33 dependent ALI during sepsis also occurs via IL-5 upregulation in pulmonary ILC2s, and the IL-5 neutralization decreases the neutrophil infiltration, and ALI during sepsis (219). Thus, an increased activation of pulmonary ILC2s during CLP-induced sepsis may contribute to the sepsis-associated ALI. However, another study has shown the protective effect of the pulmonary ILC2s during sepsis-induced ALI via preventing the endothelial cell damage in response to the IL-33 released, which via binding to the ST2 receptor, mediates the ILC2 expansion (220).

The pulmonary ILC2s produce IL-9, which prevents CASP1 activation and the pyroptosis of pulmonary endothelial cells. It reduces the sepsis-associated ALI severity. The pulmonary ILC2s increase within the first 12 h of the sepsis development along with an increase in the peritoneal ILC2s (220). However, in another study, the ILC2s (as measured by the production of IL-5 and IL-13) pre-activation via intra-tracheal IL-33 administration before the lethal *S. aureus* sepsis protects the host from ALI and death via pulmonary eosinophilia induction, which clears the pathogen from the lungs and suppresses neutrophilia (221). However, without IL-33 pre-treatment, *S. aureus* is unable to induce ILC2 proliferation and function. Hence ILC2s play both beneficial and detrimental roles in ALI and sepsis-associated mortality depending on their activation stage. It will be interesting to investigate the impact of sepsis-associated ALI in people previously affected with parasitic infections causing a rise in pulmonary ILC2s and eosinophilia. Thus, pulmonary ILCs are crucial innate immune cells of the lungs, but their relevance to the sepsis-induced ALI/ARDS is a topic for the research and future immunomodulatory therapeutics. However, a decrease in the systemic ILC population is well-described even during the early phase of the sepsis.

Alveolar Macrophages (AMs) and Sepsis-Induced ALI//ARDS

The pro-inflammatory mediators released from AMs play a crucial role in the sepsis-induced ALI via inducing neutrophil infiltration into the lungs (**Figure 3**). The interstitial-to-vascular chemotactic gradient establishment facilitates the emigration of the vascular neutrophils in the lung alveoli (**Figure 3**) (222, 223). The bacterial peritonitis-induced sepsis activates AMs and neutrophil infiltration in the lung alveoli (**Figure 3**). The neutrophil infiltration in the lung alveoli occurs via pulmonary transendothelium in response to the AM activation during sepsis (**Figure 3**) (224). The NADPH oxidase activation in the pulmonary endothelium generates superoxide anion in response to the AM activation that plays a crucial role in the transendothelial neutrophil migration during sepsis-associated ALI (224). These neutrophils are less prone to apoptotic cell death and play a significant role in the sepsis-induced severe ALI (**Figure 3**). Furthermore, these infiltrated neutrophils block pulmonary microcirculation due to their prolonged entrapment in the capillaries inducing the dead space, which further aggravates the sepsis-induced ALI (**Figure 3**) (225). These neutrophils express Mac-1 (CD11b/CD18), and the Mac-1 inhibitor decreases the incidence of disturbing pulmonary microcirculation and the sepsis-induced ALI. Also, the impaired

phagocytic activity of AMs during late stages in response to the released IL-10 during abdominal sepsis further enhances the incidence and the severity of sepsis-induced ALI (**Figure 3**) (226).

The impaired efferocytosis by AMs during the late stages of sepsis further increases the severity of ALI/ARDS due to the accumulation of dead neutrophils and other pulmonary cells (**Figure 3**) (227). However, the IFN-β treatment reverses the impaired AM function in response to the IL-10 at the late stage of sepsis and decreases the severity of sepsis-associated ALI/ARDS and the associated mortality (228). The HMG-B1 release in the cytosol of AMs during the late sepsis indicates their necrotic cell death, which further increases the ALI severity (**Figure 3**) (229). The macrophages endocytose HMG-B1 during sepsis (230). The HMG-B1 promotes pyroptosis of macrophages and endothelial cells by delivering the LPS via the receptor for advanced glycation end products (RAGE) into the cytosol, which destabilizes phagolysosome and induces CASP11 activation during lethal sepsis (231).

The CASP11 is the important inflammasome component, and HMG-B1 is known to activate NLRP3 inflammasome and the IL-1β release. The CASP11 activation causes pyroptosis via cleaving gasdermin D (GSDMD) into amino-terminal GSDMD (N-GSDMD) and carboxy-terminal GSDMD (C-GSDMD) (232, 233). The N-GSDMD is responsible for the pyroptosis. The increased lipid peroxidation (LPO) in the sepsis-associated ALI has been observed (12). This increased LPO further activates CASP11, and thus, the GSDMD to cause the pyroptosis of AMs and infiltrated monocytes and macrophages in a phospholipase C gamma 1 (PLCG1)-dependent manner (234). Also, the inflammatory IL-1β reduces the cyclic adenosine monophosphate (cAMP) and transcription factor cAMP response element-binding (CREB) in lung endothelial cells (235). This CREB blockage inhibits the VE-cadherin transcription, which induces pulmonary vascular endothelial damage to aggravate pulmonary vascular leakage and sepsis-associated ALI. Also, the treatment with rolipram (a drug inhibiting the type 4 cyclic nucleotide phosphodiesterase–mediated (PDE4-mediated) hydrolysis of cAMP) prevents sepsis-induced pulmonary vascular injury and thus the ALI via preserving the CREB-mediated VE-cadherin expression (235). Of note, the deficiency of neutrophils before sepsis also impairs the monocyte and macrophage infiltration in the lungs during both early and late stages and thus inflammatory process (236).

The iNOS induction in AMs during sepsis also causes protein leakage in the lungs and sepsis-induced ALI. The AM depletion attenuates the sepsis-induced increase in pulmonary microvascular protein leak and MPO activity that depends on the activation of iNOS (237). The increased nitric oxide ($NO^{\cdot}$) and MPO levels in BALF and lung homogenate of mice subjected to *K. pneumoniae* B5055-induced sepsis has been reported on all days in an experimental study (12). Furthermore, microRNA-199a (miR-199a) upregulation in AMs during Gram-negative bacterial sepsis also aggravates the sepsis-induced ALI, which can be prevented by the activation of sirtuin 1 [SIRT1 (Silent information regulator 2 homolog 1), an NAD^{+}-dependent class III protein deacetylase or histone deacetylase regulating cell growth, differentiation, stress resistance, oxidative damage, and metabolism] (238, 239). The induction of miR-199a in AMs during sepsis increases the release of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), the MPO activity, ALI, and the high levels of CASP3, Bax and lowers the Bcl-2 levels (238). The miR-199a inhibition during sepsis decreases the release of pro-inflammatory cytokines from AMs, MPO activity, the incidence of vascular leakage from pulmonary endothelium. The SIRT1 activation during sepsis also prevents the sepsis-induced ALI via inhibiting the NLRP3 inflammasomes in AMs and pulmonary vascular endothelial cells, which prevents the release of pro-inflammatory mediators (ICAM-1 and HMG-B1), disruption of tight and adherens junctions as indicated by the reduced lung claudin-1 and vascular endothelial-cadherin expression (240, 241).

The sepsis-associated altered AM function predisposes these mice to severe pulmonary infections and increases their mortality when challenged with Gram-negative bacteria (*P. aeruginosa*) due to the IL-1 receptor-associated kinase–M (IRAK-M) upregulation, which causes sepsis-associated immunosuppression at later stages (242). IRAK-M-mediated impaired TNF-α and iNOS expression in AMs is associated with the reduced acetylation and methylation of specific histones (AcH4 and H3K4me3) and reduced binding of RNA polymerase II to the promoters of these genes (243). However, the TLR2 and TLR4 levels remain the same in septic lungs as compared to the control group (242). Of note, diabetic rats show milder sepsis-associated ALI due to the impaired activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), increased suppressor of cytokine signaling 1 (SOCS1), and decreased MyD88 mRNA, and thus the decreased MyD88 downstream signaling in response to the TLR stimulation on AMs (244). The decreased AM activation in diabetic rats inhibits neutrophil infiltration, cyclo-oxygenase II (COX-II) expression and activity, and the pulmonary edema. The low incidence of sepsis-induced ALI has been also observed in patients with diabetes developing sepsis (245–248). However, a recent clinical study indicates that diabetes does not have any impact on sepsis-associated mortality and the 60-days mortality of ALI/ARDS (249). Diabetes may reduce the incidence of the sepsis-induce ALI/ARDS but not the associated mortality. Further studies in the direction and the establishment of AMs role in clinical patients of sepsis with diabetes about the ALI may prove helpful in a patient-specific therapeutic approach.

Hence sepsis leads to the severe ALI as compared to the ALI seen in pneumonia only patients. Furthermore, sepsis causes prolonged immunosuppressive stage in the lungs, which increases the chances of developing severe secondary pulmonary infections (hospital-acquired or community-acquired). For example, the pulmonary alveolar macrophages decrease in number in patients recovered from sepsis and show defective phagocytic function against bacterial pathogens (*E. coli* and *S. aureus*), which are frequently responsible for hospital-acquired pneumonia (169). This defective number of pulmonary alveolar macrophages stays at least for 6 months. These clinical findings (severely compromised phagocytic activity of AMs) have further been confirmed in mice subjected to secondary pneumonia caused by *E. coli* or *S. aureus* (169). These macrophages from

patients recovering from sepsis also showed increased SIRPα expression. However, the outcome of sepsis-associated ALI may also depend on several other chronic inflammatory conditions, including type 2 diabetes mellitus (T2DM). Further studies are urgently required in the field due to the high mortality of sepsis patients due to the Sepsis-induced ALI/ARDS.

CONCLUSION

Pneumonia and sepsis, both are associated with the onset of ALI/ARDS. However, the pneumonia-associated ALI is less severe and often resolves once the infection has cleared. But this resolution of ALI during sepsis has not been observed. However, the pneumonia-associated ALI resolution leaves a long-lasting impact on the host immune response to future infection. This resolution of ALI involves the transforming growth factor-β (TGF-β) generation and the activation of pulmonary regulatory T cells (Tregs) inducing the immunosuppressive environment in the lungs (169). It causes the induction of paralyzed pulmonary macrophages and DCs, which are defective in the phagocytosis of the pathogen but further secrete TGF-β responsible for the Tregs accumulation. These paralyzed DCs express an increased amount of transcription repressor called B lymphocyte-induced maturation protein-1 (Blimp-1) but a lower amount of interferon regulatory factor 4 (IRF4). Blimp-1 is essential for tolerogenic DCs. Thus, the increased expression of Blimp-1 induces a tolerogenic phenotype of DCs. Whereas, IRF-4 is for expressing the molecules required for the antigen presentation, and its lower level in paralyzed DCs decrease their antigen presentation potential.

The immunosuppressive environment in the lung following the resolution of ALI-associated with pneumonia further disposes the host to acquire secondary pulmonary infection. However, this can be avoided by following the instruction, like keeping the recovered patient in the pathogen-free environment and keeping him/her on the immune-boosting diet. For example, the study has shown that these paralyzed macrophages and DCs generated following the resolution of pneumonia-associated ALI remains active for at least 21 days post clearance of the pathogen (169). Hence the impairment in the expression of Blimp-1 and IRF-4 in other immune cells, including PECs, T, and B cells post, pneumonia should also be studied to explore the unknowns associated with the cost of resolution of ALI associated with pneumonia and the patients recovered from sepsis. ALI during sepsis proves detrimental to the host. Even the neutrophils and monocytes infiltrated into the septic lungs show the apoptosis-resistant phenotype that proves harmful to the host by further aggravating the sepsis-induced ALI. Thus, therapeutics able to induce their phagocytosis later in the sepsis will prove beneficial to prevent the sepsis-induced ALI. Keeping in mind the difference in the action of the pulmonary innate immune response during sepsis and pneumonia-induced ALI, different therapeutics should be designed as the drug or molecule worth for one may not be useful for the other. Future studies are required in the direction to prevent the sepsis or pneumonia-induced ALI by studying the pulmonary, innate immunity. For example, the discovery of ILCs in the lungs and further research in their functional role in pneumonia and sepsis-induced ALI has changed their pathogenesis and opened the door to design better and new therapeutics, including the vaccines for pneumonia.

AUTHOR CONTRIBUTIONS

VK has developed the idea, searched the literature, compiled, and wrote the article.

REFERENCES

1. Rezoagli E, Fumagalli R, Bellani G. Definition G, and epidemiology of acute respiratory distress syndrome. *Ann Transl Med.* (2017) 5:282. doi: 10.21037/atm.2017.06.62
2. Montgomery AB. Early description of ARDS. *Chest.* (1991) 99:261–2. doi: 10.1378/chest.99.1.261
3. Bernard G. Acute lung failure — our evolving understanding of ARDS. *N Engl J Med.* (2017) 377:507–9. doi: 10.1056/NEJMp1706595
4. Ashbaugh DG, Bigelow DB, Petty TL, Levine BE. Acute respiratory distress in adults. *Lancet.* (1967) 2:319–23. doi: 10.1016/S0140-6736(67)90168-7
5. Sessler CN, Bloomfield GL, Fowler AA. Current concepts of sepsis and acute lung injury. *Clin Chest Med.* (1996) 17:213–35. doi: 10.1016/S0272-5231(05)70310-5
6. Pham T, Rubenfeld GD. Fifty years of research in ARDS. The epidemiology of acute respiratory distress syndrome. A 50th birthday review. *Am J Respir Crit Care Med.* (2017) 195:860–70. doi: 10.1164/rccm.201609-1773CP
7. Schaefer L. Complexity of danger: the diverse nature of damage-associated molecular patterns. *J Biol Chem.* (2014) 289:35237–45. doi: 10.1074/jbc.R114.619304
8. Kumar V. A STING to inflammation and autoimmunity. *J leukoc Biol.* (2019) 106:171–85. doi: 10.1002/JLB.4MIR1018-397RR
9. Vijay K. Toll-like receptors in immunity and inflammatory diseases: past, present, and future. *Int Immunopharmacol.* (2018) 59:391–412. doi: 10.1016/j.intimp.2018.03.002
10. Hoving JC, Wilson GJ, Brown GD. Signalling C-type lectin receptors, microbial recognition and immunity. *Cell Microbiol.* (2014) 16:185–94. doi: 10.1111/cmi.12249
11. Franchi L, Warner N, Viani K, Nuñez G. Function of Nod-like receptors in microbial recognition and host defense. *Immunol Rev.* (2009) 227:106–28. doi: 10.1111/j.1600-065X.2008.00734.x
12. Kumar V, Chhibber S. Acute lung inflammation in *Klebsiella pneumoniae* B5055-induced pneumonia and sepsis in BALB/c mice: a comparative study. *Inflammation.* (2011) 34:452–62. doi: 10.1007/s10753-010-9253-9
13. Guirgis FW, Khadpe JD, Kuntz GM, Wears RL, Kalynych CJ, Jones AE. Persistent organ dysfunction after severe sepsis: a systematic review. *J Crit Care.* (2014) 29:320–6. doi: 10.1016/j.jcrc.2013.10.020
14. Bienenstock J. The lung as an immunologic organ. *Ann Rev Med.* (1984) 35:49–62. doi: 10.1146/annurev.me.35.020184.000405
15. Askonas BA, Humphrey JH. Formation of specific antibodies and gamma-globulin *in vitro*. A study of the synthetic ability of various tissues from rabbits immunized by different methods. *Biochem J.* (1958) 68:252–61. doi: 10.1042/bj0680252
16. Bull CG, McKee CM. Respiratory immunity in rabbits. vii. Resistance to intranasal infection in the absence of demonstrable antibodies. *Am J Epidemiol.* (1929) 9:490–9. doi: 10.1093/oxfordjournals.aje.a121662
17. Bivas-Benita M, Gillard GO, Bar L, White KA, Webby RJ, Hovav AH, et al. Airway CD8(+) T cells induced by pulmonary DNA immunization mediate protective anti-viral immunity. *Mucosal Immunol.* (2013) 6:156–66. doi: 10.1038/mi.2012.59

