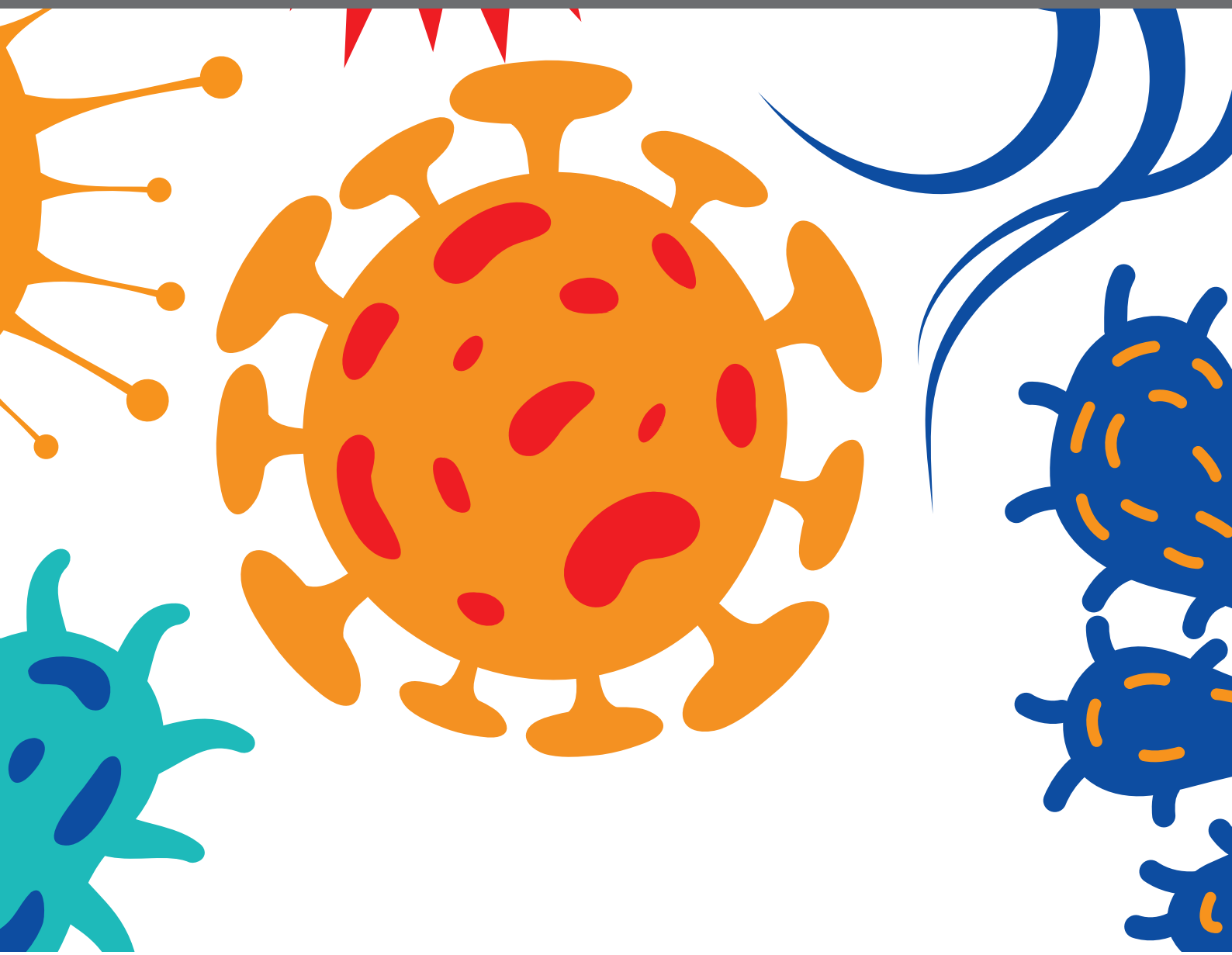
HOST-PATHOGEN INTERACTION DURING PNEUMOCOCCAL INFECTIONS

EDITED BY: Masaya Yamaguchi, Yuki Kinjo and Victor Nizet
PUBLISHED IN: Frontiers in Cellular and Infection Microbiology



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ISSN 1664-8714
ISBN 978-2-88971-888-7
DOI 10.3389/978-2-88971-888-7

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HOST-PATHOGEN INTERACTION DURING PNEUMOCOCCAL INFECTIONS

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Citation: Yamaguchi, M., Kinjo, Y., Nizet, V., eds. (2021). Host-pathogen Interaction During Pneumococcal Infections. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-888-7

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frontiers in Cellular and Infection Microbiology

EDITORIAL
published: 25 October 2021
doi: 10.3389/fcimb.2021.752959

Editorial: Host-Pathogen Interactions During Pneumococcal Infection

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Keywords: ***Streptococcus pneumoniae*, host immunity, virulence factor, inflammation, recognition mechanism**

Editorial on the Research Topic

Host-Pathogen Interaction During Pneumococcal Infections

OPEN ACCESS

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *04 August 2021*
Accepted: *11 October 2021*
Published: *25 October 2021*

Citation:
Yamaguchi M, Kinjo Y and Nizet V (2021) Editorial: Host-Pathogen Interactions During Pneumococcal Infection. Front. Cell. Infect. Microbiol. 11:752959. doi: 10.3389/fcimb.2021.752959

Streptococcus pneumoniae, a Gram-positive bacterium belonging to the mitis group, colonizes the human nasopharynx and is a leading cause of community-acquired pneumonia, meningitis, and bacteremia worldwide. Although approximately 20-40% of children carry this organism in their nasopharynx without clinical symptoms, *S. pneumoniae* is estimated to be responsible for the deaths of approximately 1,190,000 people annually from lower respiratory infections (Bogaert et al., 2004; Otsuka et al., 2013; GBD 2016 Lower Respiratory Infections Collaborators, 2018). In addition, antibiotic selective pressure has caused resistant pneumococcal clones to emerge and expand throughout the world, prompting the World Health Organization to list *S. pneumoniae* as one of 12 priority antibiotic-resistant pathogens (WHO, 2017). Data from active bacterial core surveillance obtained from 2009 to 2013 and presented by the U.S. Centers for Disease Control indicate that widespread availability of pneumococcal conjugate vaccines is effective in countering emerging antibiotic resistance (CDC, 2019). However, while such vaccines target major pneumococcal capsular serotypes, only a subset of the 100 different known serotypes are covered, applying a selective pressure for niche replacement such that the prevalence of non-vaccine serotypes of *S. pneumoniae* has been increasing worldwide (Golubchik et al., 2012). To establish novel effective control strategies, it is important to elucidate the detailed pathogenic process of pneumococcal infection.

For this *Frontiers in Cellular and Infection Microbiology* Research Topic, we collected 13 papers, including six original research studies, six review (full and mini) papers, and one perspective. Two review papers and two original studies describing fundamental microbiological characteristic of *S. pneumoniae* related to infection serve as an excellent starting point. Sanchez-Rosario and Johnson described differences between historical and modern pneumococcal growth media, highlighting key components that are necessary for cultivation/growth enhancement and their effects on bacterial phenotypes and experimental outcomes. Such precise knowledge of media used for bacterial growth is essential for pneumococcal investigators to improve the quality and comparability of ongoing research. Luck et al. reviewed the role of the pneumococcal polysaccharide capsule related to pathogenesis and the particular importance of serotype 3, with focus on capsule synthesis,

localization, and biochemical and physiological properties, as well as mechanisms of vaccine escape. On the topic of serotype replacement by pneumococcal polysaccharide vaccines, immunogenic protein antigens are being explored as an alternative to multivalent polysaccharide vaccines, with the pneumococcal surface protein A (PspA) protein a particularly promising vaccine antigen candidate (Briles et al., 2019). PspA proteins are classified into three families that are further discriminated into six clades (Hollingshead et al., 2000). In analysis of 1,939 strains isolated from cases of adult invasive pneumococcal disease in Japan, Chang et al. determined the PspA clades of 1,928 and identified new PspA clades for another four. Those findings can help in guiding the design of effective multi-valent PspA vaccines in the near future. Lastly, Ali et al. examined the role of the pneumococcal serine proteases HtrA, PrtA, SFP, and CbpG in human epithelial cell adherence and mouse nasopharyngeal colonization. Their results showed that protease deficiency reduced pneumococcal colonization, suggesting this family of virulence determinants may be useful therapeutic targets to prevent colonization and transmission.

Next, one perspective, one mini-review, and one original article that centered on novel aspects of pneumococcal host-pathogen interactions are included. Tuomanen reported that three leading bacteria agents of childhood meningitis, *S. pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*, share a similar strategy for invasion of the blood-brain barrier endothelium including utilization of the host platelet-activating factor (PAF) and laminin receptors. This commonality in pathogenic mechanism may provide a template for development of a broadly cross-protective meningitis vaccine. As part of the infectious process, host neutrophils play important roles as part of the first line of defense against pathogens (Kinjo et al., 2011; Dohrmann et al., 2016). However, excessive neutrophil activation causes destruction of host tissues. Domon and Terao summarized the way how neutrophilic inflammation can have detrimental effects in pneumococcal pneumonia, with a particular focus on neutrophil elastase, which cleaves a variety of host proteins to cause lung injury and barrier compromise. *S. pneumoniae* can degrade, transport, and metabolize various host glycans (Hobbs et al., 2018). The pneumococcal sialidase NanA is a multi-functional virulence factor shown to contribute to otitis media, meningitis, and exaggerated inflammatory responses in small animal infection models (Tong et al., 2000; Uchiyama et al., 2009; Chang et al., 2012). Evolutionary analyses reveal the encoding pneumococcal *nanA* gene is under considerable negative selection pressure (Yamaguchi et al., 2016; Yamaguchi et al., 2019; Yamaguchi et al., 2020). In our Research Topic, Tseng et al. reported a novel virulence role of NanA in pneumococcal pathogenesis, in which the desialylation it causes impairs the interaction of sialic acid-binding Ig-like lectin 5 (Siglec-5) and Toll-like receptor 2 (TLR-2), provoking excessive inflammation and cytotoxicity in infected macrophages.

Two reviews, a mini-review, and a research article explored clinicopathological correlates of disease outcome in pneumococcal infection. Increased mortality risk in pneumococcal infection can be seen in patients that experience an imbalance of cytokine responses and heightened inflammatory status (Franceschi and Campisi, 2014; Ferrucci and Fabbri, 2018). The details of this state were described by Weight et al. along with analysis of corresponding mucosal epithelial and innate immune alterations, highlighting how immunologic changes of aging strongly increase risk of adverse disease outcome. Co-infection with influenza virus is another major factor that increases the potential for severe or lethal pneumococcal infection. Sender et al. described how a preceding influenza infection modulates host immune response and how *S. pneumoniae* senses and adapts to the modified environment. Influenza virus co-infection also enhances pneumococcal transmission. Morimura et al. summarized that relationship and introduce the remarkable utility of new animal models to characterize this key factor of pneumococcal infection that poses a serious threat to public health. Finally, Murakami et al. demonstrated that cigarette smoke exposure significantly promotes pneumococcal transmission by enhancing bacterial shedding from the host, increasing the likelihood of pneumococcal colonization in contacts. This study was the first to experimentally demonstrate the importance of an environmental factor (cigarette smoke) for pneumococcal transmission.

Finally, two original papers that reported novel therapeutic candidates were reviewed. In a "drugs from bugs" approach, Li et al. showed that stimulation of macrophages with *S. pneumoniae* endopeptidase O (PepO), a multifunctional pneumococcal virulence protein, enhanced phagocytic clearance of *Staphylococcus aureus* and *S. pneumoniae*. The work by Nakakubo et al. showed that Hochu-Ekki-to, a traditional Japanese herbal medicine cocktail, accelerates clearance of pneumococcal colonization through macrophage activation and increased IL-17A production. To best address infectious challenges in the era of ever increasing antibiotic resistance, it will be necessary to continue to explore such innovative immune boosting therapeutic strategies.

In sum, the 13 papers of our Research Topic provide an exciting addition to the literature on host-pathogen interactions during pneumococcal infection, including a number of important new scientific findings and thoughtful synthesis of emerging paradigms. We hope these contributions will help investigators in the field continue to propel forward the science and clinical therapeutics of this foremost of human bacterial pathogens.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contributions to the work, and approved the final version for publication.

ACKNOWLEDGMENTS

We sincerely thank all of the authors and reviewers who contributed to our Research Topic under the difficult COVID-19 situation.

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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 17 July 2020
doi: 10.3389/fcimb.2020.00358

Streptococcus pneumoniae Endopeptidase O Promotes the Clearance of *Staphylococcus aureus* and *Streptococcus pneumoniae* via SH2 Domain-Containing Inositol Phosphatase 1-Mediated Complement Receptor 3 Upregulation

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 24 April 2020
Accepted: 10 June 2020
Published: 17 July 2020

Citation:
Li S, Zhang H, Xiao J, Yuan T, Shu Z, Min Y, Xu W, Yin Y and Zhang X (2020) Streptococcus pneumoniae Endopeptidase O Promotes the Clearance of Staphylococcus aureus and Streptococcus pneumoniae via SH2 Domain-Containing Inositol Phosphatase 1-Mediated Complement Receptor 3 Upregulation. Front. Cell. Infect. Microbiol. 10:358. doi: 10.3389/fcimb.2020.00358

Increasing evidences demonstrate that microorganism and their products protect against bacterial and viral pathogens through various mechanisms including immunomodulation. *Streptococcus pneumoniae* endopeptidase O (PepO), a pneumococcal virulence protein, has been proven to enhance the phagocytosis of *Staphylococcus aureus* and *Streptococcus pneumoniae* by macrophages in our previous study, where we detected the down regulation of SH2 domain-containing inositol phosphatase 1 (SHIP1) and the up regulation of complement receptor 3 (CR3) in PepO-stimulated macrophages. In the present study, using SHIP1 over-expression plasmid and CR3 siRNA, we proved that the down regulation of SHIP1 and the up regulation of CR3 mediate the enhanced phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-stimulated macrophages. The down regulation of SHIP1 also mediates the up regulation of CR3. To further determine whether PepO protects against respiratory pathogens, we constructed a mouse model with intranasal infection of *S. aureus* or *S. pneumoniae* and found that PepO significantly promoted their clearance. The down regulation of SHIP1 and the up regulation of CR3 also play a role in this process. This study provides a new preventive and therapeutic option for respiratory infectious diseases and lays the theoretical basis for the development of PepO as an immunomodulation agent.