18. Takaki H, Ichimiya S, Matsumoto M, Seya T. Mucosal immune response in nasal-associated lymphoid tissue upon intranasal administration by adjuvants. *J Innate Immun.* (2018) 10:515–21. doi: 10.1159/000489405
19. Song K, Bolton DL, Wei C-J, Wilson RL, Camp JV, Bao S, et al. Genetic immunization in the lung induces potent local and systemic immune responses. *Proc Natl Acad Sci.* (2010) 107:22213–8. doi: 10.1073/pnas.1015536108
20. McDermott MR, Befus AD, Bienenstock J. The structural basis for immunity in the respiratory tract. *Int Rev Exp Pathol.* (1982) 23:47–112.
21. Bienenstock J, Johnston N, Perey DY. Bronchial lymphoid tissue. I. Morphologic characteristics. *Lab Invest.* (1973) 28:686–92.
22. Bienenstock J, Johnston N, Perey DY. Bronchial lymphoid tissue. II. Functional characterisitics. *Lab Invest.* (1973) 28:693–8.
23. Richmond I, Pritchard GE, Ashcroft T, Avery A, Corris PA, Walters EH. Bronchus associated lymphoid tissue (BALT) in human lung: its distribution in smokers and non-smokers. *Thorax.* (1993) 48:1130–4. doi: 10.1136/thx.48.11.1130
24. Bienenstock J. Gut and bronchus associated lymphoid tissue: an overview. *Adv Exp Med Biol.* (1982) 149:471–7. doi: 10.1007/978-1-4684-9066-4_66
25. Kimura S, Mutoh M, Hisamoto M, Saito H, Takahashi S, Asakura T, et al. Airway M cells arise in the lower airway due to RANKL signaling and reside in the bronchiolar epithelium associated with iBALT in murine models of respiratory disease. *Front Immunol.* (2019) 10:1323. doi: 10.3389/fimmu.2019.01323
26. Corr SC, Gahan CCGM, Hill C. M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. *FEMS Immunol Med Microbiol.* (2008) 52:2–12. doi: 10.1111/j.1574-695X.2007.00359.x
27. Bockman DE, Cooper MD. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, Peyer's patches. An electron microscopic study. *Am J Anat.* (1973) 136:455–77. doi: 10.1002/aja.1001360406
28. Rácz P, Tenner-Rácz K, Myrvik QN, Fainter LK. Functional architecture of bronchial associated lymphoid tissue and lymphoepithelium in pulmonary cell-mediated reactions in the rabbit. *J Reticuloendothel Soc.* (1977) 22:59–83.
29. Tschernig T, Pabst R. Bronchus-associated lymphoid tissue (BALT) is not present in the normal adult lung but in different diseases. *Pathobiology.* (2000) 68:1–8. doi: 10.1159/000028109
30. Gould SJ, Isaacson PG. Bronchus-associated lymphoid tissue (BALT) in human fetal and infant lung. *J Pathol.* (1993) 169:229–34. doi: 10.1002/path.1711690209
31. Ersch J, Tschernig T, Stallmach T. Frequency and potential cause of bronchus-associated lymphoid tissue in fetal lungs. *Pediatr Allergy Immunol.* (2005) 16:295–8. doi: 10.1111/j.1399-3038.2005.00269.x
32. Meuwissen HJ, Hussain M. Bronchus-associated lymphoid tissue in human lung: correlation of hyperplasia with chronic pulmonary disease. *Clin Immunol Immunopathol.* (1982) 23:548–61. doi: 10.1016/0090-1229(82)90139-8
33. Sato A. Basic and clinical aspects of bronchus-associated lymphoid tissue. *Nihon Kokyuki Gakkai Zasshi.* (2000) 38:3–11.
34. Lloyd CM, Marsland BJ. Lung homeostasis: influence of age, microbes, and the immune system. (2017) 46:549–61. doi: 10.1016/j.immuni.2017.04.005
35. Gallacher DJ, Kotecha S. Respiratory microbiome of new-born infants. *Front Pediatr.* (2016) 4:10. doi: 10.3389/fped.2016.00010
36. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc Natl Acad Sci USA.* (2010) 107:11971–5. doi: 10.1073/pnas.1002601107
37. Gollwitzer ES, Saglani S, Trompette A, Yadava K, Sherburn R, McCoy KD, et al. Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat Med.* (2014) 20:642–7. doi: 10.1038/nm.3568
38. Wang J, Li F, Sun R, Gao X, Wei H, Li LJ, et al. Bacterial colonization dampens influenza-mediated acute lung injury via induction of M2 alveolar macrophages. *Nat Commun.* (2013) 4:2106. doi: 10.1038/ncomms3106
39. Murphy SL, Kochanek KD, Xu J, Arias E. Mortality in the United States, 2014. *NCHS Data Brief.* (2015) 1–8. doi: 10.1093/gao/9781884446054.article.T2271585
40. Murphy SL, Xu J, Kochanek KD, Arias E. (2018) Mortality in the United States, 2017. *NCHS Data Brief.* 328:1–8.
41. Cilloniz C, Martin-Loeches I, Garcia-Vidal C, San Jose A, Torres A. Microbial etiology of pneumonia: epidemiology, diagnosis and resistance patterns. *Int J Mol Sci.* (2016) 17:2120. doi: 10.3390/ijms17122120
42. Arnold FW, Summersgill JT, Ramirez JA. Role of atypical pathogens in the etiology of community-acquired pneumonia. *Semin Respir Crit Care Medi.* (2016) 37:819–28. doi: 10.1055/s-0036-1592121
43. Cilloniz C, Torres A, Niederman M, van der Eerden M, Chalmers J, Welte T, et al. Community-acquired pneumonia related to intracellular pathogens. *Intensive Care Med.* (2016) 42:1374–86. doi: 10.1007/s00134-016-4394-4
44. Branger J, Knapp S, Weijer S, Leemans JC, Pater JM, Speelman P, et al. Role of toll-like receptor 4 in gram-positive and gram-negative pneumonia in mice. *Infect Immun.* (2004) 72:788–94. doi: 10.1128/IAI.72.2.788-794.2004
45. Rossaint J, Zarbock A. Tissue-specific neutrophil recruitment into the lung, liver, and kidney. *J Innate Immun.* (2013) 5:348–57. doi: 10.1159/000345943
46. Maas SL, Soehnlein O, Viola JR. Organ-specific mechanisms of transendothelial neutrophil migration in the lung, liver, kidney, and aorta. *Front Immunol.* (2018) 9:2739. doi: 10.3389/fimmu.2018.02739
47. Zemans RL, Colgan SP, Downey GP. Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. *Am J Respir Cell Mol Biol.* (2009) 40:519–35. doi: 10.1165/rcmb.2008-0348TR
48. Mizgerd JP. Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs. *Semin Immunol.* (2002) 14:123–32. doi: 10.1006/smim.2001.0349
49. Goodman RB, Strieter RM, Martin DP, Steinberg KP, Milberg JA, Maunder RJ, et al. Inflammatory cytokines in patients with persistence of the acute respiratory distress syndrome. *Am J Respir Crit Care Med.* (1996) 154:602–11. doi: 10.1164/ajrccm.154.3.8810593
50. Donnelly SC, Strieter RM, Kunkel SL, Walz A, Robertson CR, Carter DC, et al. Interleukin-8 C, and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet.* (1993) 341:643–7. doi: 10.1016/0140-6736(93)90416-E
51. Koltsova EK, Ley K. The mysterious ways of the chemokine CXCL5. *Immunity.* (2010) 33:7–9. doi: 10.1016/j.immuni.2010.07.012
52. Mei J, Liu Y, Dai N, Favara M, Greene T, Jeyaseelan S, et al. CXCL5 regulates chemokine scavenging and pulmonary host defense to bacterial infection. *Immunity.* (2010) 33:106–17. doi: 10.1016/j.immuni.2010.07.009
53. Miller EJ, Cohen AB, Nagao S, Griffith D, Maunder RJ, Martin TR, et al. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am Rev Respir Dis.* (1992) 146:427–32. doi: 10.1164/ajrccm/146.2.427
54. Cai S, Batra S, Lira SA, Kolls JK, Jeyaseelan S. CXCL1 regulates pulmonary host defense to Klebsiella infection via CXCL2, CXCL5, NF-κB, and MAPKs. *J Immunol.* (2010) 185:6214–25. doi: 10.4049/jimmunol.0903843
55. Zhou X, Dai Q, Huang X. Neutrophils in acute lung injury. *Front Biosci.* (2012) 17:2278–83. doi: 10.2741/4051
56. Williams AE, Chambers RC. The mercurial nature of neutrophils: still an enigma in ARDS? Am *J Physiol Lung Cell Mol Physiol.* (2014) 306:L217–30. doi: 10.1152/ajplung.00311.2013
57. Eisele NA, Anderson DM. Host defense and the airway epithelium: frontline responses that protect against bacterial invasion and pneumonia. *J Pathog.* (2011) 2011:249802. doi: 10.4061/2011/249802
58. Leiva-Juárez MM, Kolls JK, Evans SE. Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense. *Mucosal Immunology.* (2018) 11:21–34. doi: 10.1038/mi.2017.71
59. Guadiz G, Sporn LA, Goss RA, Lawrence SO, Marder VJ, Simpson-Haidaris PJ. Polarized secretion of fibrinogen by lung epithelial cells. *Am J Respir Cell Mol Biol.* (1997) 17:60–9. doi: 10.1165/ajrcmb.17.1.2730
60. Haidaris PJ. Induction of fibrinogen biosynthesis and secretion from cultured pulmonary epithelial cells. *Blood.* (1997) 89:873–82. doi: 10.1182/blood.V89.3.873
61. Hippenstiel S, Opitz B, Schmeck B, Suttorp N. Lung epithelium as a sentinel and effector system in pneumonia – molecular mechanisms of pathogen recognition and signal transduction. *Respir Res.* (2006) 7:97. doi: 10.1186/1465-9921-7-97
62. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signalling complexes. *Nat Rev Immunol.* (2014) 14:546–58. doi: 10.1038/nri3713

63. Chaput C, Sander LE, Suttorp N, Opitz B. NOD-like receptors in lung diseases. *Front Immunol.* (2013) 4:393. doi: 10.3389/fimmu.2013.00393
64. Lee S, Suh GY, Ryter SW, Choi AM. Regulation and function of the nucleotide binding domain leucine-rich repeat-containing receptor, pyrin domain-containing-3 inflammasome in lung disease. *Am J Respir Cell Mol Biol.* (2016) 54:151–60. doi: 10.1165/rcmb.2015-0231TR
65. Cai S, Batra S, Shen L, Wakamatsu N, Jeyaseelan S. Both TRIF- and MyD88-dependent signaling contribute to host defense against pulmonary Klebsiella infection. *J Immunol.* (2009) 183:6629–38. doi: 10.4049/jimmunol.0901033
66. van Lieshout MH, Blok DC, Wieland CW, de Vos AF, van't Veer C, van der Poll T. Differential roles of MyD88 and TRIF in hematopoietic and resident cells during murine gram-negative pneumonia. *J Infect Dis.* (2012) 206:1415–23. doi: 10.1093/infdis/jis505
67. Power MR, Li B, Yamamoto M, Akira S, Lin TJ. A role of toll-IL-1 receptor domain-containing adaptor-inducing IFN-beta in the host response to *Pseudomonas aeruginosa* lung infection in mice. *J Immunol.* (2007) 178:3170–6. doi: 10.4049/jimmunol.178.5.3170
68. Jeyaseelan S, Young SK, Fessler MB, Liu Y, Malcolm KC, Yamamoto M, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)-mediated signaling contributes to innate immune responses in the lung during *Escherichia coli* pneumonia. *J Immunol.* (2007) 178:3153–60. doi: 10.4049/jimmunol.178.5.3153
69. van Lieshout MH, Florquin S, Van't Veer C, de Vos AF, van der Poll T. TIR-domain-containing adaptor-inducing interferon-β (TRIF) mediates antibacterial defense during gram-negative pneumonia by inducing interferon-x03B3. *J Innate Immun.* (2015) 7:637–46. doi: 10.1159/000430913
70. Jeyaseelan S, Young SK, Yamamoto M, Arndt PG, Akira S, Kolls JK, et al. Toll/IL-1R domain-containing adaptor protein (TIRAP) is a critical mediator of antibacterial defense in the lung against *Klebsiella pneumoniae* but not *Pseudomonas aeruginosa. J Immunol.* (2006) 177:538–47. doi: 10.4049/jimmunol.177.1.538
71. Skerrett SJ, Liggitt HD, Hajjar AM, Wilson CB. Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against *Pseudomonas aeruginosa* but not *Staphylococcus aureus. J Immunol.* (2004) 172:3377–81. doi: 10.4049/jimmunol.172.6.3377
72. Knapp S, Wieland CW, van't Veer C, Takeuchi O, Akira S, Florquin S, et al. Toll-like receptor 2 plays a role in the early inflammatory response to murine pneumococcal pneumonia but does not contribute to antibacterial defense. *J Immunol.* (2004) 172:3132–8. doi: 10.4049/jimmunol.172.5.3132
73. Gowing SD, Chow SC, Cools-Lartigue JJ, Chen CB, Najmeh S, Jiang HY, et al. Gram-positive pneumonia augments non-small cell lung cancer metastasis via host toll-like receptor 2 activation. *Int J Cancer.* (2017) 141:561–71. doi: 10.1002/ijc.30734
74. Chavarria-Smith J, Vance RE. Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. *PLoS Pathog.* (2013) 9:e1003452. doi: 10.1371/journal.ppat.1003452
75. Sandstrom A, Mitchell PS, Goers L, Mu EW, Lesser CF, Vance RE. Functional degradation: a mechanism of NLRP1 inflammasome activation by diverse pathogen enzymes. *Science.* 364:e aau1330. doi: 10.1126/science.aau1330
76. de Nardo D, de Nardo CM, Latz E. New insights into mechanisms controlling the NLRP3 inflammasome and its role in lung disease. *Am J Pathol.* (2014) 184:42–54. doi: 10.1016/j.ajpath.2013.09.007
77. Leissinger M, Kulkarni R, Zemans RL, Downey GP, Jeyaseelan S. Investigating the role of nucleotide-binding oligomerization domain-like receptors in bacterial lung infection. *Am J Respir Crit Care Med.* (2014) 189:1461–8. doi: 10.1164/rccm.201311-2103PP
78. Hirota JA, Hirota SA, Warner SM, Stefanowicz D, Shaheen F, Beck PL, et al. The airway epithelium nucleotide-binding domain and leucine-rich repeat protein 3 inflammasome is activated by urban particulate matter. *J Allergy Clin Immunol.* (2012) 129:1116–25.e6. doi: 10.1016/j.jaci.2011.11.033
79. Willingham SB, Allen IC, Bergstralh DT, Brickey WJ, Huang MT, Taxman DJ, et al. NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways. *J Immunol.* (2009) 183:2008–15. doi: 10.4049/jimmunol.0900138
80. Fang R, Uchiyama R, Sakai S, Hara H, Tsutsui H, Suda T, et al. ASC and NLRP3 maintain innate immune homeostasis in the airway through an inflammasome-independent mechanism. *Mucosal Immunol.* (2019) 12:1092–103. doi: 10.1038/s41385-019-0181-1
81. Giraud AS, Pereira PM, Thim L, Parker LM, Judd LM. TFF-2 inhibits iNOS/NO in monocytes, and nitrated protein in healing colon after colitis. *Peptides.* (2004) 25:803–9. doi: 10.1016/j.peptides.2004.01.019
82. Soriano-Izquierdo A, Gironella M, Massaguer A, May FE, Salas A, Sans M, et al. Trefoil peptide TFF2 treatment reduces VCAM-1 expression and leukocyte recruitment in experimental intestinal inflammation. *J Leukoc Biol.* (2004) 75:214–23. doi: 10.1189/jlb.0803396
83. McBerry C, Egan CE, Rani R, Yang Y, Wu D, Boespflug N, et al. Trefoil factor 2 negatively regulates type 1 immunity against Toxoplasma gondii. *J Immunol.* (2012) 189:3078–84. doi: 10.4049/jimmunol.1103374
84. Taupin D, Podolsky DK. Trefoil factors: initiators of mucosal healing. *Nat Rev Mol Cell Biol.* (2003) 4:721–32. doi: 10.1038/nrm1203
85. Hung L-Y, Oniskey TK, Sen D, Krummel MF, Vaughan AE, Cohen NA, et al. Trefoil factor 2 promotes type 2 immunity and lung repair through intrinsic roles in hematopoietic and nonhematopoietic cells. *Am J Pathol.* (2018) 188:1161–70. doi: 10.1016/j.ajpath.2018.01.020
86. Wills-Karp M, Rani R, Dienger K, Lewkowich I, Fox JG, Perkins C, et al. Trefoil factor 2 rapidly induces interleukin 33 to promote type 2 immunity during allergic asthma and hookworm infection. *J Exp Med.* (2012) 209:607–22. doi: 10.1084/jem.20110079
87. Tsuji S, Uehori J, Matsumoto M, Suzuki Y, Matsuhisa A, Toyoshima K, et al. Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. *J Biol Chem.* (2001) 276:23456–63. doi: 10.1074/jbc.M103162200
88. Watanabe T, Chibana K, Shiobara T, Tei R, Koike R, Nakamura Y, et al. Expression of intelectin-1 in bronchial epithelial cells of asthma is correlated with T-helper 2 (Type-2) related parameters and its function. *Allergy Asthma Clin Immunol.* (2017) 13:35. doi: 10.1186/s13223-017-0207-8
89. Günther J, Seyfert H-M. The first line of defence: insights into mechanisms and relevance of phagocytosis in epithelial cells. *Semin Immunopathol.* (2018) 40:555–65. doi: 10.1007/s00281-018-0701-1
90. Kumar V. Phagocytosis: phenotypically simple yet a mechanistically complex process. *Int Rev Immunol.* (2020) 39:118–50. doi: 10.1080/08830185.2020.1732958
91. Stout-Delgado HW, Vaughan SE, Shirali AC, Jaramillo RJ, Harrod KS. Impaired NLRP3 inflammasome function in elderly mice during influenza infection is rescued by treatment with nigericin. *J Immunol.* (2012) 188:2815–24. doi: 10.4049/jimmunol.1103051
92. Cho SJ, Rooney K, Choi AMK, Stout-Delgado HW. NLRP3 inflammasome activation in aged macrophages is diminished during *Streptococcus pneumoniae* infection. *Am J Physiol Lung Cell Mol Physiol.* (2018) 314:L372–87. doi: 10.1152/ajplung.00393.2017
93. Cho SJ, Plataki M, Mitzel D, Lowry G, Rooney K, Stout-Delgado H. Decreased NLRP3 inflammasome expression in aged lung may contribute to increased susceptibility to secondary *Streptococcus pneumoniae* infection. *Exp Gerontol.* (2018) 105:40–6. doi: 10.1016/j.exger.2017.11.010
94. van Lieshout MHP, de Vos AF, Dessing MC, de Porto APNA, de Boer OJ, de Beer R, et al. ASC and NLRP3 impair host defense during lethal pneumonia caused by serotype 3 Streptococcus pneumoniae in mice. *Eur J Immunol.* (2018) 48:66–79. doi: 10.1002/eji.201646554
95. Kebaier C, Chamberland RR, Allen IC, Gao X, Broglie PM, Hall JD, et al. Staphylococcus aureus alpha-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. *J Infect Dis.* (2012) 205:807–17. doi: 10.1093/infdis/jir846
96. Craven RR, Gao X, Allen IC, Gris D, Bubeck Wardenburg J, McElvania-Tekippe E, et al. Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. *PLoS ONE.* (2009) 4:e7446. doi: 10.1371/journal.pone.0007446
97. Ezekwe EA Jr, Weng C, Duncan JA. ADAM10 cell surface expression but not activity is critical for staphylococcus aureus alpha-hemolysin-mediated activation of the NLRP3 inflammasome in human monocytes. *Toxins.* (2016) 8:95. doi: 10.3390/toxins8040095
98. Paudel S, Ghimire L, Jin L, Baral P, Cai S, Jeyaseelan S. NLRC4 suppresses IL-17A-mediated neutrophil-dependent host defense through upregulation of IL-18 and induction of necroptosis during Gram-positive pneumonia. *Mucosal Immunol.* (2019) 12:247–57. doi: 10.1038/s41385-018-0088-2

99. Ghimire L, Paudel S, Jin L, Baral P, Cai S, Jeyaseelan S. NLRP6 negatively regulates pulmonary host defense in Gram-positive bacterial infection through modulating neutrophil recruitment and function. *PLoS Pathog.* (2018) 14:e1007308. doi: 10.1371/journal.ppat.1007308
100. Wu S HJ. Resveratrol alleviates *Staphylococcus aureus* pneumonia by inhibition of the NLRP3 inflammasome. *Exp Ther Med.* (2017) 14:6099–14. doi: 10.3892/etm.2017.5337
101. Cai S, Batra S, Wakamatsu N, Pacher P, Jeyaseelan S. NLRC4 inflammasome-mediated production of il-1β modulates mucosal immunity in the lung against gram-negative bacterial infection. *J Immunol.* (2012) 188:5623–35. doi: 10.4049/jimmunol.1200195
102. Cohen TS, Prince AS. Activation of inflammasome signaling mediates pathology of acute *P. aeruginosa* pneumonia. *J Clin Invest.* (2013) 123:1630-7. doi: 10.1172/JCI66142
103. Kamata H, Yamamoto K, Wasserman GA, Zabinski MC, Yuen CK, Lung WY, et al. Epithelial cell–derived secreted and transmembrane 1a signals to activated neutrophils during pneumococcal pneumonia. *Am J Respir Cell Mol Biol.* (2016) 55:407–18. doi: 10.1165/rcmb.2015-0261OC
104. Cortes G, Alvarez D, Saus C, Alberti S. Role of lung epithelial cells in defense against *Klebsiella pneumoniae* pneumonia. *Infect Immun.* (2002) 70:1075–80. doi: 10.1128/IAI.70.3.1075-1080.2002
105. de Astorza B, Cortes G, Crespi C, Saus C, Rojo JM, Alberti S. C3 promotes clearance of *Klebsiella pneumoniae* by A549 epithelial cells. *Infect Immun.* (2004) 72:1767–74. doi: 10.1128/IAI.72.3.1767-1774.2004
106. Doorduijn DJ, Rooijakkers SH, van Schaik W, Bardoel BW. Complement resistance mechanisms of *Klebsiella pneumoniae*. *Immunobiology.* (2016) 221:1102–9. doi: 10.1016/j.imbio.2016.06.014
107. Wadehra M, Iyer R, Goodglick L, Braun J. The tetraspan protein epithelial membrane protein-2 interacts with beta1 integrins and regulates adhesion. *J Biol Chem.* (2002) 277:41094–100. doi: 10.1074/jbc.M206868200
108. Dahlin K, Mager EM, Allen L, Tigue Z, Goodglick L, Wadehra M, et al. Identification of genes differentially expressed in rat alveolar type I cells. *Am J Respir Cell Mol Biol.* (2004) 31:309–16. doi: 10.1165/rcmb.2003-0423OC
109. Lin, W-C, Gowdy KM, Madenspacher JH, Zemans RL, Yamamoto K, Lyons-Cohen M, et al. Epithelial membrane protein 2 governs transepithelial migration of neutrophils into the airspace. *J Clin Invest.* (2020) 130:157–70. doi: 10.1172/JCI127144
110. Kumar V. Innate lymphoid cells: immunoregulatory cells of mucosal inflammation. *Eur J Inflamm.* (2014) 12:11–20. doi: 10.1177/1721727X1401200102
111. Kumar V. Innate lymphoid cells: new paradigm in immunology of inflammation. *Immunol Lett.* (2014) 157:23–37. doi: 10.1016/j.imlet.2013.11.003
112. Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells: 10 years on. *Cell.* (2018) 174:1054–66. doi: 10.1016/j.cell.2018.07.017
113. Serafini N, Vosshenrich CA, Di Santo JP. Transcriptional regulation of innate lymphoid cell fate. *Nat Rev Immunol.* (2015) 15:415–28. doi: 10.1038/nri3855
114. Diefenbach A, Colonna M, Koyasu S. Development, differentiation, and diversity of innate lymphoid cells. *Immunity.* (2014) 41:354–65. doi: 10.1016/j.immuni.2014.09.005
115. Moore TA, Perry M, Getsoian AG, Newstead MW, Standiford TJ. Divergent role of gamma interferon in a murine model of pulmonary versus systemic *Klebsiella pneumoniae*. *Infect Immun.* (2002) 70:6310–8. doi: 10.1128/IAI.70.11.6310-6318.2002
116. Shinozawa Y, Matsumoto T, Uchida K, Tsujimoto S, Iwakura Y, Yamaguchi K. Role of interferon-gamma in inflammatory responses in murine pulmonary infection with *Legionella pneumophila*. *J Med Microbiol.* (2002) 51:225–30. doi: 10.1099/0022-1317-51-3-225
117. Marquardt N, Kekalainen E, Chen P, Kvedaraite E, Wilson JN, Ivarsson MA, et al. Human lung natural killer cells are predominantly comprised of highly differentiated hypofunctional CD69(-)CD56(dim) cells. *J Allergy Clin Immunol.* (2017) 139:1321–30.e4. doi: 10.1016/j.jaci.2016.07.043
118. Wang J, Li F, Zheng M, Sun R, Wei H, Tian Z. Lung natural killer cells in mice: phenotype and response to respiratory infection. *Immunology.* (2012) 137:37–47. doi: 10.1111/j.1365-2567.2012.03607.x
119. Cong J, Wei H. Natural killer cells in the lungs. *Front Immunol.* (2019) 10:1416. doi: 10.3389/fimmu.2019.01416
120. Hervier B, Russick J, Cremer I, Vieillard V. NK cells in the human lungs. *Front Immunol.* (2019) 10:1263. doi: 10.3389/fimmu.2019.01263
121. Xu X, Weiss ID, Zhang HH, Singh SP, Wynn TA, Wilson MS, et al. Conventional NK cells can produce IL-22 and promote host defense in *Klebsiella pneumoniae* pneumonia. *J Immunol.* (2014) 192:1778–86. doi: 10.4049/jimmunol.1300039
122. Ivin M, Dumigan A, de Vasconcelos FN, Ebner F, Borroni M, Kavirayani A, et al. Natural killer cell-intrinsic type I IFN signaling controls *Klebsiella pneumoniae* growth during lung infection. *PLoS Pathog.* (2017) 13:e1006696. doi: 10.1371/journal.ppat.1006696
123. Elhaik-Goldman S, Kafka D, Yossef R, Hadad U, Elkabets M, Vallon-Eberhard A, et al. The natural cytotoxicity receptor 1 contribution to early clearance of *Streptococcus pneumoniae* and to natural killer-macrophage cross talk. *PLoS ONE.* (2011) 6:e23472. doi: 10.1371/journal.pone.0023472
124. Panda SK, Colonna M. Innate lymphoid cells in mucosal immunity. *Front Immunol.* (2019) 10:861. doi: 10.3389/fimmu.2019.00861
125. Barlow JL, McKenzie ANJ. Innate lymphoid cells of the lung. *Annu Rev Physiol.* (2019) 81:429-52. doi: 10.1146/annurev-physiol-020518-114630
126. Shikhagaie MM, Björklund ÅK, Mjösberg J, Erjefält JS, Cornelissen AS, Ros XR, et al. Neuropilin-1 is expressed on lymphoid tissue residing LTi-like group 3 innate lymphoid cells and associated with ectopic lymphoid aggregates. *Cell Rep.* (2017) 18:1761–3. doi: 10.1016/j.celrep.2017.01.063
127. Lügering A, Ross M, Sieker M, Heidemann J, Williams IR, Domschke W, et al. CCR6 identifies lymphoid tissue inducer cells within cryptopatches. *Clin Exp Immunol.* (2010) 160:440–9. doi: 10.1111/j.1365-2249.2010.04103.x
128. Rangel-Moreno J, Carragher DM, de la Luz Garcia-Hernandez M, Hwang JY, Kusser K, Hartson L, et al. The development of inducible bronchus-associated lymphoid tissue depends on IL-17. *Nat Immunol.* (2011) 12:639–46. doi: 10.1038/ni.2053
129. Silver JS, Kearley J, Copenhaver AM, Sanden C, Mori M, Yu L, et al. Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat Immunol.* (2016) 17:626–35. doi: 10.1038/ni.3443
130. Ohne Y, Silver JS, Thompson-Snipes L, Collet MA, Blanck JP, Cantarel BL, et al. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat Immunol.* (2016) 17:646–55. doi: 10.1038/ni.3447
131. Bal SM, Bernink JH, Nagasawa M, Groot J, Shikhagaie MM, Golebski K, et al. IL-1beta XR, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat immunol.* (2016) 17:636–45. doi: 10.1038/ni.3444
132. Ruan S, McKinley L, Zheng M, Rudner X, D'Souza A, Kolls JK, et al. Interleukin-12 and host defense against murine pneumocystis pneumonia. *Infect Immun.* (2008) 76:2130–7. doi: 10.1128/IAI.00065-08
133. Nguyen, Q-T, Furuya Y, Roberts S, Metzger DW. Role of interleukin-12 in protection against pulmonary infection with methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother.* (2015) 59:6308–16. doi: 10.1128/AAC.00968-15
134. Denney L, Byrne AJ, Shea TJ, Buckley JS, Pease JE, Herledan GM, et al. Pulmonary epithelial cell-derived cytokine TGF-beta1 is a critical cofactor for enhanced innate lymphoid cell function. *Immunity.* (2015) 43:945–58. doi: 10.1016/j.immuni.2015.10.012
135. van Maele L, Carnoy C, Cayet D, Ivanov S, Porte R, Deruy E, et al. Activation of Type 3 innate lymphoid cells and interleukin 22 secretion in the lungs during Streptococcus pneumoniae infection. *J Infect Dis.* (2014) 210:493–503. doi: 10.1093/infdis/jiu106
136. Robinson KM, Ramanan K, Clay ME, McHugh KJ, Rich HE, Alcorn JF. Novel protective mechanism for interleukin-33 at the mucosal barrier during influenza-associated bacterial superinfection. *Mucosal Immunol.* (2018) 11:199–208. doi: 10.1038/mi.2017.32
137. Saluzzo S, Gorki AD, Rana BMJ, Martins R, Scanlon S, Starkl P, et al. First-breath-induced type 2 pathways shape the lung immune environment. *Cell Rep.* (2017) 18:1893–905. doi: 10.1016/j.celrep.2017.01.071
138. Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration of flagellin protects mice from Streptococcus pneumoniae lung infection. *Infect Immun.* (2010) 78:4226–33. doi: 10.1128/IAI.00224-10
139. Munoz-Wolf N, Rial A, Fougeron D, Tabareau J, Sirard JC, Chabalgoity JA. Sublingual flagellin protects against acute pneumococcal pneumonia in a

TLR5-dependent and NLRC4-independent fashion. *Future Microbiol.* (2016) 11:1167–77. doi: 10.2217/fmb-2016-0045
140. Porte R, Fougeron D, Munoz-Wolf N, Tabareau J, Georgel AF, Wallet F, et al. A toll-like receptor 5 agonist improves the efficacy of antibiotics in treatment of primary and influenza virus-associated pneumococcal mouse infections. *Antimicrob Agents Chemother.* (2015) 59:6064–72. doi: 10.1128/AAC.01210-15
141. Xiong H, Keith JW, Samilo DW, Carter RA, Leiner IM, Pamer EG. Innate lymphocyte/Ly6C(hi) monocyte crosstalk promotes *Klebsiella pneumoniae* clearance. *Cell.* (2016) 165:679–89. doi: 10.1016/j.cell.2016.03.017
142. Gray J, Oehrle K, Worthen G, Alenghat T, Whitsett J, Deshmukh H. Intestinal commensal bacteria mediate lung mucosal immunity and promote resistance of newborn mice to infection. *Sci Transl Med.* (2017) 9:aaf9412. doi: 10.1126/scitranslmed.aaf9412
143. Tamburini S, Clemente JC. Gut microbiota: neonatal gut microbiota induces lung immunity against pneumonia. *Nat Rev Gastroenterol Hepatol.* (2017) 14:263–4. doi: 10.1038/nrgastro.2017.34
144. Gauguet S, D'Ortona S, Ahnger-Pier K, Duan B, Surana NK, Lu R, et al. Intestinal microbiota of mice influences resistance to *Staphylococcus aureus* pneumonia. *Infect Immun.* (2015) 83:4003–14. doi: 10.1128/IAI.00037-15
145. Kopf M SS, Nobs SP. The development and function of lung-resident macrophages and dendritic cells. *Nat Immunol.* (2015) 16:36–44. doi: 10.1038/ni.3052
146. Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. Pulmonary macrophages: key players in the innate defence of the airways. *Thorax.* (2015) 70:1189–96. doi: 10.1136/thoraxjnl-2015-207020
147. Puttur F, Gregory LG, Lloyd CM. Airway macrophages as the guardians of tissue repair in the lung. *Immunol Cell Biol.* (2019) 97:246–57. doi: 10.1111/imcb.12235
148. Cai B, Kasikara C, Doran AC, Ramakrishnan R, Birge RB, Tabas I. MerTK signaling in macrophages promotes the synthesis of inflammation resolution mediators by suppressing CaMKII activity. *Sci Signal.* (2018) 11:eaar3721. doi: 10.1126/scisignal.aar3721
149. Byrne AJ, Maher TM, Lloyd CM. Pulmonary macrophages: a new therapeutic pathway in fibrosing lung disease? *Trends Mol Med.* (2016) 22:303–16. doi: 10.1016/j.molmed.2016.02.004
150. Davies LC, Jenkins SJ, Allen JE, Taylor PR. Tissue-resident macrophages. *Nat Immunol.* (2013) 14:986–95. doi: 10.1038/ni.2705
151. Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD, Gordon S. Macrophage receptors and immune recognition. *Annu Rev Immunol.* (2005) 23:901–44. doi: 10.1146/annurev.immunol.23.021704.115816
152. Kazuo T, Suzuki Y, Yoshimura K, Yasui H, Karayama M, Hozumi H, et al. Macrophage mannose receptor CD206 predicts prognosis in community-acquired pneumonia. *Sci Rep.* (2019) 9:18750. doi: 10.1038/s41598-019-55289-2
153. Cakarova L, Marsh LM, Wilhelm J, Mayer K, Grimminger F, Seeger W, et al. Macrophage tumor necrosis factor-alpha induces epithelial expression of granulocyte-macrophage colony-stimulating factor: impact on alveolar epithelial repair. *Am J Respir Crit Care Med.* (2009) 180:521–32. doi: 10.1164/rccm.200812-1837OC
154. Taut K, Winter C, Briles DE, Paton JC, Christman JW, Maus R, et al. Macrophage turnover kinetics in the lungs of mice infected with streptococcus pneumoniae. *Am J Respir Cell Mol Biol.* (2008) 38:105–13. doi: 10.1165/rcmb.2007-0132OC
155. Medeiros AI, Serezani CH, Lee SP, Peters-Golden M. Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling. *J Exp Med.* (2009) 206:61–8. doi: 10.1084/jem.20082058
156. Aronoff DM, Canetti C, Peters-Golden M. Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. *J Immunol.* (2004) 173:559–65. doi: 10.4049/jimmunol.173.1.559
157. Salina AC, Souza TP, Serezani CH, Medeiros AI. Efferocytosis-induced prostaglandin E2 production impairs alveolar macrophage effector functions during *Streptococcus pneumoniae* infection. *Innate immun.* (2017) 23:219–27. doi: 10.1177/1753425916684934
158. Lauw FN, Branger J, Florquin S, Speelman P, van Deventer SJH, Akira S, et al. IL-18 improves the early antimicrobial host response to pneumococcal pneumonia. *J Immunol.* (2002) 168:372–8. doi: 10.4049/jimmunol.168.1.372
159. Schultz MJ, Knapp S, Florquin S, Pater J, Takeda K, Akira S, et al. Interleukin-18 impairs the pulmonary host response to Pseudomonas aeruginosa. *Infect Immun.* (2003) 71:1630–4. doi: 10.1128/IAI.71.4.1630-1634.2003
160. Nepal S, Tiruppathi C, Tsukasaki Y, Farahany J, Mittal M, Rehman J, et al. STAT6 induces expression of Gas6 in macrophages to clear apoptotic neutrophils and resolve inflammation. *Proc Natl Acad Sci USA.* (2019) 116:16513–8. doi: 10.1073/pnas.1821601116
161. Sham HP, Walker KH, Abdulnour RE, Krishnamoorthy N, Douda DN, Norris PC, et al. 15-epi-Lipoxin A4, Resolvin D2, and Resolvin D3 induce NF-kappaB regulators in bacterial pneumonia. *J Immunol.* (2018) 200:2757–66. doi: 10.4049/jimmunol.1602090
162. El Kebir D, József L, Pan W, Wang L, Petasis NA, Serhan CN, et al. 15-epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury. *Am J Respir Crit Care Med.* (2009) 180:311–9. doi: 10.1164/rccm.200810-1601OC
163. El Kebir D, Filep JG. Modulation of neutrophil apoptosis and the resolution of inflammation through β2 integrins. *Front Immunol.* (2013) 4:60. doi: 10.3389/fimmu.2013.00060
164. Reed JC. Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, therapeutic opportunities. *Cell Death Differ.* (2006) 13:1378–86. doi: 10.1038/sj.cdd.4401975
165. Brenner D, Mak TW. Mitochondrial cell death effectors. *Curr opin Cell Biol.* (2009) 21:871–7. doi: 10.1016/j.ceb.2009.09.004
166. Mayadas TN, Cullere X. Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol.* (2005) 26:388–95. doi: 10.1016/j.it.2005.05.002
167. Jozsef L, Zouki C, Petasis NA, Serhan CN, Filep JG. Lipoxin A4 and aspirin-triggered 15-epi-lipoxin A4 inhibit peroxynitrite formation, NF-kappa B and AP-1 activation, and IL-8 gene expression in human leukocytes. *Proc Natl Acad Sci USA.* (2002) 99:13266–71. doi: 10.1073/pnas.202296999
168. Liu H, Zhou K, Liao L, Zhang T, Yang M, Sun C. Lipoxin A4 receptor agonist BML-111 induces autophagy in alveolar macrophages and protects from acute lung injury by activating MAPK signaling. *Respir Res.* (2018) 19:243. doi: 10.1186/s12931-018-0937-2
169. Roquilly A, McWilliam HEG, Jacqueline C, Tian Z, Cinotti R, Rimbert M, et al. Local modulation of antigen-presenting cell development after resolution of pneumonia induces long-term susceptibility to secondary infections. *Immunity.* (2017) 47:135–47.e5. doi: 10.1016/j.immuni.2017.06.021
170. Barclay AN, Brown MH. The SIRP family of receptors and immune regulation. *Nat Rev Immunol.* (2006) 6:457–64. doi: 10.1038/nri1859
171. Veillette A, Chen J. SIRPα-CD47 immune checkpoint blockade in anticancer therapy. *Trends Immunol.* (2018) 39:173–84. doi: 10.1016/j.it.2017.12.005
172. Roquilly A, Jacqueline C, Davieau M, Mollé A, Sadek A, Fourgeux C, et al. Alveolar macrophages are epigenetically altered after inflammation, leading to long-term lung immunoparalysis. *Nat Immunol.* (2020) 21:636–48. doi: 10.1038/s41590-020-0673-x
173. Schliehe C, Flynn EK, Vilagos B, Richson U, Swaminanthan S, Bosnjak B, et al. The methyltransferase Setdb2 mediates virus-induced susceptibility to bacterial superinfection. *Nat Immunol.* (2015) 16:67–74. doi: 10.1038/ni.3046
174. Mildner A, Chapnik E, Manor O, Yona S, Kim KW, Aychek T, et al. Mononuclear phagocyte miRNome analysis identifies miR-142 as critical regulator of murine dendritic cell homeostasis. *Blood.* (2013) 121:1016–27. doi: 10.1182/blood-2012-07-445999
175. Kimball AS, Davis FM, denDekker A, Joshi AD, Schaller MA, Bermick J, et al. The histone methyltransferase Setdb2 modulates macrophage phenotype and uric acid production in diabetic wound repair. *Immunity.* (2019) 51:258–71.e5. doi: 10.1016/j.immuni.2019.06.015
176. Sun Y, Oravecz-Wilson K, Bridges S, McEachin R, Wu J, Kim SH, et al. miR-142 controls metabolic reprogramming that regulates dendritic cell activation. *J Clin Invest.* (2019) 129:2029–42. doi: 10.1172/JCI123839
177. Herold S, Mayer K, Lohmeyer J. Acute lung injury: how macrophages orchestrate resolution of inflammation and tissue repair. *Front Immunol.* (2011) 2:65. doi: 10.3389/fimmu.2011.00065
178. Hayakawa K, Wang X, Lo EH. CD200 increases alternatively activated macrophages through cAMP-response element binding protein - C/EBP-beta signaling. *J Neurochem.* (2016) 136:900–6. doi: 10.1111/jnc.13492