Keywords: PepO, SHIP1, CR3, *S. aureus*, *S. pneumoniae*

INTRODUCTION

Infectious diseases account for 6 in 10 threats to global health in 2019 according to the world health organization (WHO), among which antimicrobial resistance has aroused increasing attention. Antibiotic therapy has rescued millions of lives and markedly reduced morbidity and mortality worldwide. Paradoxically, antibiotic therapy simultaneously increases susceptibility to a range of infection and colonization of antimicrobial-resistant pathogens (Buffie and Pamer, 2013; Ng et al., 2013; Theriot et al., 2014; Schubert et al., 2015; Langdon et al., 2016; Fjalstad et al., 2018). To combat antimicrobial-resistant pathogens, many approaches including limits on antibiotic use and development of more effective antibiotics have been implemented. However, the antimicrobial resistance is still growing. There is an urgent need to develop new and more effective ways to combat antimicrobial-resistant pathogens.

An exciting discovery is that administration of protective commensal bacterial species shows potential to reduce antimicrobial-resistant infections (Pamer, 2016; Thiemann et al., 2017; Keith and Pamer, 2019). Several studies have suggested that reestablishment with normal intestinal microbiota reduces colonization by vancomycin-resistant enterococci (VRE), antimicrobial-resistant *Klebsiella pneumoniae* and *Escherichia coli* (Ubeda et al., 2013; Singh et al., 2014; Caballero et al., 2015; Stripling et al., 2015). Many studies have shown that administration of several commensal bacterial species protects against *Clostridium difficile* infection (van Nood et al., 2013; Buffie et al., 2015; Lewis and Pamer, 2017; Deng et al., 2019). However, there are many concerns about developing the live microorganism into preventive or therapeutic agents for their potential pathogenicity, possibility of acquiring antibiotic resistance, and difficult guarantee of purity, uniformity, and effectiveness (Pamer, 2016; Zitvogel et al., 2017). Identifying a single component owing the beneficial effects of live microorganism may be the solution to the above problems (Zitvogel et al., 2017). Several studies have proven that some microbial products protect against viral and bacterial pathogens through various mechanisms including immunomodulation (Steed et al., 2017; Webster et al., 2017; Jacobson et al., 2018).

Streptococcus pneumoniae endopeptidase O (PepO) is a ubiquitously expressed pneumococcal virulence protein (Agarwal et al., 2013). Our previous work has proven that PepO enhances the phagocytic function of macrophages in a miR-155 dependent manner (Yao et al., 2017), and macrophages play an important role in the clearance of respiratory pathogens (Lovewell et al., 2014; Byrne et al., 2015; Eichinger et al., 2015; Lemon et al., 2015), indicating that PepO may protect against respiratory pathogens partially through immunomodulation. Even so, the exact molecular mechanisms involved in this process are still unclear. In the previous study, we detected the down regulation of SH2 domain-containing inositol phosphatase 1 (SHIP1) in PepO-stimulated macrophages and proved that SHIP1 down regulation was targeted by miR-155. Several studies have shown that SHIP1 negatively regulates the phagocytic function of macrophages via various mechanisms including inhibiting the release of proinflammatory cytokines and degrading phosphatidylinositol-3,4,5,-trisphosphate at the phagocytic cup (Horan et al., 2007; Cremer et al., 2009). However, whether SHIP1 down regulation is correlated with the phagocytosis by PepO-stimulated macrophages and the related mechanisms remain to be proven.

SHIP1 has been proven to inhibit phagocytic activity mediated by complement receptor 3 (CR3) (Horan et al., 2007). However, we detected the increased expression of CR3 in PepO-stimulated macrophages. Therefore, we speculated that SHIP1 may participate in the regulation of phagocytosis by PepO-stimulated macrophages via modulating CR3 expression level. To test this speculation, we transfected macrophages with pHBLV-CMV-SHIP1 plasmid for over-expression of SHIP1 or with CR3 siRNA for knock down of CR3 and then explored the effect of PepO on these cells' phagocytic function. In the present study, we showed that the enhanced phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-stimulated macrophages was mediated by the down regulation of SHIP1 and the up regulation of CR3. SHIP1 down regulation also mediated CR3 up regulation. To determine whether PepO protects against respiratory pathogens, we constructed a mouse model with intranasal infection of *S. aureus* or *S. pneumoniae* and found that PepO significantly promoted their clearance. The enhanced clearance of *S. aureus* and *S. pneumoniae* also correlated with the down regulation of SHIP1 and the up regulation of CR3. This study provides a new preventive and therapeutic option for respiratory infectious diseases and lays the theoretical basis for the development of PepO as an immunomodulation agent.

MATERIALS AND METHODS

Mice

Specific-pathogen-free male and female, 6–8 weeks old C57BL/6 mice were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China) and maintained at Chongqing Medical University. All mice were maintained with sterile water and mouse chow *ad libitum* under barrier conditions. All experimental procedures were approved by the Ethics Committee of Chongqing Medical University.

Bacterial Strains

D39, a standard strain of *S. pneumoniae*, was purchased from the American Type Culture Collection (ATCC). A standard strain of *S. aureus* numbered ATCC 29213 was obtained from the Children's Hospital of Chongqing Medical University. The strains were seeded on the Columbia sheep blood agar (PANGTONG, Chongqing, China) and cultured in 5% CO_2 at 37°C overnight. Then the bacteria were suspended in phosphate buffer solution (PBS) with a final OD_{600} value of 0.5.

Preparation of PepO Protein

Preparation of PepO protein has been described in details previously (Yao et al., 2017). The Ni^{2+}-charged column chromatograph used for PepO purification was purchased from GE Healthcare (Buckinghamshire, United Kingdom). Polymyxin B agarose used for lipopolysaccharide (LPS) removal was purchased from Genscript Corp. (New Jersey, USA). The PepO

TABLE 1 | Primers used in this study.

Primer name	Nucleotide sequence (5′-3′)
SHIP1 forward	ACTTTGCTGGAGTGTCCGT
SHIP1 reverse	TTGGGCAGAATCCTGTAAG
CR3 forward	GAGGCCCCCAGGACTTTAAC
CR3 reverse	CTTCTTGGTGAGCGGGTTCA
si SHIP1	UGCAAGAAGUCACCAGCAU
	AUGCUGGUGACUUCUUGCA
si CR3	GCAUCACCAUGAGUGCCAU
	AUGGCACUCAUGGUGAUGC

preparation contained no detectable LPS when it was detected by the Limulus Amoebocyte Lyase assay, and the concentrations of rPepO preparation were determined by BCA assay.

Cell Culture

Four days after intraperitoneal injection of 1 ml paroline, 3–5 male C57BL/6 mice were sacrificed for peritoneal exudate macrophages (PEMs) isolation. Male mice were used to collect more cells. The cells were harvested by peritoneal lavage with 13 ml sterile PBS containing 5 mM EDTA. After centrifugation and washing with Dulbecco's modified Eagle's medium (DMEM) (HyClone, Barrington, IL, USA), the total viable cell numbers were determined with the use of a Neubauer chamber and 5% trypan blue solution. Then the cells were seeded on 6-well cell culture plate (8×10^6/well) or 24-well cell culture plate (5×10^5/well) and cultured in 5% CO_2 at 37°C for 1 h. After removal of suspension cells, the remaining adherent cells were cultured in DMEM supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin-streptomycin (HyClone, Barrington, IL, USA) and treated as described below. Flow cytometry analysis was used to determine the purity of cultured cells. The results showed that the percentage of macrophages was above 90% (data not shown), so we did not detect its purity in the latter experiments.

Cell Transfection Assay

The siRNAs (siSHIP1, siCR3, and scrambled siRNA) were purchased from Chongqing LaiBoSi Biotechnology Co., Ltd. (Chongqing, China). Their sequences are listed in **Table 1**. Plasmids pHBLV-CMV-SHIP1 and pHBLV-CMV-MCS were purchased from HanHeng Biotechnology Co., Ltd. (Shanghai, China). PEMs were transfected with siRNA or plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacture's instruction. After incubation of siRNA or plasmid DNA with Lipofectamine 2000 in appropriate proportions and fixed volumes for 20 min, the liposome-DNA mixture was added into PEMs cultured in serum-free and antibiotic-free DMEM. Five hours later, the cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin for another 24 h.

Quantitative PCR (Q-PCR) Analysis

Total RNA was extracted from transfected PEMs with the use of RNAiso Plus reagent (TaKaRa) and reversely transcribed into cDNA with the use of PrimeScript RT reagent Kit (TaKaRa) according to the manufacture's instruction. For quantitative analysis, the mixture of cDNA, primers, and TB Green premix EX TaqII (TaKaRa) was amplified in a Bio-Rad real-time PCR machine at an annealing temperature of 60°C and for an extension time of 10 s. The amplification for GAPDH was used as endogenous reference. After completion of the amplification, the Bio-Rad software was used to analyze the PCR products. The relative ΔΔ CT values were used for determining quantification and the relative expressions of SHIP1 and CR3 in vector plasmid and scrambled siRNA groups were used as controls. The primers used are listed in **Table 1**.

Western Blot Analysis

After washing with pre-cold PBS, transfected PEMs were lysed with the mixture of RIPA (BiYunTian, Shanghai, China) and SDS loading buffer. Then the samples were collected, boiled for 10 min, and centrifuged at 12,000 g for 10 min to remove cell debris. An equal volume of protein was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% defatted milk at 37°C for 2 h, the membrane was probed with anti-SHIP1 (Millipore, Bedford, MA) or anti-CR3 (Novusbio, CO, USA) monoclonal antibody at 4°C overnight. After washing for 3 times, the membrane was incubated with corresponding horseradish peroxidase-labeled secondary goat anti-mouse or goat anti-rabbit antibodies at 37°C for 1 h followed by 4 washing procedures. The antigen-antibody complexes were detected using a Bio-Rad chemiluminescence detection system, and the protein expression was quantified using Quantity one software.

Phagocytosis Assay

For quantitative analysis, the transfected PEMs were stimulated with PepO for 24 h and infected with *S. aureus* at a MOI of 1:50 or D39 at a MOI of 1:100 at 37°C for 30 min, a time point when there is no bacterial death. After washing with PBS for 3 times, 100 μl pre-cold double distilled water (ddH_2O) was added. The samples were collected and placed at 4°C for 15 min to fully burst the PEMs. Following a series of dilution, the bacteria were seeded on the Columbia sheep blood agar and cultured in 5% CO_2 at 37°C overnight. The bacterial colonies were counted. For qualitative analysis, the transfected PEMs were stimulated with PepO for 24 h and infected with FITC-labeled *S. aureus* at a MOI of 1:50 or FITC-labeled D39 at a MOI of 1:100 at 37°C for 30 min. After washing with PBS for 3 times, PEMs were fixed with 0.4 ml 4% paraformaldehyde for 15 min followed by 3 washing procedures and stained with DAPI for 10 min followed by another 3 washing procedures. The phagocytic bacteria were observed under a Nikon Eclipse 80i microscope equipped with a Nikon Intensilight C-HGFI.

Flow Cytometry Analysis

PEMs were collected and washed twice with pre-chilled PBS. After blocking with CD16/32 antibody for 20 min, cells were

stained with APC-labeled CD11b antibody in the dark for 30 min. After washing with PBS for 2 times, the cells were resuspended with PBS for analysis.

Intranasal Infection of Mice

Female C57BL/6 mice were used for infection experiments because they are more susceptible to *S. pneumoniae*. They were anesthetized with pentobarbital and held in a supine position with the head down. Thirty microliter PBS containing a single compound (30 μg pHBLV-CMV-SHIP1 or pHBLV-CMV-MCS plasmid, 0.4 nmol siCR3 or scrambled siRNA, 10 μg PepO, 1×10^8 CFUs of *S. aureus* or D39) was dropped slowly into their nares with a micropipette at an interval of 24 h. Plasmids pHBLV-CMV-SHIP1 and pHBLV-CMV-MCS, siCR3, and scrambled siRNA were used for *in vivo* transient transfection (Oh et al., 2001; Darcan-Nicolaisen et al., 2009). Twenty-four or forty-eight hours later, the mice were sacrificed for collection of nasal lavage fluids, lung tissues, and blood. The number of viable organisms were determined by counting bacterial colony-forming units (CFUs) on the Columbia sheep blood agar.

Statistical Analysis

All analyses were performed with the use of Prism 5 statistical software (La Jolla, CA, USA). The data are shown as mean ± standard deviation (SD). Difference between groups was determined by two-way ANOVA test or student's *t*-test. For all experiments, difference with $P < 0.05$ was considered significant.

RESULTS

The Down Regulation of SHIP1 Mediates the Enhanced Phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-Stimulated Macrophages

Our previous study has proven that PepO stimulation leads to the down regulation of SHIP1 in macrophages (Yao et al., 2017). It is still unclear that whether the down regulation of SHIP1 mediates the enhanced phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-stimulated macrophages. To answer this question, we transfected PEMs with pHBLV-CMV-SHIP1 plasmid for over-expression of SHIP1, then the effect of PepO on the phagocytic function of macrophages was determined. **Figure 1A** shows the effective over-expression of SHIP1 in transfected PEMs without PepO treatment. The number of phagocytosed *S. aureus* and D39 within PepO-treated macrophages was significantly smaller in the SHIP1 over-expression group than in the control group, with more obvious decrease of D39 number (**Figures 1B,D**). In addition, macrophages which internalized FITC labeled *S. aureus* and D39 were shown in **Figures 1C,E**. The percentage of FITC positive macrophages was significantly decreased in the SHIP1 over-expression group with PepO treatment compared with the control group, with a similar percentage to the medium group. The quantification was shown in the right panels of **Figures 1C,E**. Interestingly, **Figure 1B** shows an increased tendency in the bacterial number of SHIP1 over-expression group in the absence of PepO. We detected CR3 level in macrophages with SHIP1 over-expression and found that it was slightly increased in the absence of PepO (**Figure 3B**), which to some extent explained this increased tendency. However, in the absence of PepO, CR3 level in macrophages with SHIP1 over-expression did not decrease as expected, which suggests that CR3 expression regulation by SHIP1 should be more complicated. Taken together, these results indicate that the down regulation of SHIP1 mediates the enhanced phagocytosis of *S. aureus* and D39 by PepO-stimulated macrophages.