179. Koning N, van Eijk M, Pouwels W, Brouwer MS, Voehringer D, Huitinga I, et al. Expression of the inhibitory CD200 receptor is associated with alternative macrophage activation. *J Innate Immun.* (2010) 2:195–200. doi: 10.1159/000252803
180. Wright GJ, Puklavec MJ, Willis AC, Hoek RM, Sedgwick JD, Brown MH, et al. Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. *Immunity.* (2000) 13:233–42. doi: 10.1016/S1074-7613(00)00023-6
181. Ocaña-Guzman R, Vázquez-Bolaños L, Sada-Ovalle I. Receptors that inhibit macrophage activation: mechanisms and signals of regulation and tolerance. *J Immunol Res.* (2018) 2018:8695157. doi: 10.1155/2018/8695157
182. Westphalen K, Gusarova GA, Islam MN, Subramanian M, Cohen TS, Prince AS, et al. Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity. *Nature.* (2014) 506:503–6. doi: 10.1038/nature12902
183. Sarieddine MZ, Scheckenbach KE, Foglia B, Maass K, Garcia I, Kwak BR, et al. Connexin43 modulates neutrophil recruitment to the lung. *J Cell Mol Med.* (2009) 13:4560–70. doi: 10.1111/j.1582-4934.2008.00654.x
184. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, et al. Incidence LD, and outcomes of acute lung injury. *N Engl J Med.* (2005) 353:1685–93. doi: 10.1056/NEJMoa050333
185. Rubenfeld, Herridge GD. Epidemiology MS, and outcomes of acute lung injury. *Chest.* (2007) 131:554–62. doi: 10.1378/chest.06-1976
186. Stapleton RD, Wang BM, Hudson LD, Rubenfeld GD, Caldwell ES, Steinberg, et al. Causes KP, timing of death in patients with ARDS. *Chest.* 128:525–32. doi: 10.1378/chest.128.2.525
187. Englert JA, Bobba C, Baron RM. Integrating molecular pathogenesis and clinical translation in sepsis-induced acute respiratory distress syndrome. *JCI Insight.* (2019) 4:e124061. doi: 10.1172/jci.insight.124061
188. Huppert LA, Matthay MA, Ware LB. Pathogenesis of acute respiratory distress syndrome. *Semin Respir Crit Care Med.* (2019) 40:31–9. doi: 10.1055/s-0039-1683996
189. Tsokos M. Immunohistochemical detection of sepsis-induced lung injury in human autopsy material. *Leg Med (Tokyo).* (2003) 5:73–86. doi: 10.1016/S1344-6223(03)00010-5
190. Calfee CS, Janz DR, Bernard GR, May AK, Kangelaris KN, Matthay MA, et al. Distinct molecular phenotypes of direct vs indirect ARDS in single-center and multicenter studies. *Chest.* (2015) 147:1539–48. doi: 10.1378/chest.14-2454
191. Aziz M, Ode Y, Zhou M, Ochani M, Holodick NE, Rothstein TL, et al. B-1a cells protect mice from sepsis-induced acute lung injury. *Mol Med.* (2018) 24:26. doi: 10.1186/s10020-018-0029-2
192. Ness TL, Hogaboam CM, Strieter RM, Kunkel SL. Immunomodulatory role of CXCR2 during experimental septic peritonitis. *J Immunol.* (2003) 171:3775–84. doi: 10.4049/jimmunol.171.7.3775
193. Standiford TJ, Kunkel SL, Lukacs NW, Greenberger MJ, Danforth JM, Kunkel RG, et al. Macrophage inflammatory protein-1 alpha mediates lung leukocyte recruitment, lung capillary leak, and early mortality in murine endotoxemia. *J Immunol.* (1995) 155:1515–24.
194. Walter JM, Wilson J, Ware LB. Biomarkers in acute respiratory distress syndrome: from pathobiology to improving patient care. *Expert Rev Respir Med.* (2014) 8:573–86. doi: 10.1586/17476348.2014.924073
195. Kellner M, Noonepalle S, Lu Q, Srivastava A, Zemskov E, Black SM. ROS signaling in the pathogenesis of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). *Adv Exp Med Biol.* (2017) 967:105–37. doi: 10.1007/978-3-319-63245-2_8
196. Chawla LS, Fink M, Goldstein SL, Opal S, Gómez A, Murray P, et al. The epithelium as a target in sepsis. *Shock.* (2016) 45:249–58. doi: 10.1097/SHK.0000000000000518
197. Cohen TS, DiPaolo BC, Gray Lawrence G, Margulies SS. Sepsis enhances epithelial permeability with stretch in an actin dependent manner. *PLoS ONE.* (2012) 7:e38748. doi: 10.1371/journal.pone.0038748
198. Sheppard D. Modulation of acute lung injury by integrins. *Proc Am Thorac Soc.* (2012) 9:126–9. doi: 10.1513/pats.201112-052AW
199. Su G, Atakilit A, Li JT, Wu N, Bhattacharya M, Zhu J, et al. Absence of integrin αvβ3 enhances vascular leak in mice by inhibiting endothelial cortical actin formation. *Am J Respir Crit Care Med.* (2012) 185:58–66. doi: 10.1164/rccm.201108-1381OC
200. Zhou G, Dada LA, Wu M, Kelly A, Trejo H, Zhou Q, et al. Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1. *Am J Physiol Lung Cell Mol Physiol.* (2009) 297:L1120–30. doi: 10.1152/ajplung.00007.2009
201. Kitamura Y, Hashimoto S, Mizuta N, Kobayashi A, Kooguchi K, Fujiwara I, et al. Fas/FasL-dependent apoptosis of alveolar cells after lipopolysaccharide-induced lung injury in mice. *Am J Respir Crit Care Med.* (2001) 163:762–9. doi: 10.1164/ajrccm.163.3.2003065
202. Perl M, Chung C-S, Lomas-Neira J, Rachel T-M, Biffl WL, Cioffi WG, et al. Silencing of fas, but not caspase-8, in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhagic shock and sepsis. *Am J Pathol.* (2005) 167:1545–59. doi: 10.1016/S0002-9440(10)61240-0
203. Drouin SM, Kildsgaard J, Haviland J, Zabner J, Jia HP, McCray PB, et al. Expression of the complement Anaphylatoxin C3a and C5a receptors on bronchial epithelial and smooth muscle cells in models of sepsis and asthma. *J Immunol.* (2001) 166:2025–32. doi: 10.4049/jimmunol.166.3.2025
204. Bosmann M, Ward PA. Role of C3, C5 and anaphylatoxin receptors in acute lung injury and in sepsis. *Adv Exp Med Biol.* (2012) 946:147–59. doi: 10.1007/978-1-4614-0106-3_9
205. Russkamp NF, Ruemmler R, Roewe J, Moore BB, Ward PA, Bosmann M. Experimental design of complement component 5a-induced acute lung injury (C5a-ALI): a role of CC-chemokine receptor type 5 during immune activation by anaphylatoxin. *FASEB J.* (2015) 29:3762–72. doi: 10.1096/fj.15-271635
206. Juss JK, House D, Amour A, Begg M, Herre J, Storisteanu DML, et al. Acute respiratory distress syndrome neutrophils have a distinct phenotype and are resistant to phosphoinositide 3-kinase inhibition. *Am J Respir Crit Care Med.* (2016) 194:961–73. doi: 10.1164/rccm.201509-1818OC
207. Fialkow L FFL, Bozzetti MC, Milani AR, Rodrigues Filho EM, Ladniuk RM, Pierozan P, et al. Neutrophil apoptosis: a marker of disease severity in sepsis and sepsis-induced acute respiratory distress syndrome. *Crit Care.* (2006) 10:R155. doi: 10.1186/cc5090
208. Dorward DA, Felton JM, Robb CT, Craven T, Kipari T, Walsh TS, et al. The cyclin-dependent kinase inhibitor AT7519 accelerates neutrophil apoptosis in sepsis-related acute respiratory distress syndrome. *Thorax.* (2017) 72:182–85. doi: 10.1136/thoraxjnl-2016-209229
209. Galiacy S, Planus E, Lepetit H, Féréol S, Laurent V, Ware L, et al. Keratinocyte growth factor promotes cell motility during alveolar epithelial repair *in vitro*. *Exp Cell Res.* (2003) 283:215–29. doi: 10.1016/S0014-4827(02)00049-6
210. McAuley DF, Cross LM, Hamid U, Gardner E, Elborn JS, Cullen KM, et al. Keratinocyte growth factor for the treatment of the acute respiratory distress syndrome (KARE): a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet Respir Med.* (2017) 5:484–91. doi: 10.1016/S2213-2600(17)30171-6
211. Ray P, Devaux Y, Stolz DB, Yarlagadda M, Watkins SC, Lu Y, et al. Inducible expression of keratinocyte growth factor (KGF) in mice inhibits lung epithelial cell death induced by hyperoxia. *Proc Natl Acad Sci USA.* (2003) 100:6098–103. doi: 10.1073/pnas.1031851100
212. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CGK, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* (2011) 12:1045–54. doi: 10.1038/ni.2131
213. de Grove KC, Provoost S, Verhamme FM, Bracke KR, Joos GF, Maes T, et al. characterization and quantification of innate lymphoid cell subsets in human lung. *PLoS ONE.* (2016) 11:e0145961. doi: 10.1371/journal.pone.0145961
214. Cruz-Zárate D, Cabrera-Rivera GL, Ruiz-Sánchez BP, Serafín-López J, Chacón-Salinas R, López-Macías C, et al. Innate lymphoid cells have decreased HLA-DR expression but retain their responsiveness to TLR ligands during sepsis. *J Immunol.* (2018) 201:3401–10. doi: 10.4049/jimmunol.1800735
215. Huang Y, Mao K, Chen X, Sun M-A, Kawabe T, Li W, et al. S1P-dependent interorgan trafficking of group 2 innate lymphoid cells supports host defense. *Science (New York, N.Y.).* (2018) 359:114–119. doi: 10.1126/science.aam5809
216. Winkler MS, Märtz KB, Nierhaus A, Daum G, Schwedhelm E, Kluge S, et al. Loss of sphingosine 1-phosphate (S1P) in septic shock is predominantly caused by decreased levels of high-density lipoproteins (HDL). *J Intensive Care.* (2019) 7:23. doi: 10.1186/s40560-019-0376-2

217. Germain RN, Huang Y. ILC2s - resident lymphocytes pre-adapted to a specific tissue or migratory effectors that adapt to where they move? *Curr Opin Immunol.* (2019) 56:76–81. doi: 10.1016/j.coi.2018.11.001
218. Chun TT, Chung C-S, Fallon EA, Hutchins NA, Clarke E, Rossi A-L, et al. Group 2 innate lymphoid cells (ILC2s) are key mediators of the inflammatory response in polymicrobial sepsis. *Am J Pathol.* (2018) 188:2097–108. doi: 10.1016/j.ajpath.2018.05.009
219. Xu H, Xu J, Xu L, Jin S, Turnquist HR, Hoffman R, et al. Interleukin-33 contributes to ILC2 activation and early inflammation-associated lung injury during abdominal sepsis. *Immunol Cell Biol.* (2018) 96:935–47. doi: 10.1111/imcb.12159
220. Lai D, Tang J, Chen L, Fan EK, Scott MJ, Li Y, et al. Group 2 innate lymphoid cells protect lung endothelial cells from pyroptosis in sepsis. *Cell Death Dis.* (2018) 9:369. doi: 10.1038/s41419-018-0412-5
221. Krishack PA, Louviere TJ, Decker TS, Kuzel TG, Greenberg JA, Camacho DF, et al. Protection against *Staphylococcus aureus* bacteremia-induced mortality depends on ILC2s and eosinophils. *JCI Insight.* (2019) 4:e124168. doi: 10.1172/jci.insight.124168
222. Puneet P, Moochhala S, Bhatia M. Chemokines in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol.* (2005) 288:L3–15. doi: 10.1152/ajplung.00405.2003
223. Wagner JG, Driscoll KE, Roth RA. Inhibition of pulmonary neutrophil trafficking during endotoxemia is dependent on the stimulus for migration. *Am J Respir Cell Mol Biol.* (1999) 20:769–76. doi: 10.1165/ajrcmb.20.4.3481
224. Wang Z, Rui T, Yang M, Valiyeva F, Kvietys PR. Alveolar macrophages from septic mice promote polymorphonuclear leukocyte transendothelial migration via an endothelial cell Src kinase/NADPH oxidase pathway. *J Immunol.* (2008) 181:8735–44. doi: 10.4049/jimmunol.181.12.8735
225. Park I, Kim M, Choe K, Song E, Seo H, Hwang Y, et al. Neutrophils disturb pulmonary microcirculation in sepsis-induced acute lung injury. *Eur Respir J.* (2019) 53:1800786. doi: 10.1183/13993003.00786-2018
226. Reddy RC, Chen GH, Newstead MW, Moore T, Zeng X, Tateda K, et al. Alveolar macrophage deactivation in murine septic peritonitis: role of interleukin 10. *Infect Immun.* (2001) 69:1394–401. doi: 10.1128/IAI.69.3.1394-1401.2001
227. Mahida R, Thickett D. Impaired alveolar macrophage efferocytosis in ARDS causes accumulation of apoptotic neutrophils & prolonged inflammation. *Eur Respir J.* (2018) 52:PA4283. doi: 10.1183/13993003.congress-2018.PA4283
228. Hiruma T, Tsuyuzaki H, Uchida K, Trapnell BC, Yamamura Y, Kusakabe Y, et al. IFN-β improves sepsis-related alveolar macrophage dysfunction and postseptic acute respiratory distress syndrome-related mortality. *Am J Respir Cell Mol Biol.* (2018) 59:45–55. doi: 10.1165/rcmb.2017-0261OC
229. Pahuja M, Tran C, Wang H, Yin K. Alveolar macrophage suppression in sepsis is associated with high mobility group box 1 transmigration. *Shock.* (2008) 29:754–60. doi: 10.1097/shk.0b013e31815d0c8f
230. Xu J, Jiang Y, Wang J, Shi X, Liu Q, Liu Z, et al. Macrophage endocytosis of high-mobility group box 1 triggers pyroptosis. *Cell Death Differ.* (2014) 21:1229–39. doi: 10.1038/cdd.2014.40
231. Deng M, Tang Y, Li W, Wang X, Zhang R, Zhang X, et al. The endotoxin delivery protein HMGB1 mediates caspase-11-dependent lethality in sepsis. *Immunity.* (2018) 49:740–53.e7. doi: 10.1016/j.immuni.2018.08.016
232. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. *Nature.* (2015) 526:666–71. doi: 10.1038/nature15541
233. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death. *Nature.* (2015) 526:660–5. doi: 10.1038/nature15514
234. Kang R, Zeng L, Zhu S, Xie Y, Liu J, Wen Q, et al. Lipid peroxidation drives gasdermin d-mediated pyroptosis in lethal polymicrobial sepsis. *Cell Host Microbe.* (2018) 24:97–108.e4. doi: 10.1016/j.chom.2018.05.009
235. Xiong S, Hong Z, Huang LS, Tsukasaki Y, Nepal S, Di A, et al. IL-1β suppression of VE-cadherin transcription underlies sepsis-induced inflammatory lung injury. *J Clin Invest.* (2020) 130:3684–98. doi: 10.1172/JCI136908
236. Janardhan KS, Sandhu SK, Singh B. Neutrophil depletion inhibits early and late monocyte/macrophage increase in lung inflammation. *Front Biosci.* (2006) 11:1569–76. doi: 10.2741/1904
237. Farley KS, Wang LF, Razavi HM, Law C, Rohan M, McCormack DG, et al. Effects of macrophage inducible nitric oxide synthase in murine septic lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2006) 290:L1164–72. doi: 10.1152/ajplung.00248.2005
238. Liu Y, Guan H, Zhang J-L, Zheng Z, Wang T, Tao K, et al. Acute downregulation of miR-199a attenuates sepsis-induced acute lung injury by targeting SIRT1. *Am J Physiol Cell Physiol.* (2018) 314:C449–55. doi: 10.1152/ajpcell.00173.2017
239. Finkel T, Deng C-X, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. *Nature.* (2009) 460:587–91. doi: 10.1038/nature08197
240. Gao R, Ma Z, Hu Y, Chen J, Shetty S, Fu J. Sirt1 restrains lung inflammasome activation in a murine model of sepsis. *Am J Physiol Lung Cell Mol Physiol.* (2015) 308:L847–53. doi: 10.1152/ajplung.00274.2014
241. Li Y, Yang X, He Y, Wang W, Zhang J, Zhang W, et al. Negative regulation of NLRP3 inflammasome by SIRT1 in vascular endothelial cells. *Immunobiology.* (2017) 222:552–61. doi: 10.1016/j.imbio.2016.11.002
242. Deng JC, Cheng G, Newstead MW, Zeng X, Kobayashi K, Flavell RA, et al. Sepsis-induced suppression of lung innate immunity is mediated by IRAK-M. *J Clin Invest.* (2006) 116:2532–42. doi: 10.1172/JCI28054
243. Lyn-Kew K, Rich E, Zeng X, Wen H, Kunkel SL, Newstead MW, et al. IRAK-M regulates chromatin remodeling in lung macrophages during experimental sepsis. *PLoS ONE.* (2010) 5:e11145. doi: 10.1371/journal.pone.0011145
244. Filgueiras JLR, Martins JO, Serezani CH, Capelozzi VL, Montes MBA, Jancar S. Sepsis-induced acute lung injury (ALI) is milder in diabetic rats and correlates with impaired NFkB activation. *PLoS ONE.* (2012) 7:e44987. doi: 10.1371/journal.pone.0044987
245. Honiden S, Gong MN. Diabetes, insulin, and development of acute lung injury. *Crit Care Med.* (2009) 37:2455–64. doi: 10.1097/CCM.0b013e3181a0fea5
246. Esper AM, Moss M, Martin GS. The effect of diabetes mellitus on organ dysfunction with sepsis: an epidemiological study. *Crit Care.* (2009) 13:R18. doi: 10.1186/cc7717
247. Gu W-J, Wan Y-D, Tie H-T, Kan C, Sun T-W. Risk of acute lung injury/acute respiratory distress syndrome in critically ill adult patients with pre-existing diabetes: a meta-analysis. *PLoS ONE.* (2014) 9:e90426. doi: 10.1371/journal.pone.0090426
248. Yu S, Christiani DC, Thompson BT, Bajwa EK, Gong MN. Role of diabetes in the development of acute respiratory distress syndrome. *Crit Care Med.* (2013) 41:2720–32. doi: 10.1097/CCM.0b013e318298a2eb
249. Ji M, Chen M, Hong X, Chen T, Zhang N. The effect of diabetes on the risk and mortality of acute lung injury/acute respiratory distress syndrome: a meta-analysis. *Medicine.* (2019) 98:e15095. doi: 10.1097/MD.0000000000015095

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oprC Impairs Host Defense by Increasing the Quorum-Sensing-Mediated Virulence of *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, found widely in the wild, causes infections in the lungs and several other organs in healthy people but more often in immunocompromised individuals. *P. aeruginosa* infection leads to inflammasome assembly, pyroptosis, and cytokine release in the host. OprC is one of the bacterial porins abundant in the outer membrane vesicles responsible for channel-forming and copper binding. Recent research has revealed that OprC transports copper, an essential trace element involved in various physiological processes, into bacteria during copper deficiency. Here, we found that *oprC* deletion severely impaired bacterial motility and quorum-sensing systems, as well as lowered levels of lipopolysaccharide and pyocyanin in *P. aeruginosa*. In addition, *oprC* deficiency impeded the stimulation of TLR2 and TLR4 and inflammasome activation, resulting in decreases in proinflammatory cytokines and improved disease phenotypes, such as attenuated bacterial loads, lowered lung barrier damage, and longer mouse survival. Moreover, *oprC* deficiency significantly alleviated pyroptosis in macrophages. Mechanistically, *oprC* gene may impact quorum-sensing systems in *P. aeruginosa* to alter pyroptosis and inflammatory responses in cells and mice through the STAT3/NF-κB signaling pathway. Our findings characterize OprC as a critical virulence regulator, providing the groundwork for further dissection of the pathogenic mechanism of OprC as a potential therapeutic target of *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, *oprC*, virulence, pyroptosis, STAT3/NF-κB

INTRODUCTION

The Gram-negative bacterium *Pseudomonas aeruginosa* is an important opportunistic pathogen that causes severe major cause of acute and chronic lung diseases in mammals. *P. aeruginosa* is the primary cause of acute and chronic lung infection, resulting in high mortality in patients with underlying conditions, such as cystic fibrosis (1). Upon *P. aeruginosa* infection, the pattern recognition receptors (PRRs) on the cell membrane of hosts recognize the corresponding pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagellin

(2). Activated PRRs, including toll-like receptors (TLRs) and Nod-like receptors (NLRs), facilitate inflammasome assembly, caspase autocleavage, and mature IL-1β formation, as well as a type of rapid inflammatory cell death termed pyroptosis (3). Gasdermin D (GSDMD) is found as the pyroptosis executioner, which is activated by both caspase-1 and caspase-11/4/5 cleavage (4). Upon GSDMD activation, the pore in the plasma membrane causes cell lysis due to GSDMD oligomerization and ultimately IL-1β release, which is a highly inflammatory event (5).

Pseudomonas aeruginosa is notoriously resistant to antibiotics, which is facilitated by multiple factors including the highly impermeable outer membrane, the multiple drug efflux system (6, 7), mobile genetic elements (MGE) (8), etc. Furthermore, the list of multidrug-resistant (MDR) *P. aeruginosa* strains is rapidly growing, and new antibiotic development is urgently needed. Therefore, a thorough understanding of the pathogenic mechanisms of its virulence factors and their interactions with the host is required in order to invent new therapeutic strategies to control the infections by the MDR *P. aeruginosa* strains (9). These bacteria can survive under various growth conditions with vesicles from their outer membrane (OMV). A previous study (10) described the proteomic profiles of OMVs of *P. aeruginosa* biofilms and found that the outer membrane proteins OprC, OprD, OprE, OprF, OprH, and OprG were significant components of the OMV. OprC is one of the outer membrane porins responsible for channel-forming and copper binding (11). Then, researchers focused on the relationship between MDR and OprC in *P. aeruginosa* and revealed (12–14) that OprC was unrelated to meropenem, ceftazidime susceptibility, and imipenem diffusion.

Recent studies showed that the *oprC* expression level is involved in copper homeostasis (15). The essential trace element copper is the cofactor of oxidoreductases in *P. aeruginosa*. The copper enzymes, such as cytochrome c oxidase, lysyl oxidase, and ferroxidase, possess crucial physiological functions. Although copper is generally bound to proteins, an excess of free copper is harmful to the cell due to its redox properties (16). To maintain copper homeostasis, organisms generate a set of cytoplasmic copper-sensing regulators and transporters, including OprC. Research has shown that OprC-bound Azurin (a copper-containing redox protein released by *P. aeruginosa*) is essential for copper transport under copper-limited conditions (17).

Here, we analyzed how *oprC* deficiency affects *P. aeruginosa* pathogenicity compared to the wild type strain. We noticed that *oprC* deficiency reduced quorum sensing potential and impaired motility in the bacterium. Furthermore, infection by *oprC* deficiency strain diminished inflammasome activation, cytokine secretion, and transcription factor activity, as well as a significantly lower pyroptosis in host cells. Our findings revealed a novel crucial function of *oprC* in controlling pathogenic virulence activity, providing a basis to further advance the pathogenesis details of *oprC*.

MATERIALS AND METHODS

Mice

C57BL/6J mice (6–8 weeks) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of North Dakota and were performed in accordance with the animal care and institutional guidelines. The experimental procedures for animals, including treatment, care, and endpoint, strictly followed the Animal Research: Reporting *in vivo* Experiment guidelines (18).

Cell Lines

Murine macrophage MH-S cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Roswell Park Memorial Institute 1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) incubated in a 5% CO_2 environment at 37°C (19).

Inhibitor Treatment

STAT3 inhibitor V, stattic (sc-202818), and BAY (sc-202490) were obtained from Santa Cruz Biotechnology, USA. Stattic inhibits the activation of the STAT3 transcription factor by blocking phosphorylation and dimerization events. Stattic was resuspended in dimethyl sulfoxide (DMSO) to generate a 50 mM stock solution. A working solution (500 μM) was generated by diluting the stock solution in PBS (final concentration of DMSO: 1%). MH-S cells were treated with 10 μM of the specific STAT3 Inhibitor V, stattic, 30 min before infection. PBS/DMSO was added to each untreated well in order to perform vehicle controls (final concentration of DMSO, 1% in PBS). BAY inhibits the activation of NF-κB and the phosphorylation of Iκ-Bα. BAY was dissolved in DMSO to generate a 10 mM stock solution and diluted (1:1,000) in fresh medium before use. MH-S cells were treated with 10 μM BAY for 1 h before infection. DMSO was added to each untreated well as vehicle controls (20).

Bacteria Preparation and Infection Experiments

The wild type *P. aeruginosa* strain PAO1, the Δ*oprC* mutant, and the complemented strain (Δ*oprC*/p-*oprC*) were described previously (17). Bacteria were grown for about 16 h in LB broth at 37°C with 220 rpm shaking. The bacteria were pelleted by centrifugation at 5,000 g. Cells were changed to antibiotic-free medium and infected by bacteria in a multiplicity of infection (MOI) of a 10:1 bacterium-cell ratio for 2 h. Mice were anesthetized with 45 mg/kg ketamine and intranasally instilled 2×10^7 clonal-forming units (CFU) of PAO1 in 50 μL phosphate-buffered saline. Mice were monitored for symptoms and killed when they were moribund (18).

ELISA and LDH Assay

Mouse TNF-α, IL-6, and IL-1β uncoated ELISA kits from Invitrogen (San Diego, CA) were used to measure cytokine concentration. Pierce LDH Cytotoxicity Assay Kit was used for the quantification of LDH released from the cell. Culture supernatants were collected at the indicated times after infection for ELISA and LDH analysis in accordance with the manufacturer's instructions (21).

Immunoblotting

Mouse Abs against p-p65 (p-NFκB p65 Antibody [Ser 536]: sc-136548), ASC (ASC Antibody [B-3]: sc-514414), caspase-1(caspase-1 p10 Antibody [M-20]: sc-514), and β-Actin (β-Actin Antibody [C4]: sc-47778) were obtained from Santa Cruz Biotechnology (Dallas, TX). Rabbit Abs against p65 (NF-κB p65 [D14E12] XP® Rabbit mAb #8242), STAT3 (Stat3 [D3Z2G] Rabbit mAb #12640), and p-STAT3 (Phospho-Stat3 [Tyr705] [D3A7] XP® Rabbit mAb #9145) were obtained from Cell Signaling Technology (Danvers, MA). Gasdermin-D (Anti-GSDMD antibody [EPR19828] ab209845) was obtained from Abcam. NLRC4 (Cat# PA5-88997) was obtained from Invitrogen (Carlsbad, CA). NLRP3 Rabbit pAb (Cat# A12694) was obtained from ABclonal (Woburn, MA). The samples derived from cells and lung homogenates were lysed in RIPA buffer, separated by electrophoresis on SDS-PAGE gels, and transferred to nitrocellulose transfer membranes (GE Amersham Biosciences, Pittsburgh, PA). Proteins were detected by western blotting using primary Abs at a concentration of 1:200 (Santa Cruz Biotechnology) or 1:1,000 (Cell Signaling Technology, Abcam, Invitrogen, and ABclonal) and were incubated overnight. Labeling of the first Abs was detected using relevant secondary Abs conjugated to HRP (Rabbit anti-Mouse IgG [H+L] Secondary Antibody, HRP; Goat anti-Rabbit IgG [H+L] Secondary Antibody, HRP, Invitrogen), which were detected using ECL reagents (GE Health) (22).

RNA Isolation and Quantitative Reverse Transcription-PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. For the mRNA assay, a total of 2 μg of DNA-free RNA was subjected to first-strand cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qRT-PCR assay was performed using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) and gene-specific primers (synthesized by Integrated Eurofins Genomics) in a CFX Connect Real-Time PCR Detection System (Bio-Rad). The separate well $2^{-\Delta\Delta Ct}$ cycle threshold method was used to determine the relative quantitative expression of individual mRNAs. Mammalian mRNAs were expressed as the fold difference to *β-actin*. Bacterial mRNAs were expressed as the fold difference to *16S* (23, 24).

Histological Analysis

Lung tissues of three independent mice were fixed in 10% formalin (Fisher Scientific), soaked in 30% sucrose, and then embedded in optimal cutting temperature (OCT) compound. Six-micrometer sections were cut, stained by standard H&E protocol, and examined for differences in morphology after infection. The lung injury score for each sample was determined by neutrophil accumulation in the alveolar and interstitial space, formation of hyaline membranes, presence of proteinaceous debris in the alveolar space, and thickening of the alveolar wall. Each of these parameters was scored on a scale of 0 (absent) to 3 (severe) and summed to generate the lung injury score (25, 26).

Swimming and Swarming

LB containing 0.3% (wt/vol) Difco agar (BD) was used for the swimming test. BM2 (62 mM potassium phosphate buffer [pH 7], 2 mM $MgSO_4$, 10 μM $FeSO_4$, 0.4% [wt/vol] glucose, and 0.1% [wt/vol] casamino acids) containing 0.5% (wt/vol) Difco agar was used for the swarming test. One microliter overnight LB cultures were introduced into the center of the agar plate by puncturing into the agar but without touching the base of the plates. The plates were incubated at 37°C for 24 h with the right side up. The diameter of the motility trace was measured (27).

Twitching

LB medium supplemented with 1% (wt/vol) agar was inoculated by a tip stabbed through the agar to the agar-plastic interface, with 1 μL of cultures grown in LB broth. After 60 h of incubation, twitching motility was determined by measuring the diameters of the twitching zones stained by a 0.1% crystal violet solution (28).

Measurement of Pyocyanin Production

Bacteria cultures were grown at 37°C, 220 rpm. Supernatants were collected after centrifugation at 10,000 rpm for 10 min and then filter sterilized. 4.5 mL of chloroform was added to 7.5 mL of supernatant and vortexed. Samples were centrifuged for 10 min at 10,000 rpm. The resulting blue layer at the bottom was transferred to a new tube. 1.5 mL of 0.2 M HCl was added to each tube and vortexed. Samples were centrifuged for 2 min at 10,000 rpm, and 1 mL from the pink layer was transferred to cuvettes. Spectrophotometric measurements were done at 520 nm. 0.2 M HCl was used as a blank. Pyocyanin concentration (μL/mL) was calculated by multiplying the value at 520 nm with 17.072 and then multiplying it again by 1.5 (27).

Immunofluorescence

Collected lungs were embedded in OCT and were immediately frozen. Six-micrometer sections were cut using Leica CM1520 Cryostat. OCT was removed from cryosections in PBS, and the samples were fixed using 4% paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature. Permeabilization and blocking were done in 5% BSA in PBS containing 0.25% Triton X-100. The expression of Claudin-1, ZO-1, TLR4, NLRP3, NLRC4, ASC, caspase-1, p-STAT3, and p-NFκB p65 was determined by immunofluorescence. Abs Claudin-1 (Invitrogen, Cat# 71-7800), ZO-1 (Proteintech, Cat# 66452-1-lg), TLR4 [Santa Cruz Biotechnology, TLR4 Antibody (25): sc-293072], NLRP3 (ABclonal, Cat# A12694), NLRC4 (Invitrogen, Cat# PA5-88997), ASC (Santa Cruz Biotechnology, ASC Antibody [B-3]: sc-514414), caspase-1 (Santa Cruz Biotechnology, caspase-1 p10 Antibody [M-20]: sc-514), p-NFκB p65 (Santa Cruz Biotechnology, p-NFκB p65 Antibody [Ser 536]: sc-136548), and p-STAT3 (Cell Signaling Technology, Phospho-Stat3 [Tyr705] [D3A7] XP® Rabbit mAb #9145) were used as primary antibodies at a 1:100 dilution. Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Cat# A-11034, Invitrogen), or Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (Cat# A-11005, Invitrogen) was used at a 1:1,000 dilution as secondary antibodies. Cell nuclei were stained with DAPI solution

(1 μg/mL DAPI in PBS). Slides were visualized with an Olympus FV3000 confocal laser scanning microscope. Quantification analysis was performed by Fiji (19).

LPS Quantification Assay

Bacteria cultures were grown at 37°C, 220 rpm, until an OD600 of 0.5 was reached. Supernatants were collected after centrifugation at 10,000 rpm for 10 min and then filter sterilized. Diluted supernatants (1:4) were used for LPS measurement by Pierce LAL Chromogenic Endotoxin Quantitation Kit (Cat#88282 Thermo Scientific) in accordance with the manufacturer's instructions.

Protease Assay

Bacteria were grown at 37°C, 220 rpm overnight. Supernatants were collected after centrifugation at 4,000 rpm for 30 min. 0.1 mL azocasein solution (30 mg dissolved in 1 mL water), 3 mL phosphate buffer (50 mM, pH 7.5), and 0.1 mL supernatant were mixed and incubated at 37°C for 1 h. Adding 0.5 mL 20% trichloroacetic acid (TCA) to stop the reaction. Supernatants were collected by centrifugation at 12,000 g for 10 min. Two hundred microliters supernatants were added to the microtiter plate for absorbance measurement at 366 nm (29).

Alginate Assay

After bacteria had been cultured in 37°C shaker overnight, bacterial cultures were mixed with equal volume of 0.85% saline and centrifugated at 4,000 rpm for 30 min to collect the supernatants. The supernatants were mixed with equal volume of 2% cetylpyridinium chloride. The precipitates were collected by centrifugation at 4,000 rpm for 30 min. The precipitates were dissolved in 1 M HCl solution, precipitated with isopropanol, and dissolved again in the 0.85% saline. Fifty microliters samples were mixed with 200 μl of borate-sulfuric acid reagent (10 mM H_3BO_3 in concentrated H_2SO_4) and 50 μl of carbazole reagent (0.1% in ethanol) before incubation at 100°C for 10 min. Two hundred microliters of supernatants were transferred to the microtiter plate and absorbance at 550 nm was determined spectrophotometrically (30).

Rhamnolipid Assay

Bacteria were grown in 5 mL LB-MOPS medium (dissolve 25 g LB powder and 10 g MOPS in 1 L deionized water, adjust pH to 7.2 using NaOH) overnight at 37°C, 220 rpm. After centrifugation at 4,000 rpm for 30 min to collect supernatants, 1N HCl was added to 4 mL supernatants to adjust pH to 2.3. Mixing 4 mL supernatant with 4 mL ethyl acetate and vertexing vigorously. After centrifugation at 500 rpm for 1 min, the upper phases were transferred to new tubes and evaporated to dryness. Methylene blue solution (Cat#1808, Sigma-Aldrich) was diluted 1:25 in deionized water and was adjusted to 8.6 pH by adding 15 μl 50 mM borax buffer. Four milliliters chloroform and 400 μl diluted methylene blue solution were added to the tubes containing the dry extracts and vortexed vigorously. After incubation at room temperature for 15 min, 1 mL chloroform phase and 500 μl 0.2 N HCl were added to 2 mL microcentrifuge tube and vortexed 20 s. The tubes were centrifuged at 500 rpm for 1 min. Two hundred microliters upper phases were transferred to the microtiter plate for absorbance measurement at 638 nm against an 0.2 N HCl blank (31).

Growth Curves

The bacteria cultures were diluted when an OD_{600} value of 0.05 was obtained. The growth curves were performed in polystyrene microtiter plates by adding 100 μL cultures and incubated at 37°C. The optical densities at OD_{600} were recorded every 1 h (32).

Flow Cytometry

Single cells were obtained from lungs digested by collagenase. The cells were stained for 1 h with abs PE Rat Anti-Mouse F4/80 (BD Pharmingen Cat# 565410), PE/Cy7 Anti-Mouse/Human CD11b (BioLegend Cat# 101215), PerCP/Cyanine5.5 Anti-Mouse CD45 (BioLegend Cat# 103132), and FITC Anti-Mouse Ly-6G/Ly-6C (Gr-1) (BioLegend Cat# 108406) diluted in PBS at a 1: 1,000. For compensation, single stained samples were set. Cells were analyzed on BD FACSymphony (BD). Data were generated using FlowJo V10 (Treestar, Stanford, CA).

Statistical Analysis

Survival differences and growth curves were analyzed by the Kolmogorov-Smirnov test. In all other cases, one-way ANOVA with a *post-hoc* Tukey test was performed. For all statistical analyses, the statistical package R 3.6.0 was used.