The Up Regulation of CR3 Mediates the Enhanced Phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-Stimulated Macrophages

Our previous study has proven that PepO stimulation leads to the up regulation of CR3 in macrophages. To determine whether the up regulation of CR3 mediates the enhanced phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-stimulated macrophages, we transfected PEMs with CR3 siRNA for knock-down of CR3, then the effect of PepO on the phagocytic function of macrophages was determined. **Figure 2A** shows the effective knock-down of CR3 in PEMs. As shown in **Figures 2B,D**, the number of phagocytosed *S. aureus* and D39 within PepO-treated macrophages was significantly smaller in the CR3 siRNA group than in the control group, with more obvious decrease of D39 number. Moreover, the percentage of FITC positive macrophages was significantly decreased in the CR3 siRNA group with PepO treatment, as compared with the control group (**Figures 2C,E**). The quantification was shown in the right panels of **Figures 2C,E**. Unexpectedly, we did not see any effect with CR3 knock down in the absence of PepO. A possible reason for this finding maybe that other signaling pathways responsible for phagocytosis compensate for CR3 knock down in macrophages, and that the phagocytosed bacteria in the absence of PepO are relatively less. These results indicate that the up regulation of CR3 mediates the enhanced phagocytosis of *S. aureus* and D39 by PepO-stimulated macrophages.

The Down Regulation of SHIP1 Mediates the Up Regulation of CR3 in PepO-Stimulated Macrophages

Previous studies have shown that SHIP1 negatively regulates the activity of CR3 (Dianne et al., 2001; Horan et al., 2007). It is still unclear that whether the expression of CR3 is regulated by SHIP1 in the current system. To answer this question, PEMs were transfected with pHBLV-CMV-SHIP1 plasmid or SHIP1 siRNA followed by PepO stimulation, then the expression of CR3 was determined. **Figures 3A,B** shows that both CR3 transcripts and protein were decreased in macrophages with SHIP1 over-expression in the presence of PepO. In contrast, they were increased in macrophages with SHIP1 knock down in the presence of PepO (**Figures 3C,D**). The quantification of the protein levels of SHIP1 and CR3 was shown in the lower panels of **Figures 3B,D**. We also used flow cytometry analysis to measure CR3 levels on transfected macrophages with PepO treatment. As shown in **Figures 3E,F**, CR3 level was decreased

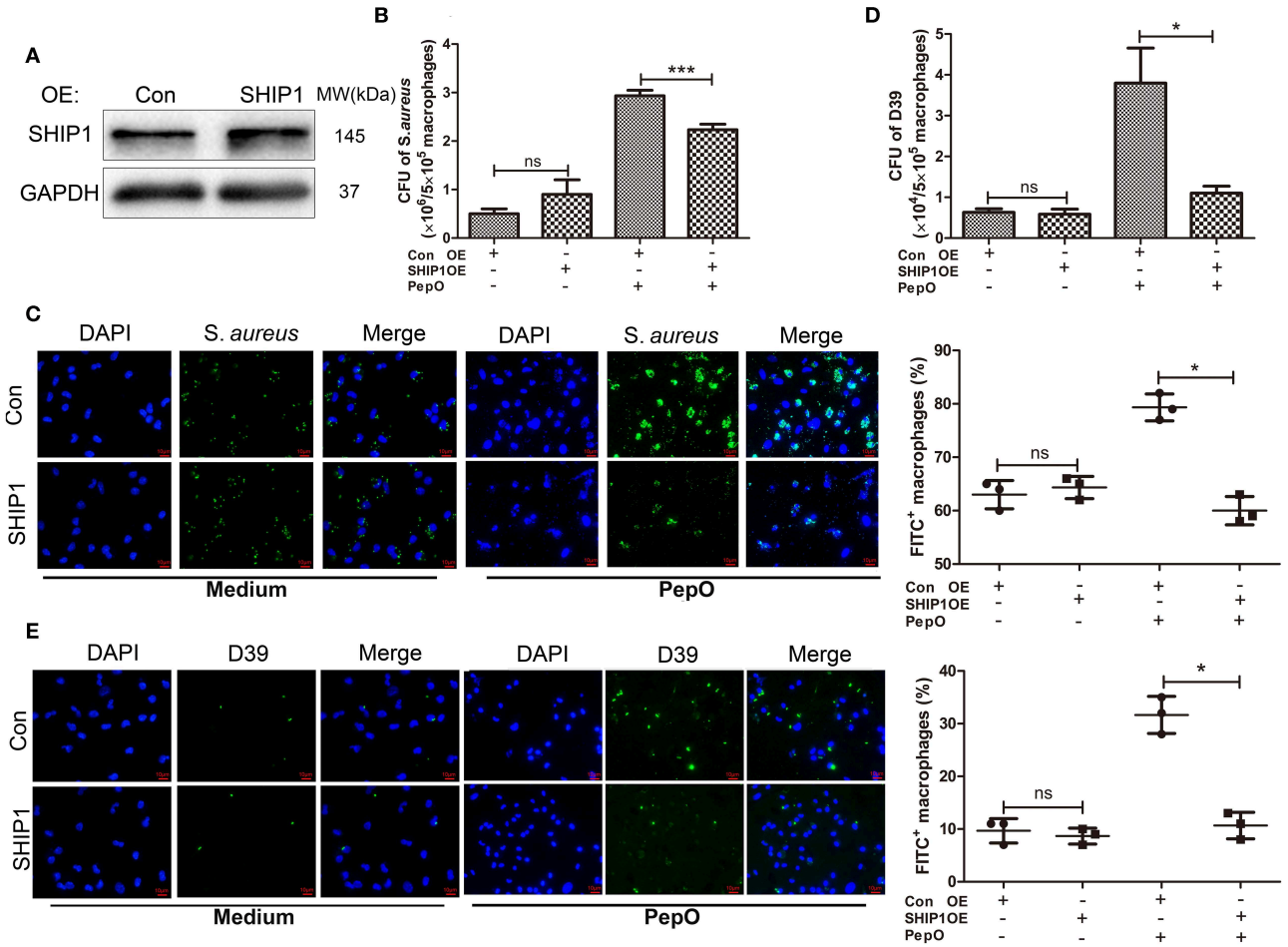

FIGURE 1 | The down regulation of SHIP1 mediates the enhanced phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-stimulated macrophages. **(A)** Immunoblot showed the effective over-expression of SHIP1 in PEMs. **(B,D)** PEMs were transfected with pHBLV-CMV-SHIP1 plasmid or pHBLV-CMV-MCS control plasmid for 24 h followed by PepO stimulation for another 24 h and infection of *S. aureus* **(B)** or D39 **(D)** for 30 min. The number of phagocytosed bacteria was determined. Data are shown as averaged (±SD) bacterial number of three independent repeated experiments. **(C,E)** PEMs were treated as described in **(B)** and infected with FITC-labeled *S. aureus* **(C)** or FITC-labeled D39 **(E)** for 30 min. Representative pictures (original magnification, ×40) from one of the three experiments are shown. The quantification is shown as the averaged (±SD) percent of $FITC^+$ macrophages from three independent repeated experiments. Scale bar, 10 μm. Statistical analysis was performed by two-way ANOVA test. ***$p < 0.001$, *$p < 0.05$, ns, not significant; OE, over-expression; Con, control.

in SHIP1 over-expression group while increased in SHIP1 siRNA group compared with the control group, which is consistent with the western blot analysis. These results indicate that SHIP1 negatively regulates the expression of CR3 in PepO-stimulated macrophages, and the down regulation of SHIP1 mediates the up regulation of CR3 in our experiments.

PepO Promotes the Clearance of *S. aureus* and *S. pneumoniae* in C57BL/6 Mice

Many studies have shown that macrophages play a dominant role in the clearance of respiratory pathogens (Lovewell et al., 2014; Byrne et al., 2015; Eichinger et al., 2015; Lemon et al., 2015). PepO can enhance the phagocytic function of macrophages, indicating that it owes the potential as an immunomodulation agent. Whether it protects against respiratory pathogens remains to be determined. To address this question, female C57BL/6 mice were administrated intranasally with PepO followed by an intranasal infection of *S. aureus* or D39, a standard strain of *S. pneumoniae*. Twenty-four or forty-eight hours later, the mice were sacrificed and their nasal lavage fluids and lungs were harvested for determination of the number of viable *S. aureus* or D39. As shown in **Figures 4A–D**, the number of viable *S. aureus* or D39 in nasal lavage fluids and lung tissues was significantly smaller in the PepO group than in the PBS group at each time point, with the highest decrease occurring in the lung tissues of PepO group at 48 h. These results indicate that PepO promotes the clearance of *S. aureus* and D39 and alleviates their infection in C57BL/6 mice.

Our above results have shown that the enhanced phagocytosis of *S. aureus* and D39 by PepO-stimulated macrophages depends

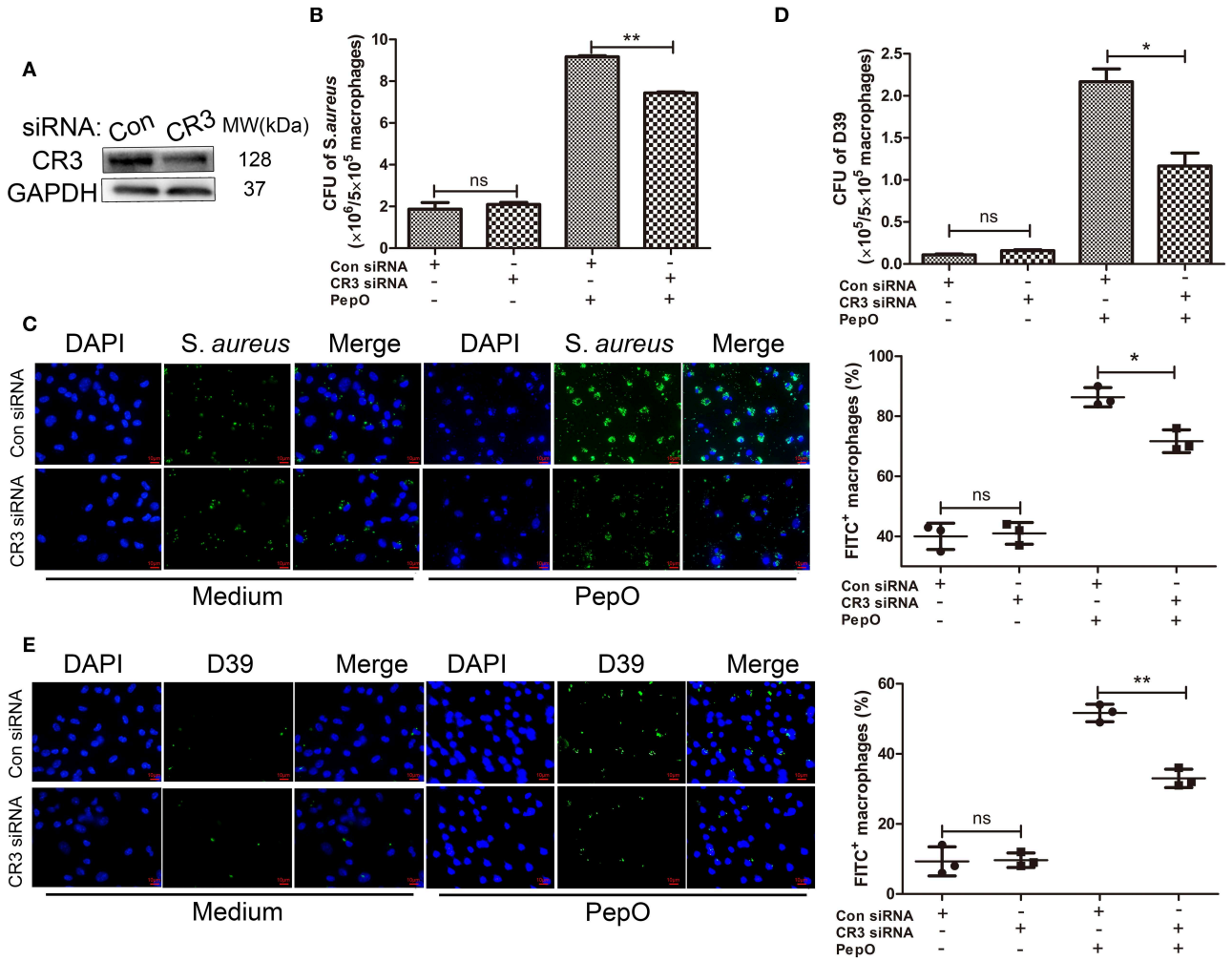

FIGURE 2 | The up regulation of CR3 mediates the enhanced phagocytosis of *S. aureus* and *S. pneumoniae* by PepO-stimulated macrophages. **(A)** Immunoblot showed the effective knockdown of CR3 in PEMs. **(B,D)** PEMs were transfected with CR3 siRNA or scrambled siRNA for 24 h followed by PepO stimulation for another 24 h and infection of *S. aureus* **(B)** or D39 **(D)** for 30 min. The number of phagocytosed bacteria was determined. Data are shown as averaged (±SD) bacterial number of three independent repeated experiments. **(C,E)** PEMs were treated as described in **(B)** and infected with FITC-labeled *S. aureus* **(C)** or FITC-labeled D39 **(E)** for 30 min. Representative pictures (original magnification, ×40) from one of the three experiments are shown. The quantification is shown as averaged (±SD) percent of $FITC^+$ macrophages from three independent repeated experiments. Scale bar, 10 μm. Statistical analysis was performed by two-way ANOVA test. $^{**}p < 0.01$, $^{*}p < 0.05$, ns, not significant; Con, control.

on the down regulation of SHIP1 and the up regulation of CR3 (Yao et al., 2017). To determine whether the enhanced clearance of *S. aureus* and D39 in PepO-treated mice is associated with SHIP1 and CR3, we detected the expression of SHIP1 and CR3 in cells from bronchoalveolar lavage fluids. **Figure 4E** shows that the SHIP1 protein was down regulated in the PepO group compared with the PBS group, while the CR3 protein was up regulated in the PepO group compared with the PBS group both after 24 and 48 h PepO treatment. The quantification of SHIP1 and CR3 levels was shown in the right panel of **Figure 4E**. These results suggest that SHIP1 and CR3 are associated with the enhanced clearance of *S. aureus* and D39 in PepO-treated mice.

The Down Regulation of SHIP1 Plays a Role in the Enhanced Clearance of *S. aureus* and *S. pneumoniae* in PepO-Treated Mice

To determine whether the down regulation of SHIP1 is necessary for the enhanced clearance of *S. aureus* and D39 in PepO-treated mice, female C57BL/6 mice were transiently transfected with pHBLV-CMV-SHIP1 plasmid via intranasal administration, then the effect of PepO on the clearance of *S. aureus* and D39 was examined. SHIP1 level in cells from bronchoalveolar lavage fluids was measured by western blot analysis, and the effective over-expression of SHIP1 was

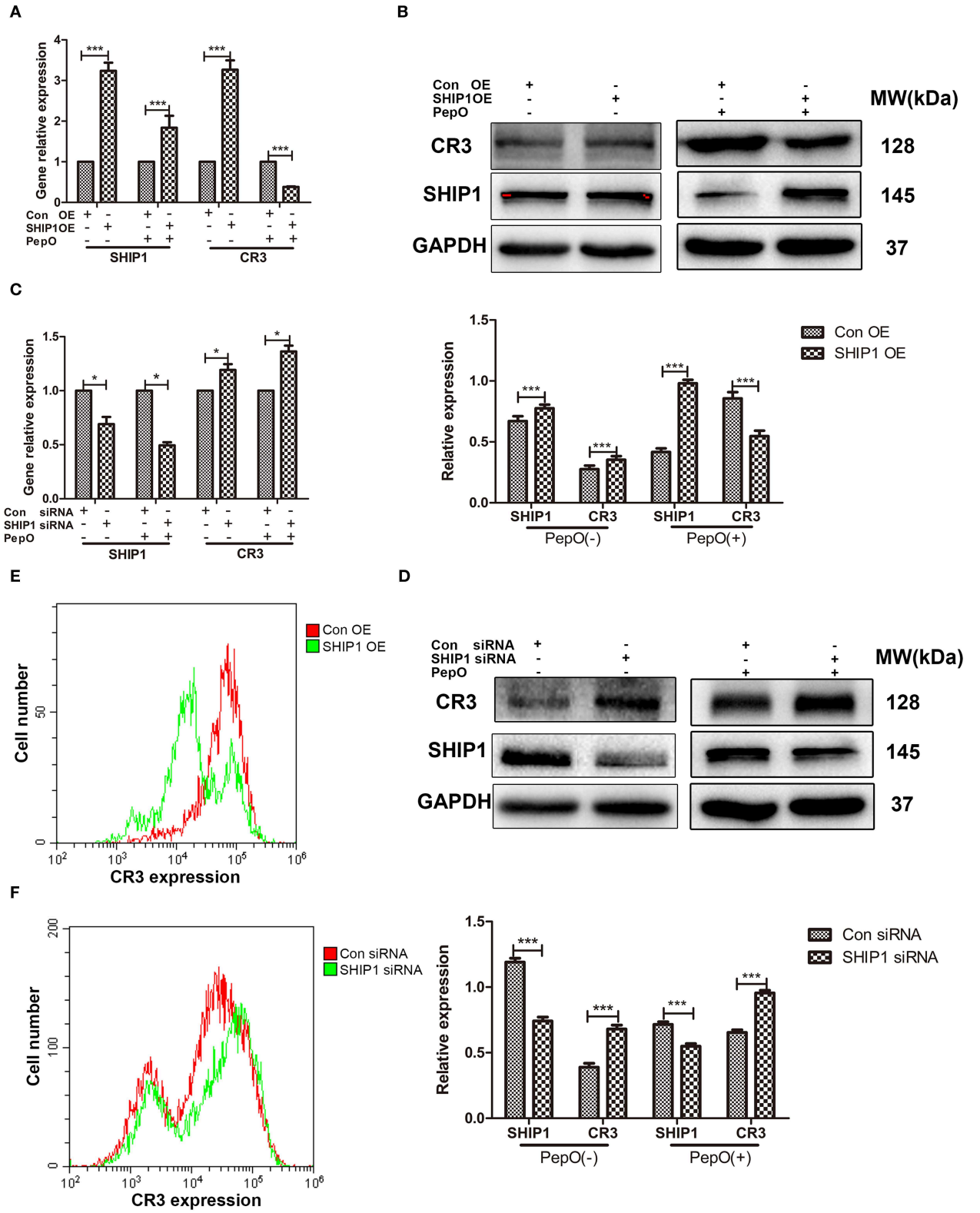

FIGURE 3 | Regulation of CR3 expression by SHIP1 in PepO-stimulated PEMs. **(A–F)** PEMs were transfected with pHBLV-CMV-SHIP1 plasmid, pHBLV-CMV-MCS control plasmid **(A,B,E)**, SHIP1 siRNA, or scrambled siRNA **(C,D,F)** for 24 h followed by PepO or medium stimulation for another 24 h. SHIP1 and CR3 transcripts were determined by Q-PCR analysis, and SHIP1 protein was determined by WB analysis. CR3 protein was determined by WB and flow cytometry analysis. Representative bands from three independent repeated experiments are shown. Data are shown as mean (±SD) of three independent repeated experiments. Statistical analysis was performed by two-way ANOVA test. ***$p < 0.001$, *$p < 0.05$, Con, control; OE, over-expression.

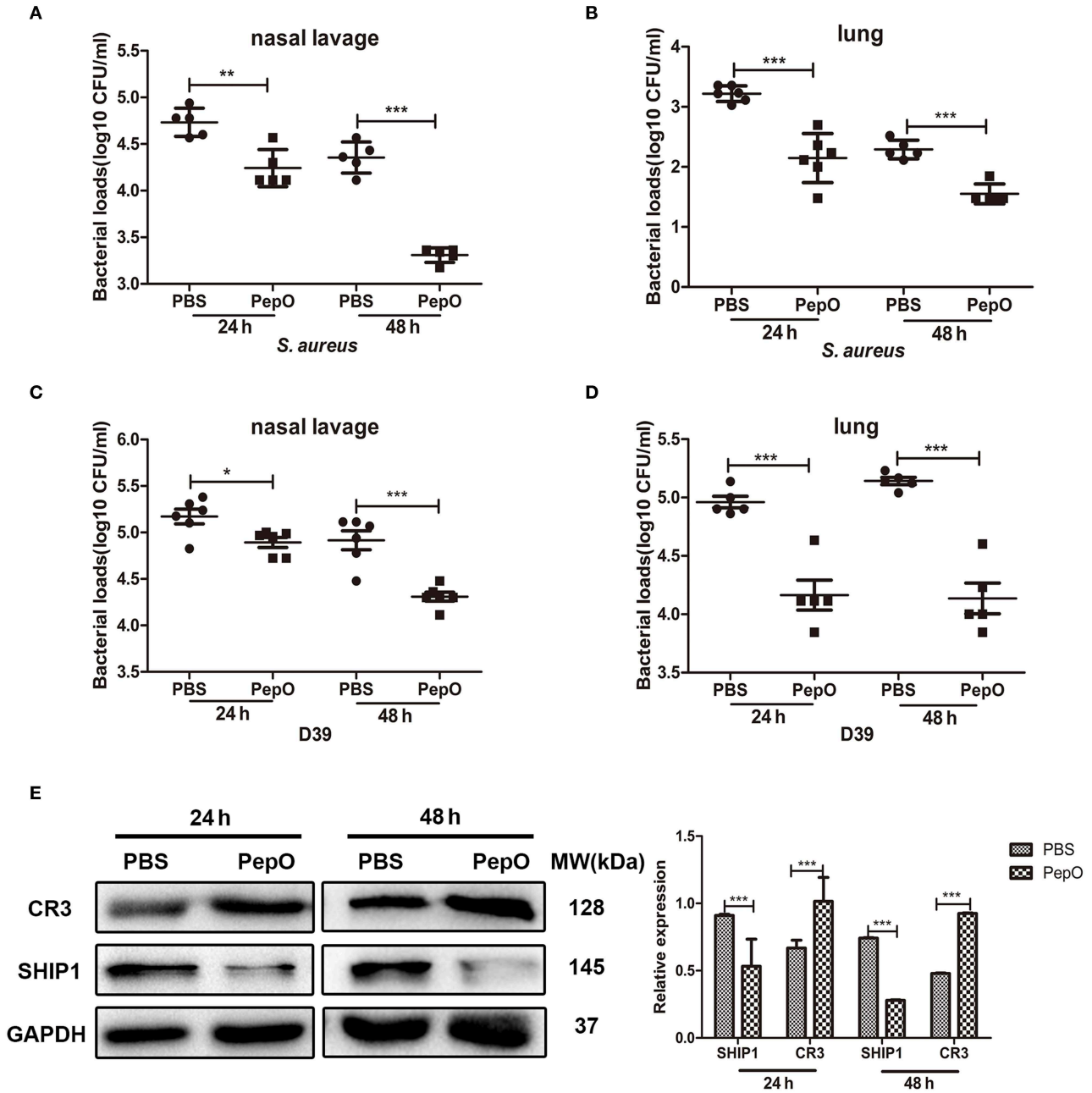

FIGURE 4 | PepO promotes the clearance of *S. aureus* and *S. pneumoniae* in C57BL/6 mice. **(A–D)** C57BL/6 mice were intranasally administrated with PepO or an equivalent volume of PBS for 24 h followed by an intranasal infection of *S. aureus* **(A,B)** or D39 **(C,D)** for 24 or 48 h. The number of viable *S. aureus* or D39 in nasal lavage liquids and lungs was determined. Data are shown as averaged (±SD) bacterial loads of at least five mice per group from one representative experiment of two. **(E)** Immunoblot showed the expression of SHIP1 and CR3 in cells from bronchoalveolar lavage fluids after 24 and 48 h PepO treatment. Representative bands from three independent repeated experiments are shown. Statistical analysis was performed by student's *t*-test. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

shown in **Figure 5A**. As shown in **Figures 5B,D**, the bacterial loads in nasal lavage fluids were significantly increased in the SHIP1 over-expression group at each time point with PepO treatment, as compared with the control group. The bacterial loads of *S. aureus* in lung tissues at 48 h were also significantly higher in the SHIP1 over-expression group than in the control group with PepO treatment. Although there was no significant difference in bacterial loads of D39 in lung tissues between the two groups with PepO treatment, the bacterial loads in SHIP1 over-expression group showed an increased tendency compared with the control group (**Figures 5C,E**). These results indicate that the down regulation of SHIP1 plays a role in the enhanced clearance of *S. aureus* and D39 in PepO-treated mice.

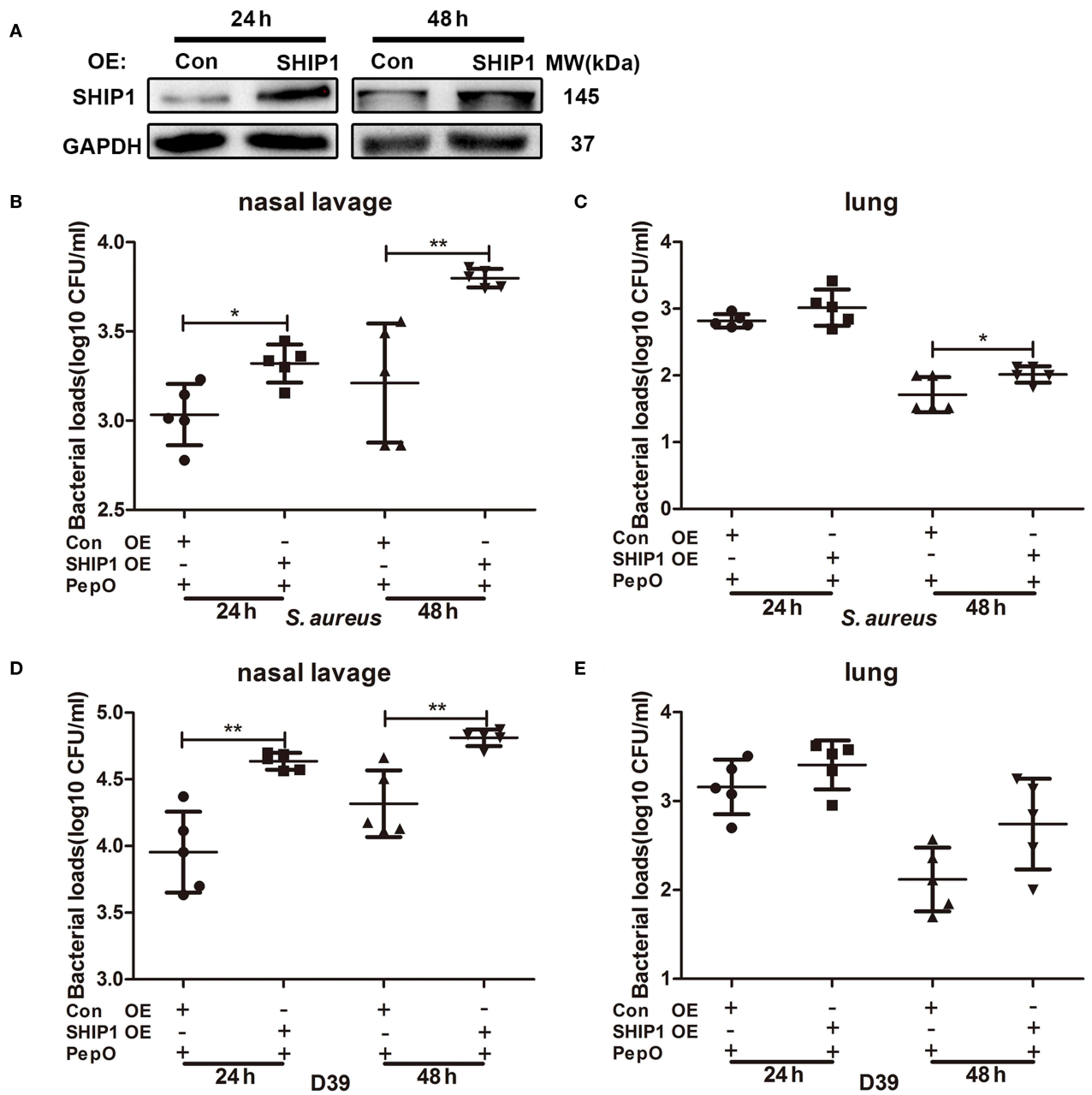

FIGURE 5 | The enhanced clearance of *S. aureus* and *S. pneumoniae* in PepO-treated mice is associated with the down regulation of SHIP1. **(A)** C57BL/6 mice were transiently transfected with pHBLV-CMV-SHIP1 plasmid or pHBLV-CMV-MCS control plasmid via intranasal administration for 24 and 48 h. The effective over-expression of SHIP1 was determined by WB analysis. **(B–E)** After transfected with pHBLV-CMV-SHIP1 plasmid or pHBLV-CMV-MCS control plasmid, mice were intranasally administrated with PepO for another 24 h followed by an intranasal infection of *S. aureus* **(B,C)** or D39 **(D,E)** for 24 and 48 h. The number of viable *S. aureus* or D39 in nasal lavage fluids and lungs were determined. Data are shown as averaged (±SD) bacterial loads of at least five mice per group from one representative experiment of two. Statistical analysis was performed by student's *t*-test. $^{**}p < 0.01$, $^{*}p < 0.05$, OE, over-expression; Con, control.