RESULTS

oprC Deficiency Impacts Bacterial Motility

To investigate the effects of *oprC* deficiency on bacterial physiologic and/or pathogenic characteristics, we compared the swarming, swimming, and twitching motility between PAO1, Δ*oprC*, and Δ*oprC*/p-*oprC* strains (17). **Supplemental Figure 1** shows decreased mRNA expression of Δ*oprC* compared to PAO1 ($p = 2.10e-05$) and Δ*oprC*/p-*oprC* ($p = 6.90e-10$) strains. Swarming of *P. aeruginosa* is a multicellular motility action relating to the quorum-sensing system (QS) (33–35). QS signals may modulate the expression and production of hundreds of virulence factors and regulate multiple downstream effects (36). As shown in **Figure 1A**, Δ*oprC* lost the dendritic branch features on BM2 swarming plates, and the diameter of the swarming zone was reduced by more than three quarters compared to PAO1 and complemented strains. We examined the swimming motility on swimming plates to assess the individual cell motility by rotating flagella (37). The swimming zone diameter of Δ*oprC* was half of that of the WT strain (**Figure 1B**). Next, we also examined the twitching motility related to type IV pili (37). **Figure 1C** illustrates decreased twitching motility of Δ*oprC* compared to PAO1 ($p = 7.54e-03$) and complemented strains ($p = 3.65e-03$). However, no apparent change in growth was induced by the *oprC*-deficient mutation (**Figure 1D**). Altogether, these findings suggest that *oprC*-deficient mutation impaired bacterial motility.

oprC Is Involved in Virulence Regulation

The QS system is highly involved in competence, antibiotic production, biofilm formation, bacterial motility, and virulence factor secretion (36, 38). Given the bacterial motility changes of

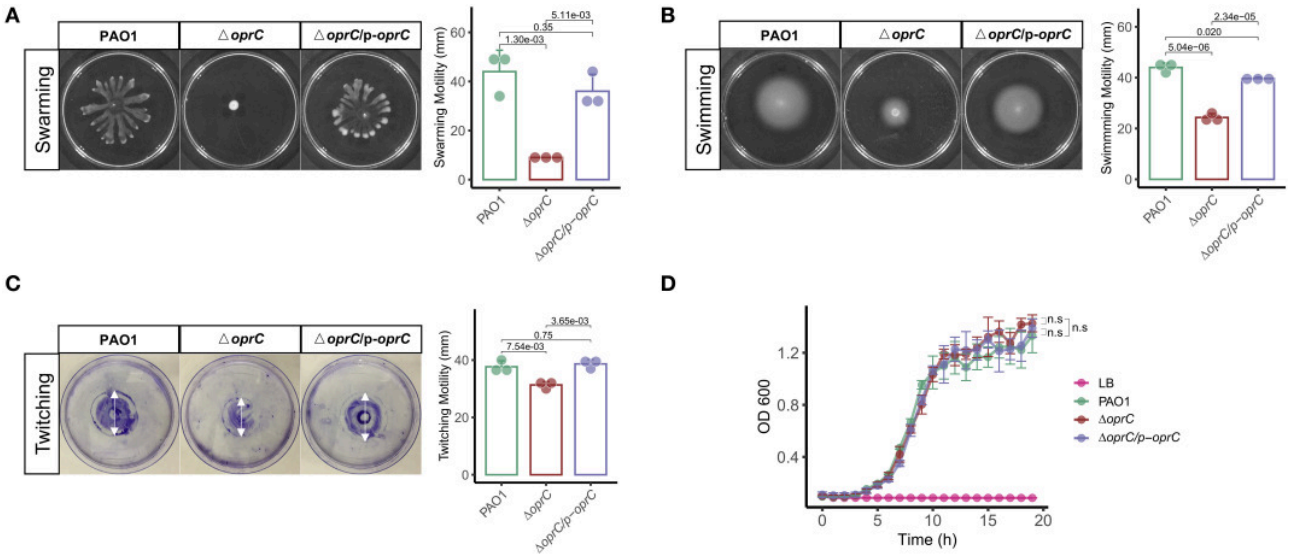

FIGURE 1 | Altered bacterial motility in the Δ*oprC* strain. After the bacteria were incubated at 37°C for 24 h, **(A)** swarming motility ($n = 3$) and **(B)** swimming motility ($n = 3$) were assessed in a BM2 plate containing 0.5% agar and an LB plate containing 0.3% agar, respectively. **(C)** Twitching motility ($n = 3$) after incubating for 60 h at 37°C. **(D)** Bacterial growth curves ($n = 4$) on LB media are shown. Error bars represent the mean ± s.d. Kolmogorov-Smirnov test was performed for bacteria growth curves analysis. One-way ANOVA with a *post-hoc* Tukey test was performed for comparison of means of groups in other cases.

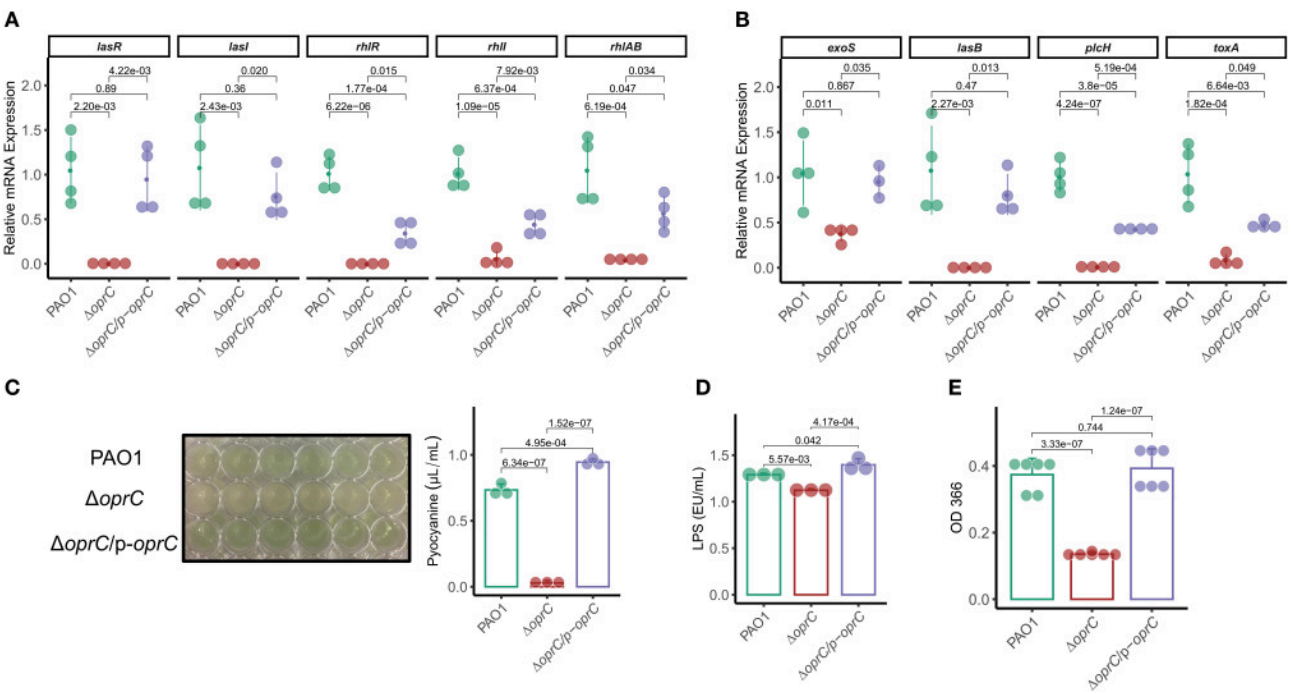

FIGURE 2 | Δ*oprC* exhibited increased toxin production and upregulated virulence-related gene expression. **(A,B)** The expression levels of quorum-sensing and virulence genes were assessed by qRT-PCR ($n = 3$–4). **(C)** Quantification of pyocyanin production in *P. aeruginosa* ($n = 3$). **(D)** Quantification of LPS production in *P. aeruginosa* ($n = 3$). **(E)** Exoproteases activity of bacteria determined by the azocasein assay ($n = 6$). Error bars represent the mean ± s.d. One-way ANOVA with a *post-hoc* Tukey test was performed for comparison of means of groups.

Δ*oprC*, we reasoned that QS system might be affected by the deletion mutation. We then measured expression levels of the genes known to be involved in the QS system. The *oprC*-deficient mutation significantly downregulated the expression of multiple QS system genes: *lasR* ($p = 2.20e\text{-}03$), *lasI* ($p = 2.43e\text{-}03$), *rhlR* ($p = 6.22e\text{-}06$), *rhlI* ($p = 1.09e\text{-}05$), and *rhlAB* ($p = 6.19e\text{-}04$;

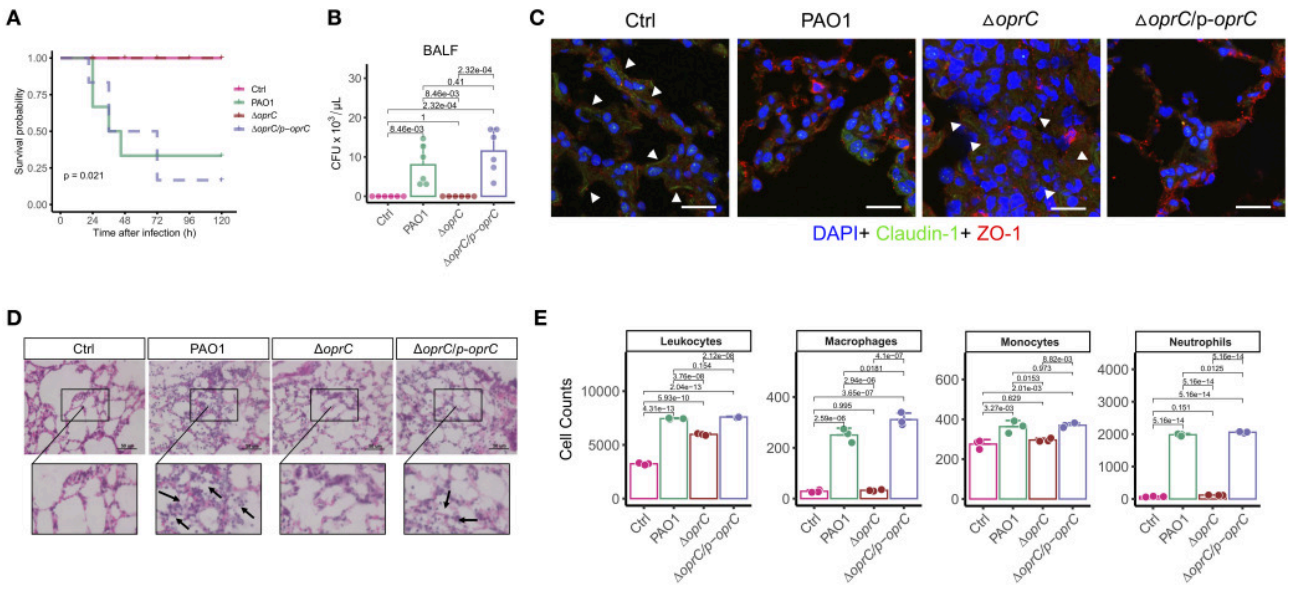

FIGURE 3 | Δ*oprC* infection decreased mouse mortality and lung damage following *P. aeruginosa* infection. **(A)** C57BL6 mice were intranasally challenged with wild type PAO1, Δ*oprC*, and complemented strain at 2 × 10^7 CFU in 25 μL PBS; moribund mice were killed to obtain survival data ($n = 6$). **(B)** Bacterial burdens in the BALF were determined 24 h after bacterial infection ($n = 6$). **(C)** Representative images of immunofluorescence staining of the lungs infected with bacteria for Claudin-1 co-stained with ZO-1 and DAPI ($n = 3$). Arrowheads show the membrane localization of Claudin-1. Scale bars, 20 μm. **(D)** Representative histological views of the lungs of mice 24 h after intranasal infection with bacteria by H&E staining (insets show the enlarged views) ($n = 3$). Arrows show examples of neutrophil infiltration areas. Scale bars, 50 μm. **(E)** Quantification of leukocytes, macrophages, monocytes, and neutrophils numbers per 30,000 cells collected from lung infected with bacteria for 24 h. Error bars represent the mean ± s.d. Kolmogorov-Smirnov test was performed for survival curves analysis. One-way ANOVA with a *post-hoc* Tukey test was performed for comparison of means of groups in other cases.

Figure 2A). In addition, the expression of major virulence genes, such as *exoS, lasB, plcH,* and *toxA*, in the mutant strain was significantly decreased compared to PAO1 (**Figure 2B**). Next, we examined QS regulated virulence factors (38), pyocyanin (PCN), LPS, exoproteases, alginates, and rhamnolipids. PCN, a blue-green pigment mediating tissue damage and necrosis during lung infection, is one of the exotoxins secreted by *P. aeruginosa* (39). PCN secretion was drastically reduced in Δ*oprC* ($p = 6.34e\text{-}07$) compared to PAO1 and was reversed by *oprC* complementation (**Figure 2C**). LPS, also known as lipoglycans and endotoxins, were significantly reduced in the Δ*oprC* strain compared to PAO1 ($p = 5.57e\text{-}03$) and complemented strains ($p = 4.17e\text{-}04$; **Figure 2D**). The release of exoproteases, helping to dismantle the tissue connection (40), showed a similar pattern as shown in **Figure 2E**. Also, the productions of alginates and rhamnolipids (**Supplemental Figures 2A,B**) were decreased in the mutant group. Collectively, these results support that the *oprC* deletion mutant affects virulence regulation and toxin secretion of *P. aeruginosa*.

oprC Deficiency Attenuates Mouse Mortality and Lung Damage Following *P. aeruginosa* Infection

Due to the significant alterations in motility and virulence, we hypothesized that OprC potently affected the host-pathogen interaction. In an acute lung infection model, the *oprC*-deficient mutation completely protected the mice from death after infection compared to the PAO1 and the complemented strain ($p = 0.021$, **Figure 3A**). Mice infected with Δ*oprC* strain showed only lethargy within 12 h post-infection but recovered within 24 h post-infection, resulting in no death. Bacterial burdens were markedly decreased in the Δ*oprC* strain-challenged group compared to the PAO1 group at 24 h post-infection in bronchoalveolar lavage fluid (BALF; $p = 8.46e\text{-}03$), blood ($p = 8.10e\text{-}03$), and lungs ($p = 2.14e\text{-}04$; **Figure 3B** and **Supplemental Figures 3A,B**). In contrast to the Δ*oprC* strain-challenged group, there was no marked difference between PAO1 and complemented groups. As shown in **Figure 3C**, we noticed that change of Claudin-1 in localization from membrane to cytosol hampered the integrity of tight junctions in the PAO1 group (41), suggesting that PAO1 infection caused more severe lung barrier damages than ΔoprC strain. Also, the degree of lung inflammation in the Δ*oprC* strain-infected mice was significantly lower than that in the PAO1-infected mice (**Figure 3D** and **Supplemental Figure 3C**). **Figure 3E** showed more leukocytes, macrophages, monocytes, and neutrophils in PAO1- and Δ*oprC*/p-*oprC* strain-infected lungs. Gating strategies for flow cytometry were shown in **Supplemental Figure 3D**. Overall results demonstrate that *oprC* plays an important role in *P. aeruginosa* lethality in mice.

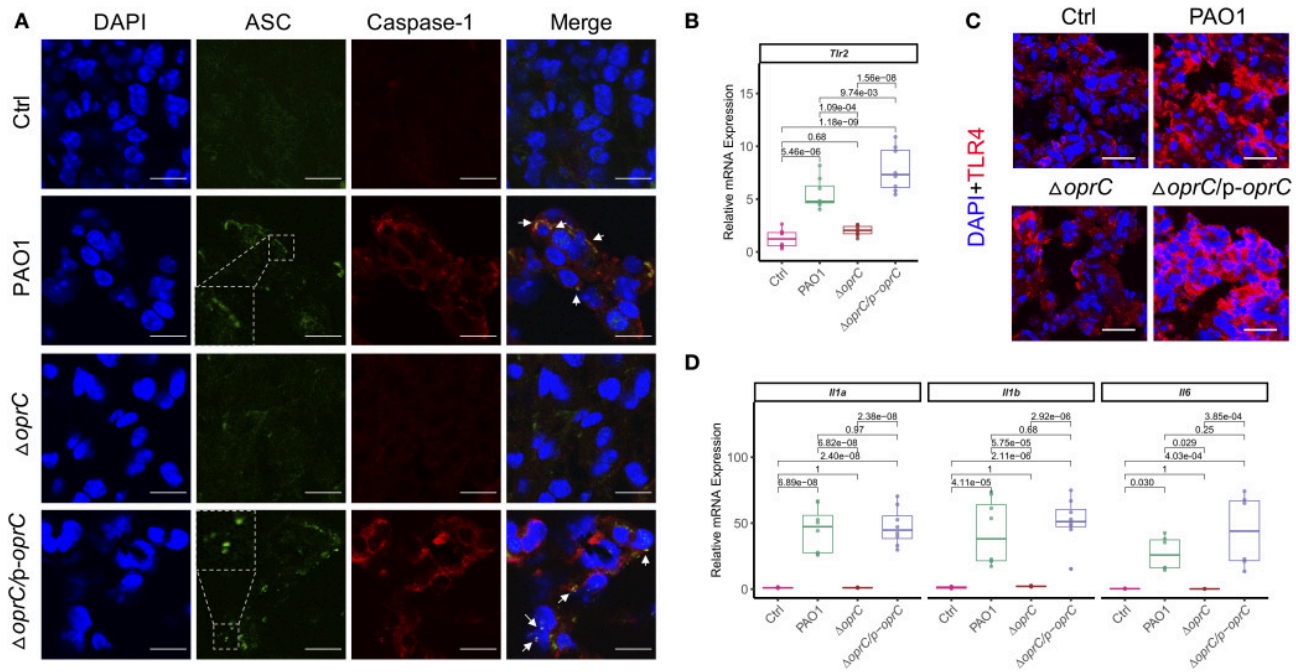

FIGURE 4 | *oprC* deficiency dampened inflammasome formation and proinflammatory cytokines production upon *P. aeruginosa* infection. **(A)** Representative images of immunofluorescence staining of the lungs infected with bacteria for ASC co-stained with caspase-1 and DAPI ($n = 3$). Insets indicate the ASC speck-like structures. Arrows show the colocalization between ASC and caspase-1. Scale bars, 10 μm. **(B)** The RNAs were isolated from the infected lungs using TRIzol and reverse-transcribed into cDNA. The expression of *Tlr2* was assessed by qRT-PCR ($n = 8$). **(C)** Representative images of immunofluorescence staining of the lungs infected with bacteria for TLR4 co-stained with DAPI ($n = 3$–5). Scale bars, 20 μm. **(D)** The RNAs were isolated from the infected lungs using TRIzol and reverse-transcribed into cDNA. The gene expression levels of *Il1a*, *Il1b*, and *Il6* were assessed by qRT-PCR ($n = 8$). Error bars represent the mean ± s.d. One-way ANOVA with a *post-hoc* Tukey test was performed for comparison of means of groups.