The Up Regulation of CR3 Plays a Role in the Enhanced Clearance of *S. aureus* and *S. pneumoniae* in PepO-Treated Mice

To determine whether the up regulation of CR3 is necessary for the enhanced clearance of *S. aureus* and D39 in PepO-treated mice, female C57BL/6 mice were transiently transfected with CR3 siRNA via intranasal administration, then the effect of PepO on the clearance of *S. aureus* and D39 was examined. CR3 level in cells from bronchoalveolar lavage fluids was measured by western blot analysis, and the effective knock-down of CR3 was shown in **Figure 6A**. **Figures 6B–E** shows that the bacterial loads in nasal lavage fluids and lung tissues were significantly higher in the CR3

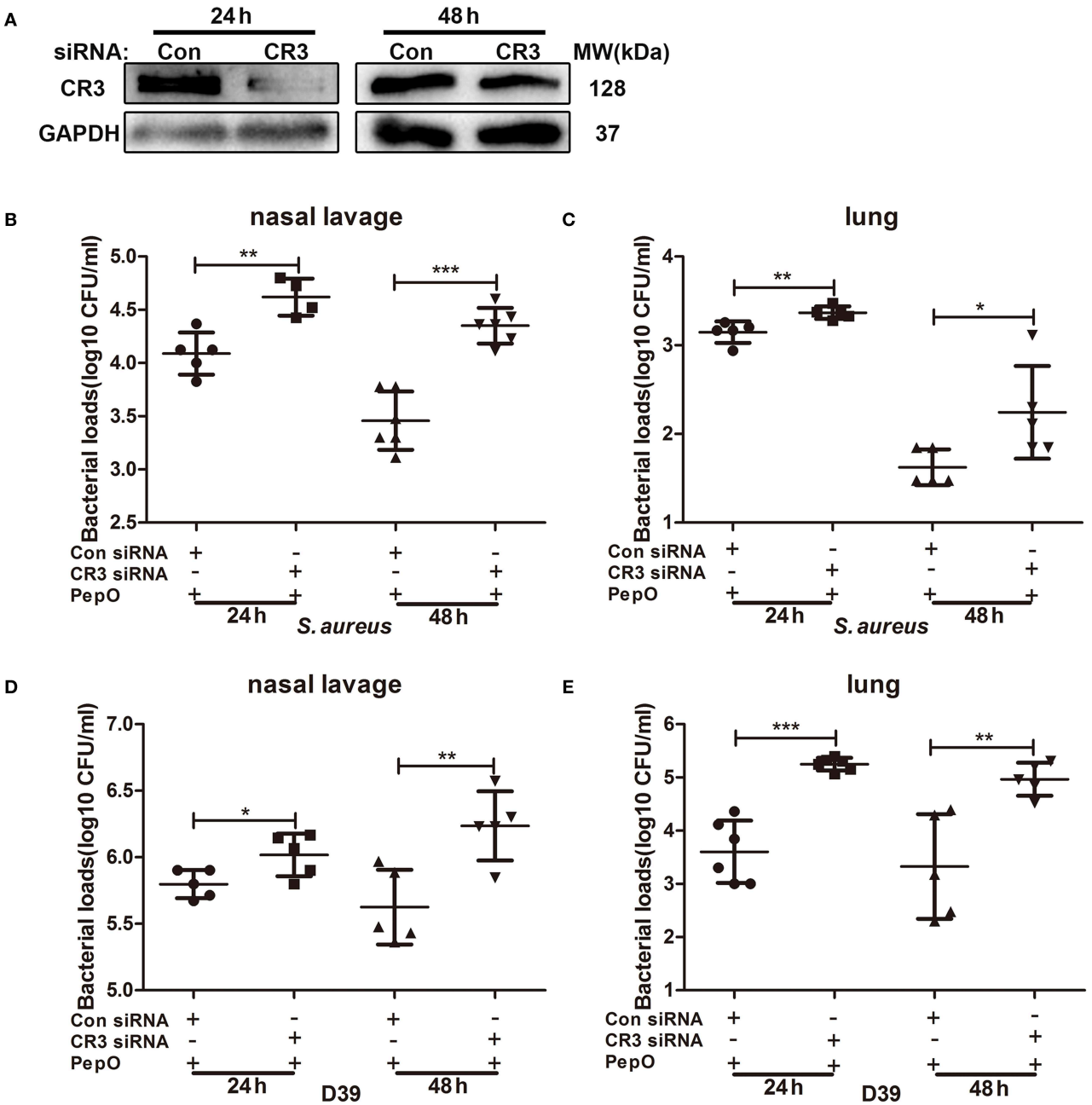

FIGURE 6 | The enhanced clearance of *S. aureus* and *S. pneumoniae* in PepO-treated mice is associated with the up regulation of CR3. **(A)** C57BL/6 mice were transiently transfected with CR3 siRNA or scrambled siRNA via intranasal administration for 24 and 48 h. The effective knock down of CR3 was determined by WB analysis. **(B–E)** After transfected with CR3 siRNA or scrambled siRNA, mice were intranasally administrated with PepO for another 24 h followed by an intranasal infection of *S. aureus* **(B,C)** or D39 **(D,E)** for 24 and 48 h. The number of viable *S. aureus* or D39 in nasal lavage fluids and lungs were determined. Data are shown as averaged (±SD) bacterial loads of at least five mice per group from one representative experiment of two. Statistical analysis was performed by student's *t*-test. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, Con, control.

siRNA group than in the control group at each time point, with the highest increase occurring in the lung tissues of CR3 siRNA group at 24 h. Taken together, these results indicate that the up regulation of CR3 plays a role in the enhanced clearance of *S. aureus* and D39 in PepO-treated mice.

DISCUSSION

Our previous study has detected the down regulation of SHIP1 and the up regulation of CR3 in PepO-stimulated macrophages. However, the relationship between their

changed expression and the enhanced phagocytic function of PepO-stimulated macrophages has not been proven. In this study, we provide evidence that PepO enhances the phagocytic function of macrophages in a SHIP1 and CR3 dependent manner. The down regulation of SHIP1 mediates the up regulation of CR3 in PepO-stimulated macrophages. Furthermore, PepO promotes the clearance of *S. aureus* and D39 from nasopharynx and lungs, and the down regulation of SHIP1 and the up regulation of CR3 also play a role in this process.

At least to our knowledge, in this study we first proved the regulation of CR3 expression by SHIP1. The regulation of CR3 activity by SHIP1 has long been investigated in previous studies (Dianne et al., 2001; Horan et al., 2007), while less attention has been given to the regulation of CR3 expression. This study provides new evidence that the expression of CR3 is also regulated by SHIP1. Unexpectedly, we found that both knockdown and over-expression of SHIP1 lead to the up regulated expressions of CR3 transcripts and protein in macrophages without PepO stimulation. The possible reason may be that the regulation of CR3 expression by SHIP1 is dynamic and needs to be balanced. SHIP1 probably not merely inhibits CR3 expression. Under a certain circumstance, it may also promote CR3 expression. Numerous studies have demonstrated that SHIP1 is a multifunctional protein controlled by various regulatory inputs and regulates downstream signaling via multiple means (Pauls and Marshall, 2017). Both activation and inhibition of SHIP1 inhibit the phosphoinositide 3-kinase signaling pathway (Ong et al., 2007; Fuhler et al., 2012; Fernandes et al., 2013). Another possibility for this unexpected finding is that SHIP1 over-expression activates other signaling molecules, which may mediate the up regulation of CR3. Based on the complexity of immune cell signaling regulation by SHIP1, we speculate that the regulation of CR3 expression by SHIP1 should be a complicated process and to understand this process, further investigation is still needed.

Streptococcus pneumoniae and *S. aureus* are dominant colonization bacteria in the upper respiratory tract (Zemlickova et al., 2006; Thapa et al., 2017; Dunne et al., 2018; Lo et al., 2019). Both *S. aureus* strain and *S. pneumoniae* strain used in our study were strong pathogenic strains. PepO promotes the clearance of these strains from nasopharynx and lungs, confirming its protective effect as an immunomodulation agent. The protective effects of gastrointestinal microorganism and their metabolites against infections have long been investigated in previous studies (Ubeda et al., 2013; van Nood et al., 2013; Singh et al., 2014; Buffie et al., 2015; Caballero et al., 2015; Stripling et al., 2015; Lewis and Pamer, 2017; Deng et al., 2019), while less attention has been given to the respiratory microorganisms, especially respiratory pathogens. At least to our knowledge, this study proved for the first time that product from respiratory pathogens can also play protective roles against respiratory infections.

Our current study demonstrated the effectiveness of PepO against specific strains of *S. aureus* and *S. pneumoniae,* and whether PepO can promote the clearance of additional strains of these species including antibiotic resistant isolates (e.g., methicillin-resistant *Staphylococcus aureus* (MRSA) or antibiotic resistant *S. pneumoniae*) and additional species including gram-negative bacteria (e.g., *Pseudomonas aeruginosa* or *E. coli*) still needs to be further determined. Theoretically speaking, PepO can also promote the clearance of these organisms, because it plays this role via enhancing the phagocytosis by macrophages. Even so, further experiment data are still needed to support the hypothesis.

PepO is a multifunctional pneumococcal virulence protein. Agarwal et al., demonstrated that it facilitates host cell invasion and evasion of innate immunity through interaction with plasminogen, fibronectin, C1q, and C4BP (Agarwal et al., 2013, 2014). Our work proved that PepO promotes innate immune activation via TLR2 and TLR4 signaling pathways (Zhang et al., 2016; Yao et al., 2017; Shu et al., 2020). These conclusions seem to contradict each other. Agarwal et al., concentrated on the role of PepO as a virulence protein during pneumococcal infection, and we focused on its role as a pathogen associated molecular pattern without infection, which constitutes the dual character of virulence factor. The innate immune activation effect lays the basis for PepO as an anti-microbial agent, and this effect is associated with TLR2/4 activation, which indicates that protein with similar function may be a new source of antimicrobial agents.

In summary, this study demonstrates that PepO promotes the clearance of *S. aureus* and *S. pneumoniae* from nasopharynx and lungs, and the down regulation of SHIP1 and the up regulation of CR3 play a role in this process. Our study provides a new preventive and therapeutic option for respiratory infectious diseases and lays the theoretical basis for the development of PepO as an immunomodulation agent.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This animal study was reviewed and approved by the Ethics Committee of Chongqing Medical University.

AUTHOR CONTRIBUTIONS

HZ, YY, and XZ planned the experiments. SL, JX, YM, and ZS performed the experiments. HZ, TY, WX, and XZ analyzed the data. HZ and XZ wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation grants of China (Grant No. 81701572).

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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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frontiers in Cellular and Infection Microbiology

PERSPECTIVE
published: 15 October 2020
doi: 10.3389/fcimb.2020.585791

Perspective of a Pediatrician: Shared Pathogenesis of the Three Most Successful Pathogens of Children

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Highly successful invasive pathogens exploit host vulnerabilities by adapting tools to co-opt highly conserved host features. This is especially true when pathogens develop ligands to hijack trafficking routes or signaling patterns of host receptors. In this context, highly successful pathogens can be grouped together by the patterns of organs infected and diseases they cause. In the case of this perspective, the focus is on the historically most successful invasive bacterial pathogens of children that cause pneumonia, sepsis and meningitis: *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitidis*. This triad shares a ligand to bind to PAF receptor to enter host cells despite early defenses by innate immunity. All three also target laminin receptor to cross endothelial barriers using a common set of molecular tools that may prove to be a design for a cross-protective vaccine.

Keywords: pneumococcus, meningococcus, haemophilus, PAF receptor, laminin receptor

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *21 July 2020*
Accepted: *30 September 2020*
Published: *15 October 2020*

Citation:
Tuomanen EI (2020) Perspective of a Pediatrician: Shared Pathogenesis of the Three Most Successful Pathogens of Children. Front. Cell. Infect. Microbiol. 10:585791. doi: 10.3389/fcimb.2020.585791

INTRODUCTION

"*The challenge is to figure out why the most virulent bacterial pathogens of children cause the same pattern of disease, particularly meningitis, and share the same unusual microbial physiology, particularly autolysis and natural transformation*".

Joshua Lederberg, PhD
Nobel Laureate
Personal communication

Every pediatrician will tell you that, historically, the major invasive pathogens of children that they dread the most are *Streptococcus pneumoniae, Haemophilus influenzae*, and *Neisseria meningitidis* (Kim, 2010; Lundbo and Benfield, 2017). They share striking features of the pattern of disease. All three most commonly attack children under the age of 5 years. All three begin infection by asymptomatic carriage in the nasopharynx, spread through the respiratory tract, multiply quickly to high titer bacteremia and cross the blood brain barrier to cause meningitis (Loughren et al., 2019). It is the final step to meningitis that truly sets these three apart and begs the question, what do they "know" about host vulnerability that promotes a course of infection that is so glaringly lethal? What unusual features of microbial physiology relate to shared pathogenesis? Several major surface features that promote virulence differ between them and thus, are not likely to

explain the shared organ tropism. Their capsules serve to protect all three of these bacteria from phagocytosis but are of widely varying chemical composition. *Haemophilus* and meningococcus are Gram negative and thus have a thin cell wall and an outer membrane, while the Gram positive pneumococcus has a thick cell wall and no outer membrane. Determining why this seemingly mixed triad causes such a similar pattern of disease is a significant challenge that is only partially solved.