oprC Deficiency Dampens Inflammatory Responses After *P. aeruginosa* Infection

NLRP3 and NLRC4 of the NLR family are the most widely studied inflammasomes activated by pathogenic organisms, including *P. aeruginosa* (42, 43). Real-time reverse transcription PCR (qRT-PCR) showed attenuated *Nlrc4* ($p = 5.58e\text{-}06$) and *Nlrp3* ($p = 1.17e\text{-}08$) gene expression in the Δ*oprC* strain-challenged lungs compared to PAO1-challenged lungs, whereas there was no apparent difference between the complemented and PAO1 strain (**Supplemental Figure 4A**). Immunoblotting results demonstrated increased expression of NLRC4, NLRP3, the adaptor protein ASC, pro-caspase-1, and cleaved caspase-1 p10 in PAO1-challenged lungs rather than the Δ*oprC* strain-challenged lungs (**Supplemental Figure 4B**). Moreover, we examined ASC speck formation in the infected lungs. **Figure 4A** showed more ASC specks and colocalizations between ASC and caspase-1 observed in PAO1- and Δ*oprC*/p-*oprC*-challenged lung sections but not in the Δ*oprC* sections, indicating that the inflammasome formation was downregulated by *oprC* deficiency mutation during *P. aeruginosa* infection. TLRs often serve as canonical sensors for various microbial component detection and innate immunity elicitation. TLRs, along with their adaptor proteins, initiate signaling cascades, leading to the activation of nuclear factor-kappa B (NF-κB) controlling the expression of inflammatory cytokine genes. Hence, we assessed *Tlr2* and *Tlr4* mRNA expression in infected and control lung tissue homogenates and found that *Tlr2* expression was markedly suppressed in the Δ*oprC* strain-challenged lungs ($p = 1.09e\text{-}04$; **Figure 4B**), while the gene expression of *Tlr4* was not significantly affected (**Supplemental Figure 4C**). However, the protein expression level of TLR4 was influenced by *oprC* during infection (**Figure 4C** and **Supplemental Figure 4D**). As NF-κB signaling activated by TLRs could initiate the transcription of various inflammatory cytokines, we next examined the expression of various cytokines in infected lungs. The mRNA levels of proinflammatory cytokines, including *Il1a* ($p = 6.82e\text{-}08$), *Il1b* ($p = 5.75e\text{-}05$), *Il6* ($p = 0.029$), *Il23a* ($p = 8.33e\text{-}07$), and *Il12a* ($p = 1.68e\text{-}05$), were significantly downregulated in the Δ*oprC* strain-infected lung tissues compared to the PAO1-infected group (**Figure 4D** and **Supplemental Figure 4E**). These data suggest that the inflammatory responses in the lungs infected with the Δ*oprC* strain were attenuated compared to the PAO1 group.

oprC Deficiency Decreases Pyroptosis and STAT3/NF-κB Phosphorylation Following *P. aeruginosa* Infection

In response to inflammasome activation, GSDMD can be cleaved by caspase-1. The released N-terminal domain oligomerizes

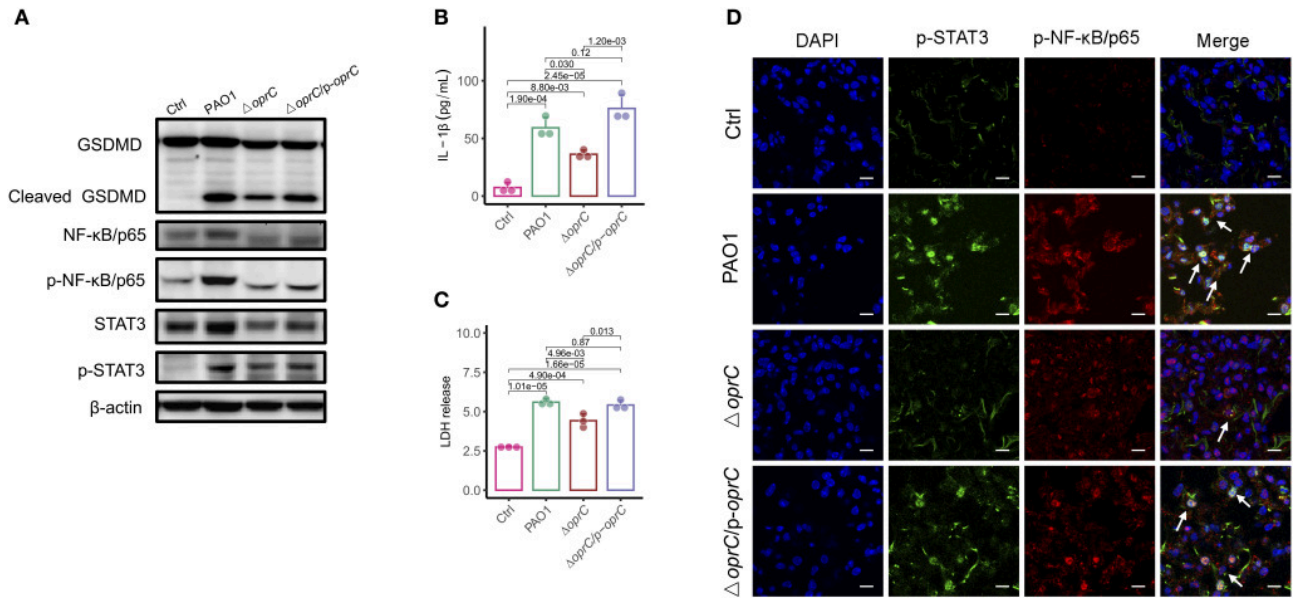

FIGURE 5 | *oprC* deficiency decreased pyroptosis and STAT3/ NF-κB phosphorylation. **(A)** MH-S cells challenged with bacteria at a multiplicity of infection (MOI) of 10 for 2 h. Immunoblotting analysis of GSDMD, NF-κB/p65, p-NF-κB/p65, STAT3, and p-STAT3. **(B,C)** Secreted IL-1β and LDH from the supernatant was assessed by ELISA and LDH assay kit after MH-S cells were infected at an MOI of 10 for 2 h ($n = 3$). **(D)** Representative images of immunofluorescence staining of the lungs infected with bacteria for p-STAT3 co-stained with p-NF-κB/p65 and DAPI ($n = 3$). Arrows indicate the colocalizations between p-STAT3 and p-NF-κB/p65. Scale bars, 10 μm. Error bars represent the mean ± s.d. One-way ANOVA with a *post-hoc* Tukey test was performed for comparison of means of groups.

and creates plasma membrane pores that lead to pyroptosis and secretion of interleukin-1β (IL-1β) (4). We then examined whether the *oprC*-deficient mutation affects GSDMD cleavage and subsequent pyroptosis in MH-S cells (mouse alveolar macrophages). **Figure 5A** shows that infection by the mutant strain still induced GSDMD cleavage and pyroptosis but to a lower extent compared to PAO1 strain infection. Since the loss of membrane integrity results in the release of lactate dehydrogenase (LDH) and IL-1β into the extracellular space (5), we measured the release of IL-1β and LDH in MH-S cells. We found that both IL-1β and LDH in the Δ*oprC* strain-infected group were significantly reduced compared to the PAO1-infected group ($p = 0.030$ and $p = 4.96e\text{-}03$, respectively; **Figures 5B,C**). However, no significant change was observed between the complemented and PAO1 strain-infected groups in IL-1β and LDH release. Immunoblotting also showed decreased cleaved caspase-1 p10 and cleaved IL-1β in Δ*oprC*-infected cells compared to the PAO1 group, which is consistent with the results from the lungs (**Supplemental Figure 5A**). Furthermore, the STAT3/NF-κB signal pathway in the host has been shown to be activated to promote proinflammatory cytokine expression against *P. aeruginosa* infection (44, 45). The protein levels of STAT3 and NF-κB/p65 in MH-S cells infected with Δ*oprC* were markedly decreased (**Figure 5A**). Immunofluorescence staining of the infected lung sections showed that phosphorylation of STAT3 and NF-κB/p65 in the Δ*oprC* strain-infected lungs was not as strong as the PAO1 strain- or complemented strain-infected lungs (**Figure 5D**). We also found more colocalization between p-STAT3 and p-NFκB/p65 in the lungs infected with PAO1 or complemented strain (**Supplemental Figure 4A**). Overall, these results suggest that pyroptosis and STAT3/NF-κB activation during *P. aeruginosa* infection are impaired in Δ*oprC* strain infection.

oprC Deficiency Attenuates Pyroptosis Dependent on Reduced STAT3/NF-κB Activation

To understand how *oprC* affects pyroptosis, we used chemical inhibitor stattic to block STAT3 phosphorylation and dimerization. We found reduced STAT3 phosphorylation, along with declined activation of NF-κB/p65 and GSDMD in the PAO1-infected and complemented-infected groups by the inhibitor, but not in the Δ*oprC* group (**Figure 6A**). Similarly, we used NF-κB inhibitor BAY to validate the data and noticed that BAY inhibited the phosphorylation of NF-κB/p65 and STAT3, as well as the GSDMD cleavage, only in the PAO1-infected and complemented-infected groups (**Figure 6B**). TNF-α is a major cytokine released by bacterial-pathogen-stimulated macrophages. STAT3 inhibitor administration significantly reduced TNF-α secretion in the Δ*oprC* group ($p = 7.4e\text{-}03$), as well as the PAO1 group ($p = 3.4e\text{-}04$) and the complemented group ($p = 5.9e\text{-}04$; **Figure 6C**). NF-κB inhibitor administration also reduced TNF-α secretion in the PAO1 group ($p = 1.3e\text{-}03$) and the complemented group ($p = 1.0e\text{-}03$) but only marginally in the Δ*oprC* group ($p = 0.061$; **Figure 6D**). In addition to TNF-α, phosphorylated NF-κB/p65 promoted expression of proinflammatory cytokines, such as IL-6 and IL-1β. We examined IL-1β secretion in the bacteria-infected MH-S cells, which showed that stattic and BAY pretreatment

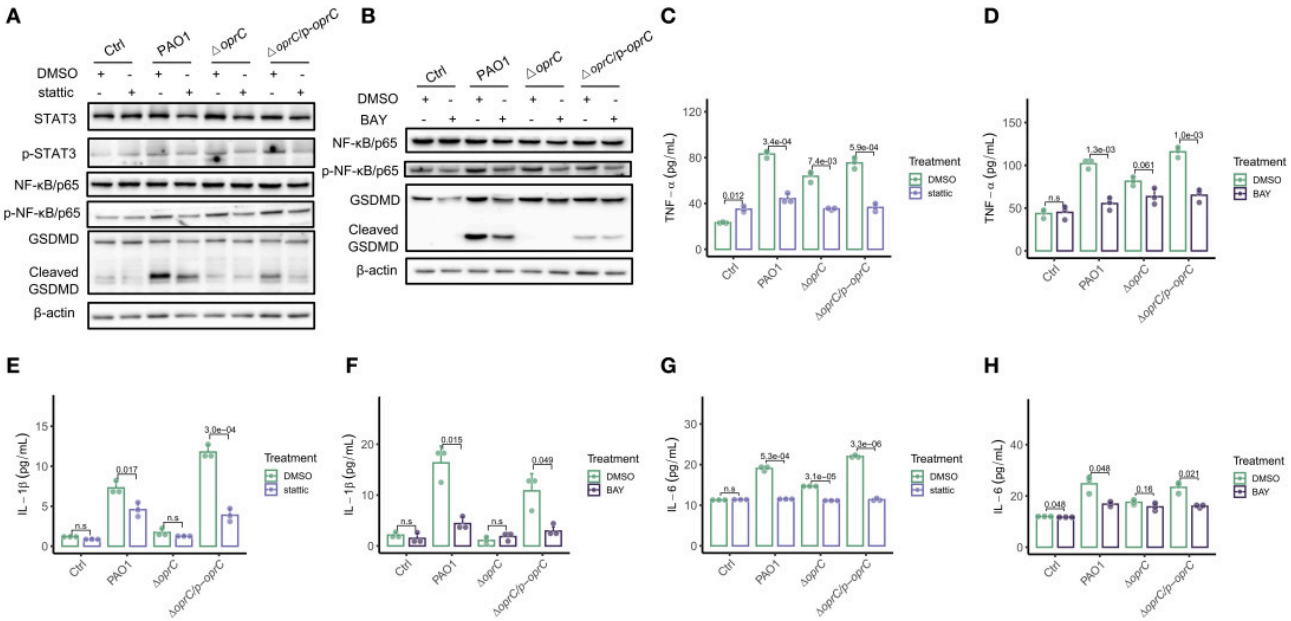

FIGURE 6 | STAT3/NF-κB inhibitors decreased pyroptosis following *P. aeruginosa* infection. MH-S cells pretreated with 10 μm stattic (the inhibitor of Stat3 activation) for 30 min, or BAY (the inhibitor of NF kappa B activation) for 1 h, were challenged with bacteria at a multiplicity of infection (MOI) of 10 for 2 h. Cell lysis and supernatants were used for western blot and ELISA analysis, respectively. **(A,B)** Western blot analysis of STAT3, p-STAT3, NF-κB/p65, p-NF-κB/p65, and GSDMD. **(C–H)** TNF-α, IL-1β, and IL-6 were assessed by ELISA ($n = 3$). Error bars represent the mean ± s.d. One-way ANOVA with a *post-hoc* Tukey test was performed for comparison of means of groups.

drastically decreased IL-1β production in MH-S cells infected with the PAO1 strain ($p = 0.017$ and $p = 0.015$, respectively) or the complemented strain ($p =$ 3.0e-04 and $p = 0.049$, respectively) but not the Δ*oprC* strain (**Figures 6E,F**). The treatment with STAT3 and NF-κB inhibitors decreased the IL-6 secretion in the PAO1-infected group ($p =$ 5.3e-04 and $p =$ 0.048, respectively) and the complemented group ($p =$ 3.3e-06 and $p = 0.021$, respectively) (**Figures 6G,H**). However, upon Δ*oprC* infection, stattic significantly reduced the IL-6 cytokine secretion back to the control level ($p =$ 3.1e-05), while there was no significant difference in the BAY-treated group ($p =$ 0.16). Collectively, these observations demonstrate that *oprC* deficiency attenuates pyroptosis, which is dependent on blunted STAT3/NF-κB activation.

DISCUSSION

Due to the growing antibiotic resistance, *P.* aeruginosa has increasingly become a major concern in hospital-acquired infections. These infections can occur in any part of the body with severe outcomes or death, imposing a heavy medical burden. The infections in the blood and lungs tend to be more severe and lead to pneumonia and/or bacteremia. The therapeutic strategies have been primarily developed based on controlling the critical virulence in order to kill pathogens, thereby reducing virulence, improving host immunity, and rescuing the infected patients.

We observed that the *oprC*-deficient mutation resulted in a change in bacterial motility. Despite no influence on bacteria growth, the *oprC* mutation diminished swarming, swimming, and twitching ability. Both multicellular swarming and individual swimming are bacterial motilities powered by rotating flagella, whereas twitching is mediated by the extension and retraction of type IV pili (37). Previous studies (46–49) showed that these three movements were positively associated with virulence factors, including the type 3 secretion system and its effectors, extracellular proteases, and iron transport.

Considering the important roles of the QS system in bacterial motility (38) and virulence modulation (50), we examined the transcription of two major QS systems, the LasR–LasI system and the RhlR–RhlI system (51). Interestingly, markedly declined expression of QS-associated genes (*lasR*, *lasI*, *rhlR*, and *rhlAB*) and typical virulence genes (*toxA*, *lasB*, *exoS*, and *plcH*) implies that *oprC* may participate in bacterial virulence regulation. Prior studies (52, 53) revealed that PCN is a crucial virulence factor of *P. aeruginosa* in the airway pathogenesis of cystic fibrosis patients. Furthermore, PCN has been shown to significantly enhance LPS-induced IL-1 and TNF-α release by monocytes (54). In this study, we noticed the marked reduction of PCN and LPS secretion in the mutant strain, as well as the further experiment results from exoproteases, alginate, and rhamnolipids, which indicated decreased virulence with *oprC* deficiency.

The changes in bacterial virulence should affect the host-pathogen interaction; however, how OprC impacts the host immune response is not well-known. Critically, our results demonstrated reduced mortality, lung barrier damage, and inflammatory responses in mice infected by the *oprC* deletion mutant. It was established that lung barrier integrity plays a

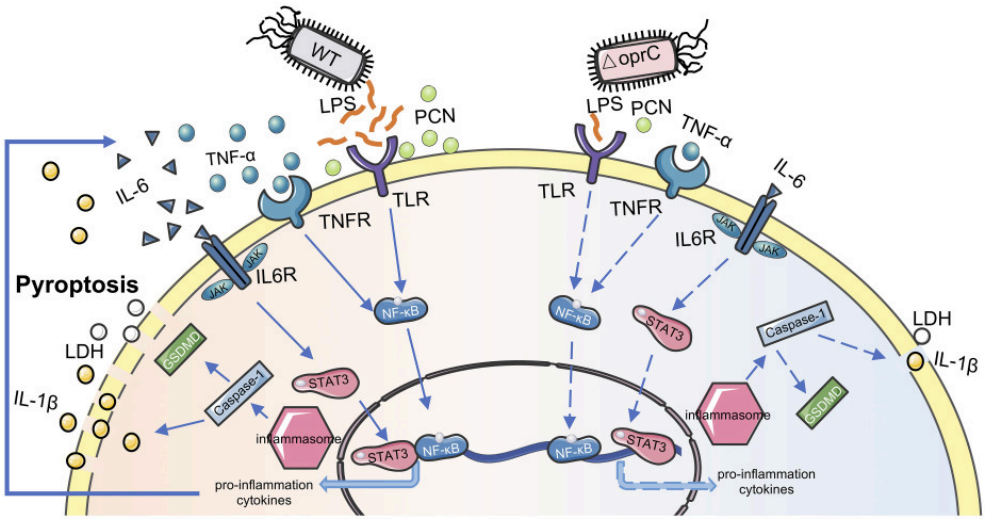

FIGURE 7 | Proposed model for *oprC*-mediated virulence regulation. Compared with *P. aeruginosa* wild type PAO1, less virulent Δ*oprC* secretes less LPS and PCN, attenuating host receptor recognition following transcription factor activation. Less proinflammation cytokine secretion and weaker membrane recognition occur during Δ*oprC* infection. Finally, *oprC* deficiency decreases the pyroptosis induced by *P. aeruginosa*.

critical role in homeostasis and immunity against pathogen invasion (55, 56). Once pathogen invades the host, the PRR will recognize the specific PAMP of the pathogen. The best-studied PRRs are the TLRs for the recognition of PAMPs of *P. aeruginosa*, including LPS, PGN, and flagellin (2). LPS recognition by TLR4 is universally attributed to triggering host defense responses against infection by Gram-negative bacteria, our data here indicated the decreased TLR4 expression in response to the *oprC*-deficient mutation of *P. aeruginosa*. Moreover, the gene or protein expression levels of inflammasomes (NLRP3 and NLRC4) and underpinning proinflammatory cytokines were assessed to probe the participation of inflammatory regulators. Consistent with previous studies (57, 58), both inflammasomes and inflammatory cytokines were activated during PAO1 infection. In contrast, *oprC* deficiency reduced inflammasome activation and the production of proinflammatory cytokines.

Generally, pyroptosis is a kind of cell death mediated by GSDMD. IL-1β and LDH can be released from the pore formed by active GSDMD. Meanwhile, IL-1β secretion is relevant to the inflammasome pathway, JAK/STAT, as well as the NF-κB signaling pathway. Our data showed the activation of GSDMD, along with the phosphorylation of STAT3 and NF-κB, caused by *P. aeruginosa*, but the activation was abolished by the *oprC* deficiency strain infection. We also observed the colocalization of p-STAT3 and p-NF-κB/p65 in PAO1-infected lungs, usually occurring in the cancer cells (59), which reflects potential interaction between STAT3 and NF-κB. NF-κB activated by TLRs can promote cytokine gene transcription, including Il-1β, and as feedback, Il-1β can in turn stimulate NF-κB activation (60). Similarly, IL-6 transcription can be regulated by the transcription factors NF-κB and STAT3. Moreover, IL-6 directly activates STAT3 (61).