CARRIAGE

Pneumococcus, *Haemophilus* and meningococcus circulate in the population by asymptomatic carriage in the nasopharynx of young children (Adegbola et al., 2014). Their mechanisms of attachment are diverse, and each has several ligand receptor interactions with the nasopharyngeal mucosa. During multiple events of carriage in early childhood, the host acquires immunity to the dominant capsular antigens and a variety of surface proteins which, in most cases, appears to be enough to limit further invasion (Segal and Pollard, 2005; Ramos-Sevillano et al., 2019). Unencapsulated strains colonize the mucosa of the upper respiratory tract quite well. The multiplicity of adherence events for each pathogen complicates the design of simple protein-based vaccines to eliminate carriage as a first step in defense. Clearly, these three pathogens start at the same physical point of entry to the host using a very different set of capabilities. Then the story changes.

SHARED INVASION STRATEGY 1: ENTER CELLS DESPITE INNATE IMMUNITY

Infection of the lower respiratory tract is the go/no go for invasive disease. While these bacteria use several different ligand/receptor interactions at any one site, it is in the respiratory tract that the three pathogens reveal they also harbor a shared invasion strategy that is effective despite innate immunity. All three bacteria decorate their surfaces with the small molecule phosphorylcholine (ChoP) (**Figure 1A**) that is added to bacterial surface components by the shared LicD protein, a ChoP transferase (Weiser et al., 1997; Weiser et al., 1998a; Zhang et al., 1999). First described for the pneumococcus, ChoP is covalently added to the teichoic acid and lipoteichoic acid of the cell wall (Briles and Tomasz, 1973). As a key bioactive adduct, ChoP on the pneumococcal cell wall serves as a non-covalent docking station for over a dozen secreted choline binding proteins that are interchanged to modulate contact interactions between the pathogen and host (Gosink et al., 2000). Rather than being added to cell wall, ChoP appears on the lipopolysaccharide of *Haemophilus* (Weiser et al., 1997) and on meningococcal lipopolysaccharide and pili (Weiser et al., 1998a).

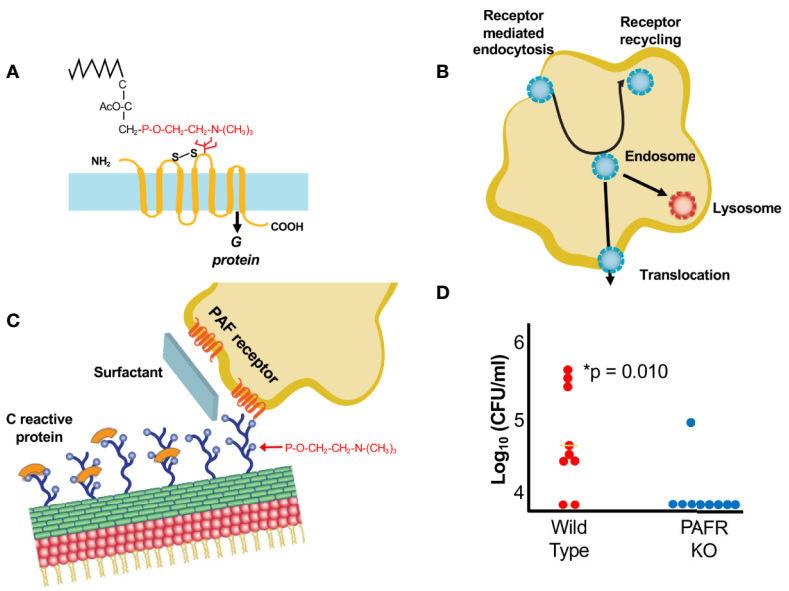

FIGURE 1 | Interactions of bacteria with ChoP-PAFR. **(A)** Platelet activating factor, an inflammatory lipid chemokine. The portion in red is ChoP which is present on bacterial surfaces while the black is the lipid backbone of PAF that is missing in the bacterial form. The 7 transmembrane PAF receptor (PAFR) is shown in yellow. **(B)** Trafficking of PAFR upon ligation by ChoP is diagrammed. Receptor mediated endocytosis engulfs the PAFR bound vesicle containing bacteria into the host cell cytoplasm. The endosome then may traffic to the lysosome for killing, recycle back to the cell surface or translocate across the cell barrier in the process of invasion. **(C)** ChoP (blue balls on cell wall) binds PAFR (red transmembrane lines on host cell). The innate immune system counteracts ChoP by C-reactive protein (orange caps covering ChoP), surfactant (broad sheet of secretions rich in ChoP) and anti-ChoP antibodies. **(D)** Mice were challenged with pneumococci intravenously and the presence bacteria in the cerebrospinal fluid was quantified at 6 h. Animals lacking PAFR (blue dots) are protected from meningitis compared to wild type (WT, red dots) (adapted from Radin et al., 2005).

Further work has expanded the list of pathogens that display ChoP to incorporate most pulmonary pathogens, including *Pseudomonas, Klebsiella, Legionella* and even mycoplasma (Clark and Weiser, 2013). Further underlining the breadth of use of this determinant, virtually all oral commensals display it on their surfaces (Gillespie et al., 1993).

The ChoP Disguise Promotes Bacterial Entry Into Cells

Expression of ChoP on the bacterial surface mimics the critical chemical determinant of the lipid chemokine platelet activating factor (PAF) (**Figure 1A**) (Cundell et al., 1995; Swords et al., 2000; Iuchi et al., 2019). The binding of PAF-ChoP to PAF receptor (PAFR) results in two outcomes: 1) it induces multiple inflammatory signals transduced by coupled G-proteins in platelets, macrophages, epithelial and endothelial cells (Chao and Olson, 1993; Izumi and Shimizu, 1995; Honda et al., 2002); and 2) the triad of invasive pathogens uses ChoP to co-opt PAFR trafficking whereby the receptor undergoes rapid internalization independent of G-protein activation (**Figure 1B**) (Chen et al., 2002; Fillon et al., 2006). Uptake of the chemokine or the bacteria by PAFR is followed by either trafficking to the lysosome *via* Rab5 and Rab7 or recycling to the cell surface (Ishii et al., 1998; Chen et al., 2002). This recirculation of PAFR from the host cell surface to the cytoplasm and back to the surface provides a shuttle for adherent bacteria to enter epithelial and endothelial cells *via* receptor mediated endocytosis (Ring et al., 1998). This trafficking involves co-localization of bacteria, PAFR and the scaffold protein *ß*-arrestin (Luttrell and Lefkowitz, 2002; Spiegel, 2003; Radin et al., 2005; Iovino et al., 2013). Bacteria in the intracellular vacuole are then subject to three fates: being killed in the lysosome, transcytosing across the cell to exit the basal surface, or recycling back to the apical surface (Ring et al., 1998).

The Host Fights Back With the Innate Immune Response

The innate immune response strongly counteracts ChoP mediated interactions between bacteria and host cells (Gould and Weiser, 2002) (**Figure 1C**). The ChoP decoration is the target of natural antibodies that are present at birth even without prior bacterial challenge (Lieberman et al., 1974; Goldenberg et al., 2004). ChoP is the determinant recognized by the first responder of the innate immune system, C-reactive protein, which by binding ChoP, serves as a competitive inhibitor (Weiser et al., 1998b; Clark and Weiser, 2013; Langereis et al., 2019). Furthermore, the lung is awash in ChoP as a major component of surfactant (Gould and Weiser, 2002). Thus, early events in establishing pneumonia are played out by tipping the balance between host recognition of ChoP as a foreign disguise on bacteria and fighting back *vs* falling for the deception that ChoP-coated bacteria mimic the beneficial proinflammatory cytokine PAF.

When Is ChoP-PAFR Operative in Infection?

Presentation of ChoP on the surfaces of all three pathogens is phase variable with greater abundance correlating with greater interactions with mucosal cells and decreased expression characterizing sustained circulation in the bloodstream (Weiser et al., 1994; Weiser et al., 1997; Serino and Virji, 2002). ChoP-PAFR is not the only mechanism of cellular entry for the three major pathogens, but it is a shared one of importance as shown by the failure of mice lacking PAFR to rapidly spread infection between organs (Rijneveld et al., 2004; Radin et al., 2005). These animals show a delayed translocation of bacteria from lung to blood and a significant defect in causing meningitis (**Figure 1D**).

Recently, evidence indicates that bacterial surface components, free from the intact bacterium, also transit barriers using the ChoP tag. Bacterial surface components are released upon lysis of bacteria by antibiotics. Using the pneumococcal cell wall as an example, ChoP on the teichoic acid enables cell wall fragments in blood to bind to PAFR on vascular endothelial cells and traffic into the brain and heart (Tuomanen et al., 1985; Fillon et al., 2006). These cell wall components are recognized by Toll-like receptor 2 and thus, are highly inflammatory (Yoshimura et al., 1999). Upon entering the brain parenchyma, ChoP cell wall induces caspase-dependent apoptosis of neurons (Braun et al., 1999; Orihuela et al., 2006). In the heart, ChoP-bearing cell wall induces death of cardiomyocytes and lethal cardiac dysfunction (Fillon et al., 2006).

In the specific clinical context of a pregnant mouse being treated for bacteremic pneumonia with antibiotics, cell wall released in the bloodstream crosses the placenta and enters the fetal brain. The interaction required for translocation of cell wall across the placenta is ChoP binding to PAFR (Humann et al., 2016). It is not as yet known if components from other bacteria decorated with ChoP also cross the placenta. While cell wall is highly inflammatory in most models, the interaction of cell wall components with embryonic neurons appears to be fundamentally different than the catastrophic death of postnatal neurons. Early in development of the fetal neocortex, neuronal progenitor cells bearing TLR2 respond to cell wall by enhancing proliferation without any cell death (Humann et al., 2016). This results in a larger pool of progenitors that constitute a wave of excess cells that migrates through all the cortical layers resulting in a 50% increase in total cell number in the neocortex. The cortical layers form normally but each layer has an abnormally high number of cells, an aberration in brain architecture that persists after birth. A bigger brain is not always a better brain. Mice born after experiencing a cell wall-induced proliferative wave of neurons during gestation exhibit abnormal social behavior, cognitive deficits and permanent changes in cortical architecture (Humann et al., 2016).

SHARED INVASION STRATEGY 2: CO-OPT RECEPTOR MEDIATED ENDOCYTOSIS TO CROSS CELL BARRIERS

Having passed from the lung into blood, the three pathogens undergo phase variation to increase capsule thickness and downregulate ChoP to effectively avoid phagocytosis resulting in high titer bacteremia, a prerequisite explaining their particular propensity to invade other organs and cause sepsis (Orihuela

et al., 2003). Bacterial titers above 10e5 cfu/ml of blood trigger translocation across substantial vascular barriers in the heart and the brain. While the three major meningeal pathogens use several strategies to transit both through and between endothelial cells, they all share the ability to exploit laminin receptor mediated endocytosis that enables entry into the brain and heart.

All three pathogens harbor a functional homolog of the pneumococcal adhesin CbpA that binds to laminin receptor of the host (**Figure 2A**) (Orihuela et al., 2003). Within the CbpA domain that binds laminin receptor, the sequence EPRNEEK forms a loop between two helices (Luo et al., 2005). Both the sequence and tertiary structure are highly conserved among pneumococci and are critical to function (Mann et al., 2014); pneumococci lacking CbpA show poor penetration into the cerebrospinal fluid in mouse models (**Figure 2B**). Although there is no sequence homology to this domain in their genomes, meningococci display pilus protein PilQ and outer membrane protein PorA and *Haemophilus* present membrane protein OmpP2 that crossreact with antibodies to CbpA and enable both bacteria to also bind to laminin receptor (Mann et al., 2014). This binding facilitates both adherence of bacteria to the cerebral vasculature and subsequent translocation across the endothelial cell cytoplasm into the brain parenchyma. Intravenous injection of CbpA-coated beads into mice followed by imaging of the brain surface through a cranial window dramatically reveals the ability of CbpA to bring particles to and through the cerebral capillary endothelium (**Figure 2C**).

Further impact of the adhesin/laminin receptor interaction is revealed by recent work that, during the course of bacteremic pneumonia, the pneumococcus can translocate into myocardial cells forming microlesions (Brown et al., 2014). It appears that the mechanism of translocation across the cardiac vascular endothelium again involves CbpA/laminin receptor facilitating live bacterial entry into cardiomyocytes. Cardiac damage is reflected by increased levels of Troponin T and abnormal electrocardiography as the bacterial microcolonies interrupt electrical transduction pathways. Necroptosis and apoptosis within the lesions lead to permanent scar formation and contribute to cardiac morbidity and mortality, underappreciated as sequela of clinical pneumonia (Reyes et al., 2017). Such cardiac injury has been associated clinically with the acute and convalescent phases of pneumonia but the mechanism of this link is only now appreciated.

Design of a Cross-Protective Vaccine

Cross-reactivity between the meningeal pathogens of the ligands for laminin receptor-mediated translocation is highlighted by the observation that induction of antibody by vaccination with CbpA conveys protection not only against pneumococcal infection but also against *Haemophilus* sepsis and otitis media and meningococcal meningitis in mouse models (Mann et al., 2014; Rowe et al., 2019). A CbpA-based vaccine is effective in preventing cardiac lesions based on blocking the shared CbpA/laminin receptor mechanism (Mann et al., 2014). Just as was the case for ChoP/PAF receptor as a generalized code for pulmonary/meningeal pathogens entering cells, CbpA/laminin receptor is a shared key to recognizing the blood brain barrier and the heart. Neurotropic pathogens as disparate as syphilis, Venezuelan equine encephalitis virus, Sinbis virus, and prions all share entry into the central nervous system *via* laminin receptor. If many laminin receptor ligands also cross react, this is a feature

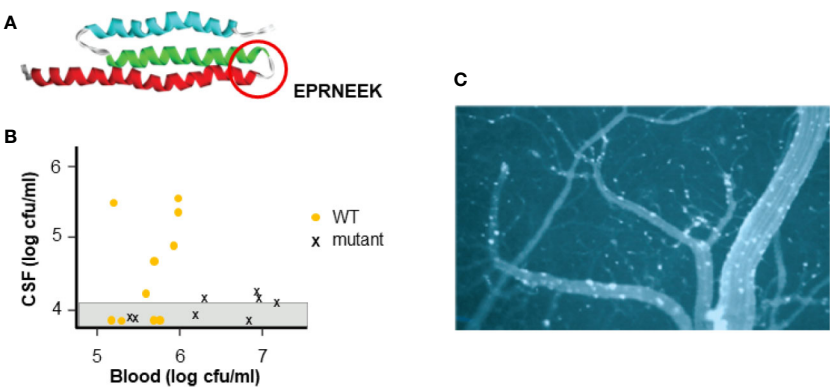

FIGURE 2 | Interaction of CbpA-like adhesin with laminin receptor. **(A)** The structure of the CbpA adhesin domain highlighting the amino acid sequence of the region required for binding to the blood brain barrier (circle) (adapted from Luo et al., 2005). **(B)** Pneumococcal mutant lacking CbpA is compared to wild type (WT) for causing meningitis. Strains were injected intravenously into mice (each symbol is one mouse) and the presence of bacteria in the blood and CSF was determined. Graph shows that WT pneumococci cross the blood brain barrier into the CSF at a blood threshold of ~10e5 cfu. Pneumococcal mutants lacking CbpA do not cross the blood brain barrier into the CSF even at 10e7 cfu in the blood. (adapted from Orihuela et al., 2003) **(C)** Beads coated with CbpA and injected intravenously in mice, adhere to cerebral capillaries (white) and cross into the brain parenchyma (dark) as viewed through a cranial window of a mouse. (adapted from Orihuela et al., 2009).

that could be exploited for a more broadly protective meningitis vaccine; how broadly protective is yet to be determined.

SUMMARY

Pathogens utilize a vast array of individual host/receptor interactions. However, particularly successful ones, like pneumococcus, *Haemophilus influenzae*, and meningococcus, they harbor a uniquely effective shared invasion strategy targeting receptor mediated endocytosis by PAFR and laminin receptor. These mechanisms to cross endothelial barriers have proven successful in causing a similar pattern of severe infections including sepsis and meningitis. Using this commonality to design a vaccine to elicit crossreactive antibodies against the bacterial ligands for laminin receptor may prove to be a broadly effective counterattack.

AUTHOR CONTRIBUTIONS

ET conceived of the concept and wrote the manuscript.

FUNDING

This research was supported by NIH grant NIAID R01 AI 128756 and ALSAC. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Conflict of Interest: The author declares 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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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 22 October 2020
doi: 10.3389/fcimb.2020.569158

Traditional Japanese Herbal Medicine Hochu-Ekki-to Promotes Pneumococcal Colonization Clearance via Macrophage Activation and Interleukin 17A Production in Mice

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Streptococcus pneumoniae may colonize the nasopharynx, and as pneumococcal colonization causes invasive diseases and the subsequent transmission, reducing bacterial burden in the nasal cavity is critical. Hochu-ekki-to (TJ-41) is a traditional Japanese herbal medicine that exerts immunomodulatory effects in host cells. In this study, we investigated the potency of TJ-41 in modulating pneumococcal colonization clearance by activating host immunity. Mice, intranasally inoculated with pneumococci, were treated orally with TJ-41. During colonization, TJ-41 treatment significantly reduced pneumococcal burden and increased macrophage population in the nasopharynx. Furthermore, interleukin 17A production was significantly enhanced after TJ-41 treatment. *In vitro* experiment using nasal-derived cells revealed that pneumococcal antigen exposure upregulated the transcription of interleukin 17A in the TJ-41-treated group compared with that in the control group. Macrophages activated by killed bacteria were significantly increased in the presence of TJ-41 in an interleukin 17A-dependent manner. Moreover, TJ-41 enhanced phagocytosis, inhibited bacterial growth, and improved the antigen-presenting capacity of macrophages. Our results demonstrate that TJ-41 accelerates the clearance of pneumococcal nasopharyngeal colonization via macrophage activation. Subsequent production of interleukin 17A provides an additional benefit to effector cells.

Keywords: traditional herbal medicine, Hochu-ekki-to (TJ-41), pneumococcal colonization, macrophage, interleukin 17, innate immunity

OPEN ACCESS

Edited by:
Victor Nizet, University of California, San Diego, United States

Reviewed by:
Justin A. Thornton, Mississippi State University, United States
Sven Hammerschmidt, University of Greifswald, Germany

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *03 June 2020*
Accepted: *22 September 2020*
Published: *22 October 2020*

Citation:
Nakakubo S, Kimura S, Mimura K, Kajiwara C, Ishii Y, Konno S and Tateda K (2020) Traditional Japanese Herbal Medicine Hochu-Ekki-to Promotes Pneumococcal Colonization Clearance via Macrophage Activation and Interleukin 17A Production in Mice. Front. Cell. Infect. Microbiol. 10:569158. doi: 10.3389/fcimb.2020.569158

INTRODUCTION

Streptococcus pneumoniae, a gram-positive coccus, is a major cause of pneumonia, meningitis, and sepsis. Despite the antibiotic era, pneumococcal diseases impose a heavy burden on global health with high morbidity and mortality rates (Weiser et al., 2018). Pneumococcal vaccines have preventive effects against pneumococcal diseases (Pilishvili et al., 2010; Bonten et al., 2015; Suga et al., 2015). However, it has been reported that diseases caused by vaccine-uncovered serotypes account for a higher portion of the total pneumococcal diseases, termed "serotype replacement" (Balsells et al., 2017). Hence, there is a need for preventive strategies for pneumococcal infections.

Pneumococci generally invade human nasopharynx and establish colonization. This is a prerequisite for invasive diseases (Bogaert et al., 2004). Asymptomatically colonized individuals may act as reservoirs for inter-individual transmission of pneumococci (Kadioglu et al., 2008; Wolter et al., 2014), warranting the control of pneumococcal nasopharyngeal colonization.

Clearance of pneumococcal cells from the nasal cavity occurs through various mechanisms. An innate immune response is initiated after the recognition of pneumococci by a sensor called pattern recognizing receptor, which has an integral role in the subsequent acquired immunity (van Rossum et al., 2005; Zhang et al., 2009). Bacterial phagocytosis caused by effector cells, especially macrophages, is followed by the processing of pneumococcal cells (Davis et al., 2011). The functions of macrophages are necessary for pneumococcal clearance; and macrophage activation reduces bacterial burden (Zhang et al., 2009; Iwanaga et al., 2015). On the contrary, it has also been reported that the clearance of pneumococci cannot be achieved in the absence of T-helper 17 response and CD4-positive T cell immunity (Malley et al., 2005; Trzcinski et al., 2008; Zhang et al., 2009). These cellular responses regulate macrophage/monocyte influx in the later phase of colonization and protect against further pneumococcal infection. Thus, research on novel preventive treatments for pneumococcal diseases should focus on these dynamic interactions within the immune system.

Hochu-ekki-to (TJ-41), a traditional Japanese herbal (kampo) medicine, is clinically used to treat patients with weakened or immunocompromised conditions owing to various diseases. It exhibits immunomodulatory and protective effects against various types of viral, bacterial, and fungal infections (Abe et al., 1999; Hossain et al., 1999; Yamaoka et al., 2001; Yan et al., 2002; Dan et al., 2013). Studies have shown that innate immunity responses are enhanced by TJ-41 treatment. However, it is not clear whether TJ-41 is effective against pneumococcal colonization by inducing host defense response.

We hypothesized that TJ-41 treatment might be a novel strategy to prevent pneumococcal diseases. In the present study, we aimed to evaluate the effect of TJ-41 in a pneumococcal colonization mouse model and investigate the underlying immunological mechanisms.

MATERIALS AND METHODS

Laboratory Animals

Specific pathogen-free, 6 to 8-week-old, female BALB/c mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). We used 6 to 10-week-old female IL-17A knockout (KO; *Il-17a*$^{-/-}$) mice in the BALB/c background, established previously by the Institute of Medical Science, University of Tokyo (Nakae et al., 2002; Ishigame et al., 2009). All mice were maintained in the Laboratory Animal Research Centre of Toho University School of Medicine. All experiments were performed according to the guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and were approved by the Institutional Animal Care and Use Committee (approval number 18-51-386).

Bacterial Strain and Growth Conditions

We used a clinical isolate of *Streptococcus pneumoniae* strain 741 (serotype 19F) that is stocked in the Department of Microbiology and Infectious Diseases (Toho University School of Medicine, Tokyo, Japan); this strain was also used in our previous mouse pneumonia models (Tateda et al., 1996; Yoshioka et al., 2016). *Streptococcus pneumoniae* "ATCC 6303" serotype 3 strain was obtained from the American Type Culture Collection (ATCC). The cells were incubated on Mueller–Hinton agar (Becton, Dickinson [BD] & Co., Sparks, MD, USA) supplemented with 5% defibrinated sheep blood at 37°C for 18–24 h. The culture was scraped from the agar and suspended in Todd–Hewitt broth (Difco, Detroit, MI, USA) supplemented with 0.5% yeast extract (Bacto™ Yeast Extract, BD) and cultured at 37°C in 5% CO_2. Unlysed cells in the log phase (7 h after incubation) were collected by centrifugation. The cells were quantified by measuring the absorbance of the cell suspension at 600 nm, and then plotting the optical density on a standard curve generated using known colony-forming unit (CFU) values. The bacterial culture was then diluted to the desired concentration.

Treatment Agent

The Kampo herbal formulation, Hochu-ekki-to extract [(TJ-41; Lot No.2170041010), provided by Tsumura Co., Tokyo], is prepared as a spray-dried powder of hot water extract composed of *Astragali radix* (4.0 g), *Atractylodis lanceae rhizoma* (4.0 g), *Ginseng radix* (4.0 g), *Angelicae radix* (3.0 g), *Bupleuri radix* (2.0 g), *Zizyphi fructus* (2.0 g), *Aurantii nobilis pericarpium* (2.0 g), *Glycyrrhizae radix* (1.5 g), *Cimicifugae rhizoma* (1.0 g), and *Zingiberis rhizoma* (0.5 g). For *in vivo* experiments, TJ-41 was dissolved in water and orally administered via gavage at a dose of 2,000 mg/kg/day, starting from 14 days before colonization. This dosage was determined based on the results of previous studies (Utsuyama et al., 2001; Yan et al., 2002). Mice in the control group were administered only water. *In vitro*, TJ-41 was mixed with Roswell Park Memorial Institute (RPMI) 1640 medium at several concentrations.

Pneumococcal Colonization Model

BALB/c mice were anesthetized intramuscularly with ketamine 50 mg/kg body weight and xylazine 10 mg/kg, and then

intranasally inoculated with *S. pneumoniae* in 10 μL of saline containing 6×10^4 CFUs for asymptomatic colonization. The colonization protocol was similar to that reported previously for developing pneumococcal colonization mouse models (Mccool and Weiser, 2004; van Rossum et al., 2005; Trzcinski et al., 2008; Zhang et al., 2009). At the indicated time points, bacterial burden in the nasal wash [400 μL in phosphate-buffered saline (PBS)] was measured by plating 10-fold serial dilutions of nasal wash onto blood agar plates. The plates were subsequently incubated at 37°C under 5% CO_2 overnight; after 24 h, CFUs were enumerated.

Cell Analysis by Flow Cytometry

The nasal tissue and nasal-associated lymphoid tissue (NALT) were harvested from mice as described previously (Asanuma et al., 1997; Wu et al., 1997). The excised tissue was minced and incubated at 37°C under 5% CO_2 for 50 min in Roswell Park Memorial Institute 1640 medium containing 2% fetal bovine serum, 0.5 mg/mL collagenase D (Roche, Basel, Switzerland), and 150 μg/mL DNase (Roche). The samples were passed through a 70-μm cell strainer (Falcon; Thermo Fisher Scientific, Waltham, MA, USA). The cells were centrifuged, and the red blood cells were lysed using BD Pharm Lyse (BD Biosciences). Cell suspensions with stain buffer (phosphate-buffered saline) containing 2% bovine serum albumin and 2 mM EDTA were incubated with an Fc-receptor-blocking antibody (anti-mouse CD16/32, clone 93) for 15 min on ice to reduce non-specific antibody binding. The cells were then washed with stain buffer and surface stained for 30 min on ice using each experimental design combination of peridinin chlorophyll protein complex/Cy5.5 anti-mouse CD11b antibody (clone M1/70), allophycocyanin (APC)/Cy7 anti-mouse/human CD11c antibody (clone N418), fluorescein isothiocyanate anti-mouse Ly6G antibody (clone 1A8), phycoerythrin anti-mouse CD86 (clone GL-1), phycoerythrin/Cy7 anti-mouse F4/80 antibody (clone BM8) (all from BioLegend), and APC anti-mouse MHC class II (I-A/I-E) antibody (clone M5/114.15.2; Tonbo Biosciences). Flow cytometry was performed with BD FACS-Canto II (BD Biosciences), and the results were analyzed using FlowJo software (TreeStar). The gating strategy for flow cytometry is illustrated in **Supplementary Figure 1**.

Intracellular Cytokine Staining of NALT Cells

Intracytoplasmic cytokine staining of NALT cells was performed using the Cytofix/Cytoperm Plus kit according to the manufacturer's protocol (BD Biosciences), as described previously (Kusaka et al., 2018). To assess intracellular cytokine expression, the cells were treated with phosphomolybdic acid (PMA; 25 ng/mL), ionomycin (1 μg/mL), and GolgiPlug (1 μg/mL; BD Biosciences) and incubated for 4 h. The cells were then stained for cell-surface markers and fixed for 20 min on ice. After washing, the cells were stained for intracytoplasmic IL-17A expression with APC anti-mouse IL-17A (clone eBio17B7) or APC IgG isotype control diluted in Perm/Wash solution (BD Biosciences) for 30 min and detected using FACS-Canto II; the results were analyzed using FlowJo software (**Supplementary Figure 1**).