The administration of transcription factor inhibitors (stattic and BAY) disrupted the positive loop and reduced the proinflammation cytokine secretion. The secretion of TNF-α, IL-1β, and IL-6 was sharply decreased after inhibitor treatment, while it was only slightly decreased in the Δ*oprC* group. Together with the alleviation of inflammation responses, the reduction of cleaved GSDMD results in the diminution of pyroptosis. Although no direct evidence was provided for STAT/NF-κB facilitating GSDMD transcription, the upregulation of NLRP3 expression by NF-κB-dependent signals (20) supports the activation of GSDMD. Given the liaison between STAT3 and NF-κB (62, 63), blocking the function of either could decrease proinflammatory cytokine production and inhibit an excessive inflammatory storm in the host. *oprC* deficiency attenuates the inflammation response following *P. aeruginosa* infection via STAT3/NF-κB phosphorylation.

In summary, our study illustrates for the first time that OprC, which has recently been implicated in copper influx in *P. aeruginosa*, regulates the critical QS virulence signals and thereby strongly impacts the host immune response. It is not clear how OprC affects the QS, which may be related to copper as copper plays essential roles in cellular homeostasis maintenance as a co-factor for multiple enzymes. Here, our results demonstrate that *oprC* regulates the critical QS virulence signals, leading to a reduction in inflammasome activation, whereas exacerbated inflammatory responses profoundly impact cell viability, lung barrier integrity, tissue injury, and ultimately survival. Lung epithelial barrier is one of the critical mechanisms in preserving homeostasis and protecting immunity against pathogen invasion (55).

We proposed a model for the OprC-mediated virulence regulation and host immune response to *P. aeruginosa* infection (**Figure 7**). OprC triggers TLR signal activation by excessive

LPS secretion, promoting NF-κB activation. Subsequently, with pore-forming protein GSDMD activated by caspase-1, pyroptosis is initiated, which represents rapid plasma-membrane rupture and release of proinflammatory intracellular contents. Cytokines released into the extracellular matrix elicit corresponding receptor recognition and transcription factor (STAT3 and NF-κB) activation. This positive feedback, often occurring after *P. aeruginosa* infection, is abolished under *oprC* deficiency conditions. *oprC* deficiency downregulates *P. aeruginosa* virulence, alleviates infection, and improves inflammation via reduced pyroptosis and STAT3/NF-κB phosphorylation. Importantly, our findings establish the critical virulence activity of *oprC* in physiological relevance in mice, shedding new light on the mechanistic understanding of *P. aeruginosa* pathogenesis and host-pathogen interaction.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the University of North Dakota.

AUTHOR CONTRIBUTIONS

PG, KG, QP, and MW designed the project and wrote the manuscript. PG, KG, QP, ZW, PL, SQ, NK, JH, HL, and MW revised the manuscript. PG performed most of the experiments with the assistance from ZW, QP, SQ, and PL. PG, KG, NK, JH, HL, and MW analyzed data. All authors contributed to the article 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.2020.01696/full#supplementary-material

REFERENCES

1. Ramos JL, Levesque RC. *Pseudomonas*. Boston, MA: Springer. (2006).
2. Cigana C, Lorè NI, Bernardini ML, Bragonzi A. Dampening host sensing and avoiding recognition in *Pseudomonas aeruginosa* pneumonia. *J Biomed Biotechnol.* (2011) 2011:852513. doi: 10.1155/2011/852513
3. Qu W, Wang Y, Wu Y, Liu Y, Chen K, Liu X, et al. Triggering receptors expressed on myeloid cells 2 promotes corneal resistance against *Pseudomonas aeruginosa* by inhibiting caspase-1-dependent pyroptosis. *Front Immunol.* (2018) 9:1121. doi: 10.3389/fimmu.2018.01121
4. Shi J, Gao W, Shao F. Pyroptosis: gasdermin-mediated programmed necrotic cell death. *Trends Biochem Sci.* (2017) 42:245–54. doi: 10.1016/j.tibs.2016.10.004
5. Evavold CL, Ruan J, Tan Y, Xia S, Wu H, Kagan JC. The pore-forming protein gasdermin D regulates interleukin-1 secretion from living macrophages. *Immunity.* (2018) 48:35–44.e6. doi: 10.1016/j.immuni.2017.11.013
6. Zhao Q, Li XZ, Srikumar R, Poole K. Contribution of outer membrane efflux protein OprM to antibiotic resistance in *Pseudomonas aeruginosa* independent of MexAB. *Antimicrob Agents Chemother.* (1998) 42:1682–8. doi: 10.1128/AAC.42.7.1682
7. Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol.* (2011) 2:65. doi: 10.3389/fmicb.2011.00065
8. Cazares A, Moore MP, Hall JPJ, Wright LL, Grimes M, Emond-Rhéault J-G, et al. A megaplasmid family driving dissemination of multidrug resistance in *Pseudomonas*. *Nat Commun.* (2020) 11:1370. doi: 10.1038/s41467-020-15081-7
9. Bassetti M, Vena A, Croxatto A, Righi E, Guery B. How to manage *Pseudomonas aeruginosa* infections. *Drugs Context.* (2018) 7:212527. doi: 10.7573/dic.212527
10. Couto N, Schooling SR, Dutcher JR, Barber J. Proteome profiles of outer membrane vesicles and extracellular matrix of *Pseudomonas aeruginosa* Biofilms. *J Proteome Res.* (2015) 14:4207–22. doi: 10.1021/acs.jproteome.5b00312
11. Yoneyama H, Nakae T. Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. *Microbiology.* (1996) 142:2137–44. doi: 10.1099/13500872-142-8-2137
12. Pérez F,Navarro D,Gimeno C G-D-LJ. Meropenem permeation through the outer membrane of *Pseudomonas aeruginosa* can involve pathways other than the OprD porin channel. *Chemotherapy.* (1996) 42:210–4. doi: 10.1159/000239444
13. Pérez FJ, Navarro D, Gimeno C, Garcia-De-Lomas J. Susceptibility of *Pseudomonas aeruginosa* isolates to ceftazidime is unrelated to the expression of the outer membrane protein OprC. *Chemotherapy.* (1997) 43:27–30. doi: 10.1159/000239531
14. Lian Z, Tianjue Y. Role of outer membrane proteins in imipenem diffusion in *Pseudomonas aeruginosa*. *Chinese Med Sci.* (1999) 14:57–60.
15. Quintana J, Novoa-Aponte L, Argüello JM. Copper homeostasis networks in the bacterium *Pseudomonas aeruginosa*. *J Biol Chem.* (2017) 292:15691–704. doi: 10.1074/jbc.M117.804492
16. Grass G, Rensing C, Solioz M. Metallic copper as an antimicrobial surface. *Appl Environ Microbiol.* (2011) 77:1541–7. doi: 10.1128/AEM.02766-10
17. Han Y, Wang T, Chen G, Pu Q, Liu Q, Zhang Y, et al. A *Pseudomonas aeruginosa* type VI secretion system regulated by CueR facilitates copper acquisition. *PLOS Pathog.* (2019) 15:e1008198. doi: 10.1371/journal.ppat.1008198
18. Li X, He S, Li R, Zhou X, Zhang S, Yu M, et al. *Pseudomonas aeruginosa* infection augments inflammation through MIR-301b repression of c-Myb-mediated immune activation and infiltration. *Nat Microbiol.* (2016) 1:16132. doi: 10.1038/nmicrobiol.2016.132
19. Wu M, Huang H, Zhang W, Kannan S, Weaver A, McKibben M, et al. Host DNA repair proteins in response to *Pseudomonas aeruginosa* in lung epithelial cells and in mice. *Infect Immun.* (2011) 79:75–87. doi: 10.1128/IAI.00815-10
20. Bauernfeind F, Horvath G, Stutz A, Alnemri ES, Speert D, Fernandes-alnemri T, et al. NF-kB activating pattern recognition and cytokine

receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol.* (2010) 183:787–91. doi: 10.4049/jimmunol.0901363
21. Wu M, Pasula R, Smith PA, Martin WJ. Mapping alveolar binding sites *in vivo* using phage peptide libraries. *Gene Ther.* (2003) 10:1429–36. doi: 10.1038/sj.gt.3302009
22. Kannan S, Audet A, Huang H, Chen L, Wu M. Cholesterol-rich membrane rafts and Lyn are involved in phagocytosis during *Pseudomonas aeruginosa* infection. *J Immunol.* (2008) 180:2396–408. doi: 10.4049/jimmunol.180.4.2396
23. Pu Q, Gan C, Li R, Li Y, Tan S, Li X, et al. Atg7 deficiency intensifies inflammasome activation and pyroptosis in *Pseudomonas sepsis*. *J Immunol.* (2017) 198:3205–13. doi: 10.4049/jimmunol.1601196
24. Lin P, Pu Q, Wu Q, Zhou C, Wang B, Schettler J, et al. High-throughput screen reveals sRNAs regulating crRNA biogenesis by targeting CRISPR leader to repress Rho termination. *Nat Commun.* 10:3728. doi: 10.1038/s41467-019-11695-8
25. Zhang Y, Zhou C, Pu Q, Wu Q, Tan S, Shao X, et al. *Pseudomonas aeruginosa* regulatory protein AnvM controls pathogenicity in anaerobic environments and impacts host defense. *MBio.* (2019) 10:e01362-19. doi: 10.1128/mBio.01362-19
26. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, et al. An official American thoracic society workshop report: Features and measurements of experimental acute lung injury in animals. *Am J Resp Cell Mol Biol.* 44:725–38. doi: 10.1165/rcmb.2009-0210ST
27. Zhao J, Yu X, Zhu M, Kang H, Ma J, Wu M, et al. Structural and molecular mechanism of CdpR involved in quorum-sensing and bacterial virulence in *Pseudomonas aeruginosa*. *PLOS Biol.* (2016) 14:e1002449. doi: 10.1371/journal.pbio.1002449
28. Yeung ATY, Torfs ECW, Jamshidi F, Bains M, Wiegand I, Hancock REW, et al. Swarming of *Pseudomonas aeruginosa* is controlled by a broad spectrum of transcriptional regulators, including MetR. *J Bacteriol.* (2009) 191:5592–602. doi: 10.1128/JB.00157-09
29. Nicodeme M, Grill J-P, Humbert G, Gaillard JL. Extracellular protease activity of different *Pseudomonas* strains: dependence of proteolytic activity on culture conditions. *J Appl Microbiol.* (2005) 99:641–8. doi: 10.1111/j.1365-2672.2005.02634.x
30. Limoli DH, Whitfield GB, Kitao T, Ivey ML, Davis MR, Grahl N, et al. *Pseudomonas aeruginosa* alginate overproduction promotes coexistence with *Staphylococcus aureus* in a model of cystic fibrosis respiratory infection. *MBio.* (2017) 8:e00186-17. doi: 10.1128/mBio.00186-17
31. Rasamiravaka T, Vandeputte O, Jaziri M. Procedure for rhamnolipids quantification using methylene-blue. *Bio-Protocol.* (2016) 6:1783. doi: 10.21769/BioProtoc.1783
32. Kollaran AM, Joge S, Kotian HS, Badal D, Prakash D, Mishra A, et al. Context-specific requirement of forty-four two-component loci in *Pseudomonas aeruginosa* swarming. *iScience.* (2019) 13:305–17. doi: 10.1016/j.isci.2019.02.028
33. Daniels R, Vanderleyden J, Michiels J. Quorum sensing and swarming migration in bacteria. *FEMS Microbiol Rev.* (2004) 28:261–89. doi: 10.1016/j.femsre.2003.09.004
34. Köhler T, Curty LK, Barja F, van Delden C, Pechère JC. Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol.* (2000) 182:5990–6. doi: 10.1128/JB.182.21.5990-5996.2000
35. Li Y, Qu H-P, Liu J-L, Wan H-Y. Correlation between group behavior and quorum sensing in *Pseudomonas aeruginosa* isolated from patients with hospital-acquired pneumonia. *J Thorac Dis.* (2014) 6:810–7. doi: 10.3978/j.issn.2072-1439.2014.03.37
36. Rutherford ST, Bassler BL. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harb Perspect Med.* (2012) 2:a012427. doi: 10.1101/cshperspect.a012427
37. Kearns DB. A field guide to bacterial swarming motility. *Nat Rev Microbiol.* (2010) 8:634–44. doi: 10.1038/nrmicro2405
38. Turkina M V., Vikström E. Bacteria-host crosstalk: sensing of the quorum in the context of *Pseudomonas aeruginosa* infections. *J Innate Immun.* (2019) 11:263–79. doi: 10.1159/000494069
39. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D. *Pseudomonas aeruginosa* pyocyanin is critical for lung infection in mice. *Infect Immun.* (2004) 72:4275–8. doi: 10.1128/IAI.72.7.4275-4278.2004
40. Beaufort N, Corvazier E, Mlanaoindrou S, de Bentzmann S, Pidard D. Disruption of the endothelial barrier by proteases from the bacterial pathogen *Pseudomonas aeruginosa*: implication of matrilysis and receptor cleavage. *PLoS ONE.* (2013) 8:e75708. doi: 10.1371/journal.pone.0075708
41. Soini Y. Claudins in lung disease. *Respir Res.* (2011) 12:70. doi: 10.1186/1456-9921-12-70
42. Lindestam Arlehamn CS, Evans TJ. *Pseudomonas aeruginosa* pilin activates the inflammasome. *Cell Microbiol.* (2011) 13:388–401. doi: 10.1111/j.1462-5822.2010.01541.x
43. Deng Q, Wang Y, Zhang Y, Li M, Li D, Huang X, et al. *Pseudomonas aeruginosa* triggers macrophage autophagy to escape intracellular killing by activation of the NLRP3 inflammasome. *Infect Immun.* (2015) 84:56–66. doi: 10.1128/IAI.00945-15
44. Li R, Fang L, Pu Q, Lin P, Hoggarth A, Huang H, et al. Lyn prevents aberrant inflammatory responses to pseudomonas infection in mammalian systems by repressing a SHIP-1-associated signaling cluster. *Signal Transduct Target Ther.* (2016) 1:16032. doi: 10.1038/sigtrans.2016.32
45. Yuan K, Huang C, Fox J, Gaid M, Weaver A, Li G, et al. Elevated inflammatory response in caveolin-1-deficient mice with *Pseudomonas aeruginosa* infection is mediated by STAT3 protein and nuclear factor κB (NF-κB). *J Biol Chem.* (2011) 286:21814–25. doi: 10.1074/jbc.M111.237628
46. Verstraeten N, Braeken K, Debkumari B, Fauvart M, Fransaer J, Vermant J, et al. Living on a surface: swarming and biofilm formation. *Trends Microbiol.* (2008) 16:496–506. doi: 10.1016/j.tim.2008.07.004
47. Josenhans C, Suerbaum S. The role of motility as a virulence factor in bacteria. *Int J Med Microbiol.* (2002) 291:605–14. doi: 10.1078/1438-4221-00173
48. Overhage J, Bains M, Brazas MD, Hancock REW. Swarming of *Pseudomonas aeruginosa* is a complex adaptation leading to increased production of virulence factors and antibiotic resistance. *J Bacteriol.* (2008) 190:2671–9. doi: 10.1128/JB.01659-07
49. Newman JW, Floyd R V, Fothergill JL. The contribution of *Pseudomonas aeruginosa* virulence factors and host factors in the establishment of urinary tract infections. *FEMS Microbiol Lett.* (2017) 364:fnx124. doi: 10.1093/femsle/fnx124
50. Lee J, Zhang L. The hierarchy quorum sensing network in *Pseudomonas aeruginosa*. *Protein Cell.* (2014) 6:26–41. doi: 10.1007/s13238-014-0100-x
51. Schuster M, Greenberg EP. A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol.* (2006) 296:73–81. doi: 10.1016/j.ijmm.2006.01.036
52. Gao L, Zhang Y, Wang Y, Qiao X, Zi J, Chen C, et al. Reduction of PCN biosynthesis by NO in *Pseudomonas aeruginosa*. *Redox Biol.* (2016) 8:252–8. doi: 10.1016/j.redox.2015.10.005
53. Caldwell CC, Chen Y, Goetzmann HS, Hao Y, Borchers MT, Hassett DJ, et al. *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis. *Am J Pathol.* (2009) 175:2473–88. doi: 10.2353/ajpath.2009.090166
54. Ulmer AJ, Pryjma J, Tarnok Z, Ernst M, Flad HD. Inhibitory and stimulatory effects of *Pseudomonas aeruginosa* pyocyanine on human T and B lymphocytes and human monocytes. *Infect Immun.* (1990) 58:808–15. doi: 10.1128/IAI.58.3.808-815.1990
55. Crane MJ, Lee KM, FitzGerald ES, Jamieson AM. Surviving deadly lung infections: innate host tolerance mechanisms in the pulmonary system. *Front Immunol.* (2018) 9:1421. doi: 10.3389/fimmu.2018.01421
56. Palazon-Riquelme P, Lopez-Castejon G. The inflammasomes, immune guardians at defence barriers. *Immunology.* (2018) 155:320–330. doi: 10.1111/imm.12989
57. Balakrishnan A, Karki R, Berwin B, Yamamoto M, Kanneganti TD. Guanylate binding proteins facilitate caspase-11-dependent pyroptosis in response to type 3 secretion system-negative *Pseudomonas aeruginosa*. *Cell Death Discov.* (2018) 4:66. doi: 10.1038/s41420-018-0068-z
58. Faure E, Mear J-B, Faure K, Normand S, Couturier-Maillard A, Grandjean T, et al. *Pseudomonas aeruginosa* type-3 secretion system dampens host defense by exploiting the NLRC4-coupled inflammasome. *Am J Respir Crit Care Med.* (2014) 189:799–811. doi: 10.1164/rccm.201307-1358OC

59. Kesanakurti D, Chetty C, Maddirela DR, Gujrati M, Rao JS. Essential role of cooperative NF-κB and Stat3 recruitment to ICAM-1 intronic consensus elements in the regulation of radiation-induced invasion and migration in glioma. *Oncogene.* (2013) 32:5144–55. doi: 10.1038/onc.2012.546
60. Yamazaki K, Gohda J, Kanayama A, Miyamoto Y, Sakurai H, Yamamoto M, et al. Two mechanistically and temporally distinct NF-κB activation pathways in IL-1 signaling. *Sci Signal.* (2009) 2:ra66. doi: 10.1126/scisignal.2000387
61. Wang Y, Van Boxel-Dezaire AHH, Cheon H, Yang J, Stark GR. STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor. *Proc Natl Acad Sci USA*. (2013) 110:16975–80. doi: 10.1073/pnas.1315862110
62. Grivennikov SI, Karin M. Dangerous liaisons: STAT3 and NF-κB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev.* (2010) 21:11–19. doi: 10.1016/j.cytogfr.2009.11.005
63. Quinton LJ, Mizgerd JP. NF-κB and STAT3 signaling hubs for lung innate immunity. *Cell Tissue Res.* (2011) 343:153–65. doi: 10.1007/s00441-010-1044-y

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