Isolation of Immune Cells From Nasal Cavity and Cell Experiments *in vitro*

The nasal tissue and NALT were harvested from mice, and the cells were collected as described above. White blood cells were isolated with Percoll solution and plated at a concentration of 5×10^5 cells/well in a total volume of 100 μL. The cells were stimulated with or without whole cell antigen (ethanol-killed *S. pneumoniae* serotype 19F) for 3 days with either TJ-41 (1 mg/mL) containing RPMI medium or medium only. Whole cell antigen (WCA) was prepared with ethanol as reported previously (Malley et al., 2001). Macrophage depletion was accomplished by treating the cells with clodronate liposome (30 μg/well; Hygieia Bioscience) 1 day before the experiment. Two hours after the administration of clodronate liposome, the culture medium was washed. Adherent cells were obtained by removing floating cells.

RNA Isolation and Gene Expression Analysis

The total RNA was isolated from the tissue or cells using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. For quantitative reverse transcription polymerase chain reaction (PCR) analysis, 1 μL of total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Data analysis was performed on the QuantStudio3 Real Time PCR System (Applied Biosystems) using the SYBR Green real-time RT-PCR technique. The following PCR primers were used: *IL-17A*, 5′-TTTAACTCCCTTGGCGCAAAA-3′ (forward) and 5′-CTTTCCCTCCGCATTGACAC-3′ (reverse); and *β-actin*, 5′-AGAGGGAAATCGTGCGTGAC-3′ (forward); and 5′-CAA TAGTGATGACCTGGCCGT-3′ (reverse). Relative fold changes in transcript levels were calculated using the $2^{-\Delta\Delta CT}$ method (where CT is the threshold cycle, 45), using the housekeeping gene that encodes β-actin as a reference standard for the amount loaded and the quality of the cDNA.

Phagocytosis Assay

Streptococcus pneumoniae culture was suspended in RPMI medium with or without TJ-41. This suspension was added to macrophages pre-treated with TJ-41 for 2 h. The macrophages and bacterial cells were co-cultured for 1 h at 37°C under 5% CO_2 in humidified air, washed twice, and treated with penicillin (20 U/mL) for 30 min. The macrophages were subsequently washed twice and lysed in distilled water. The viable counts of phagocytized *S. pneumoniae* were determined. This assay was performed using MH-S cell line, and adherent cells derived from the nasal cavity of BALB/c mice.

Bacterial Growth Inhibition Assay

Streptococcus pneumoniae culture was suspended in RPMI medium and added to macrophage cell culture (MH-S). The macrophages and bacterial cells were co-cultured for 1 h at 37°C under 5% CO_2 in humidified air, and then washed twice and treated with penicillin (20 U/mL) for 30 min. The contents in the

wells were washed twice and then replaced with medium with or without TJ-41 and incubated for 2 h. Intracellular bacterial loads were measured before (0 h) and at the end of TJ-41 treatment (2 h).

Antigen-Presenting Capacity Assay

A part of the procedure was similar to that of the bacterial growth inhibition assay. After pneumococcal uptake and incubation with or without TJ-41, macrophages (MH-S, 1×10^5 cells/well) were washed and fixed with 2% paraformaldehyde (PFA) in PBS for 20 min, and then washed three times. T cells from BALB/c mouse spleen were isolated using the MACS Pan T Cell Isolation Kit (Miltenyi Biotec). The cells (1×10^6 cells/well) were then added to fixed macrophages and cultured for up to 3 days under Th17-polarizing condition using mouse IL-6 (20 ng/mL; BioLegend), mouse TGF-β (1 ng/mL; BioLegend), anti-mouse IFN-γ Ab (1 μg/mL; BioLegend), anti-mouse IL-4 Ab (1 μg/mL; BioLegend), anti-mouse CD3 Ab (10 μg/mL; BioLegend), and anti-mouse CD28 Ab (5 μg/mL). IL-17A production from T cells, reflecting the degree of antigen presentation by macrophages, was measured using mouse ELISA kits (R&D Systems, MN, USA) according to the manufacturer's protocols.

Statistical Analysis

All results are expressed as mean ± standard deviation. Data were analyzed using GraphPad Prism 8 software (GraphPad, Inc., La Jolla, CA, USA). The differences between the treatment and control groups were tested for significance using Mann–Whitney *U*-test. Statistical significance among more than three groups was determined using the one-way analysis of variance, followed by Tukey's multiple comparison *post-hoc* test for comparisons between groups. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Pneumococcal Colony Density Is Decreased by TJ-41 Treatment

An examination of the transitional change in bacterial load in the nasal cavity of the pneumococcal colonization model revealed that it was slightly decreased, although colonization in the nasal cavity was confirmed during the period of examination (**Supplementary Figure 2**). As shown in **Figure 1**, bacterial count in the nasal wash was significantly lower in the TJ-41-treated group than in the control group on days 14 and 28 post-colonization. The results suggest that TJ-41 has a positive effect on pneumococcal colonization clearance. We confirmed that TJ-41 has no direct effect against *S. pneumoniae* (data not shown).

TJ-41 Promotes Macrophage Activation and IL-17A Production in the Nasal Cavity

The flow cytometric analysis of the nasal tissue and NALT was performed to measure the number of inflammatory cells and the percentage of IL-17A-producing T cells, respectively. A significant increase in CD86-positive macrophages, defined as activated state, was observed in the TJ-41-treated group on days 14 and 28 (**Figure 2A**). There was no difference in the number of dendritic cells between the two groups (**Figure 2B**). On day 28, the neutrophil count in the TJ-41 treated group was significantly higher than that in the control group, but the neutrophil count in both groups was substantially lower than that on day 14 (**Figure 2C**). Notably, IL-17A production in TJ-41-treated mice was considerably upregulated (**Figure 2D**). Hence, the presence of macrophages and production of IL-17A in the nasal cavity may be key to understanding its underlying mechanisms. Furthermore, we administered TJ-41 to uncolonized mice, and examined cellular response in the nasal cavity after 6 weeks. Unlike the results observed in colonized mice, neither macrophage count nor IL-17A-producing T cell proportion differed between the uncolonized mice treated with TJ-41 and uncolonized control mice (data not shown). An assessment of the difference in anti-*S. pneumoniae* antibody titer of blood between the groups revealed no significant difference (**Supplementary Figure 3**).

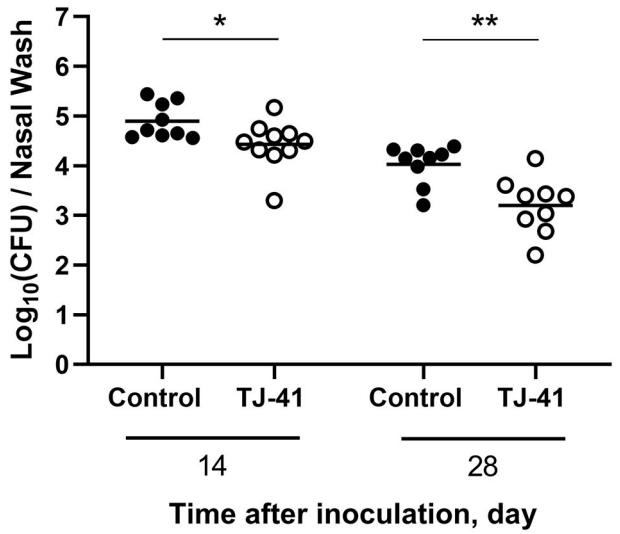

FIGURE 1 | Effect of Hochu-ekki-to (TJ-41) on nasopharyngeal bacterial count in the pneumococcal colonization mouse model. This figure shows colonization density in the nasal cavity 14 and 28 days after the inoculation of pneumococcus. Each symbol represents data from one mouse, and the horizontal bars represent values for that group ($n = 4$–5 mice in each group). Black symbols and clear symbols represent the control group and TJ-41-treated group, respectively. *$P < 0.05$ and **$P < 0.01$. Data were pooled from two independent experiments. CFU, colony forming unit.

Effect of TJ-41 on Pneumococcal Colonization Is IL-17A Dependent

To identify whether IL-17A upregulation in the TJ-41-treated group has an effect on the clearance of pneumococcal colonization, the above experiment was performed in IL-17A KO mice (**Figure 3**). Unlike the results in the wild-type mouse model, TJ-41 did not show a reducing effect on bacterial load in nasal wash on day 14 or 28 after pneumococcal colonization. Additionally, there was no difference in activated macrophage count between the TJ-41-treated and untreated

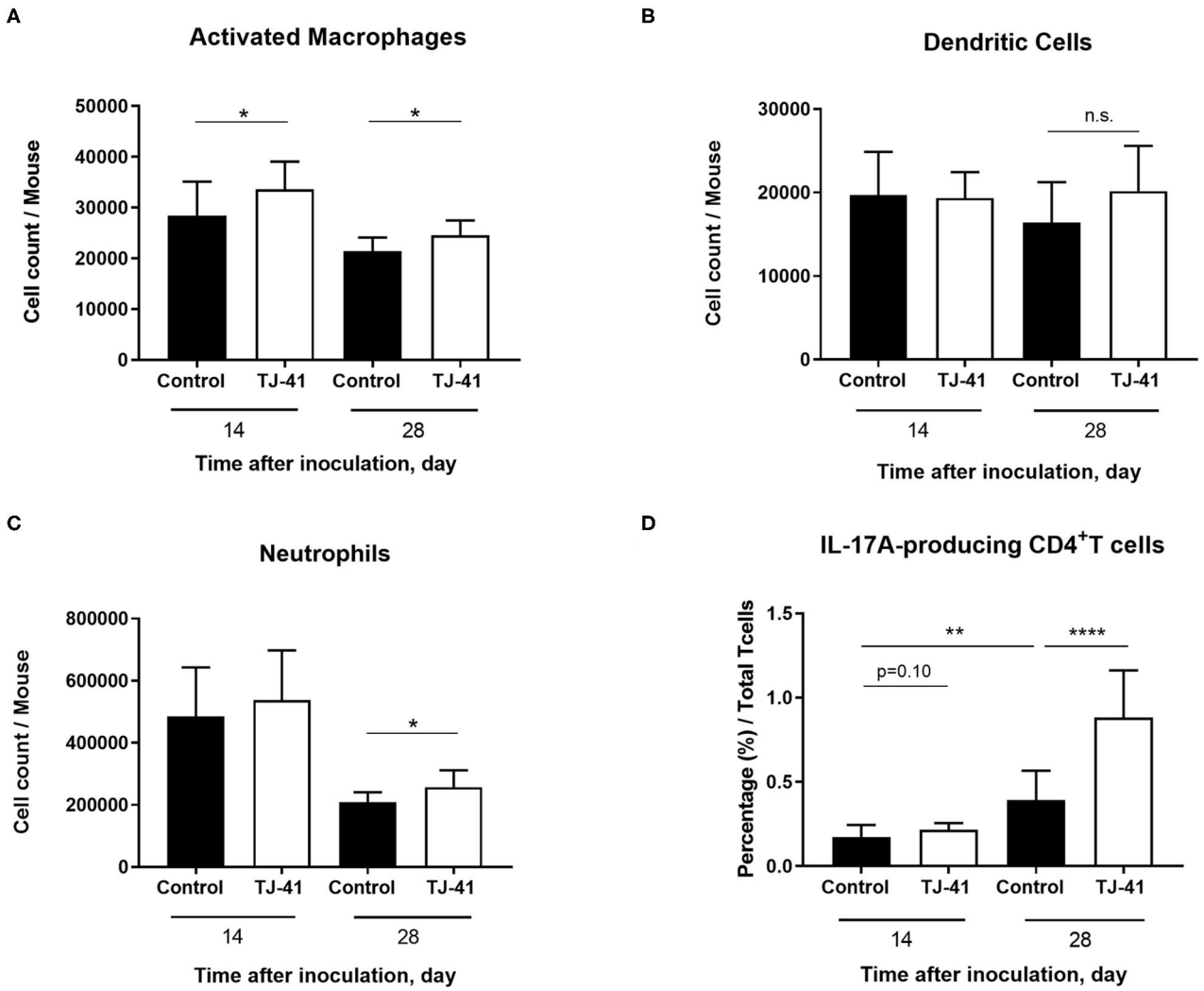

FIGURE 2 | TJ-41 treatment induces changes in interleukin 17A (IL-17A) production in T cells and accumulation of macrophages in the nasal cavity during pneumococcal colonization. The number of **(A)** activated macrophages ($CD86^+F4/80^+$ cells), **(B)** dendritic cells ($CD11c^+$MHC-classII$^+$ cells), and **(C)** neutrophils ($CD11b^+Ly6G^+$ cells) from the nasal tissue were measured 14 and 28 days after inoculation. **(D)** Percentage of IL-17A-producing T cells in the nasal associated lymphoid tissue (NALT) was assessed 14 and 28 days after pneumococcal inoculation. The bars indicate mean ± standard deviation ($n = 5$–7 mice in each group). $^*P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$. Data were pooled from two independent experiments.

groups. These results imply that IL-17A is necessary for enhanced pneumococcal clearance induced by TJ-41 in the nasal cavity.

Impact of IL-17A on Macrophage Activation and Phagocytosis in TJ-41 Treatment

The results presented in **Figures 2**, **3** raises the question whether IL-17A abundance influences macrophage activation and accumulation in the nasopharynx. Therefore, immune cells were collected from the nasal cavity *in vitro* for analysis and macrophage activation was assessed by flow cytometry. For WCA (ethanol-killed pneumococci) exposure, the TJ-41 treatment showed a significant increase in activated macrophage count in wild-type cells, but there was no significant difference in activated macrophage population between the control and TJ-41 groups of IL-17A KO cells (**Figure 4A**). Thereafter, we evaluated the phagocytic capacity of adherent cells; 75% of these cells were macrophages (confirmed by flow cytometry analysis), were obtained by removing floating cells (data not shown). In wild-type cells, macrophages in the TJ-41 group showed a higher uptake of pneumococcus than those in the control group (**Figure 4B**). No differential uptake was observed between the two groups of IL-17A KO cells (**Figure 4B**). The total count of macrophages did not significantly differ between the groups in both cell backgrounds (data not shown). These results suggest that TJ-41 treatment effect requires IL-17A production to provide an additional benefit of macrophage activation and phagocytosis.

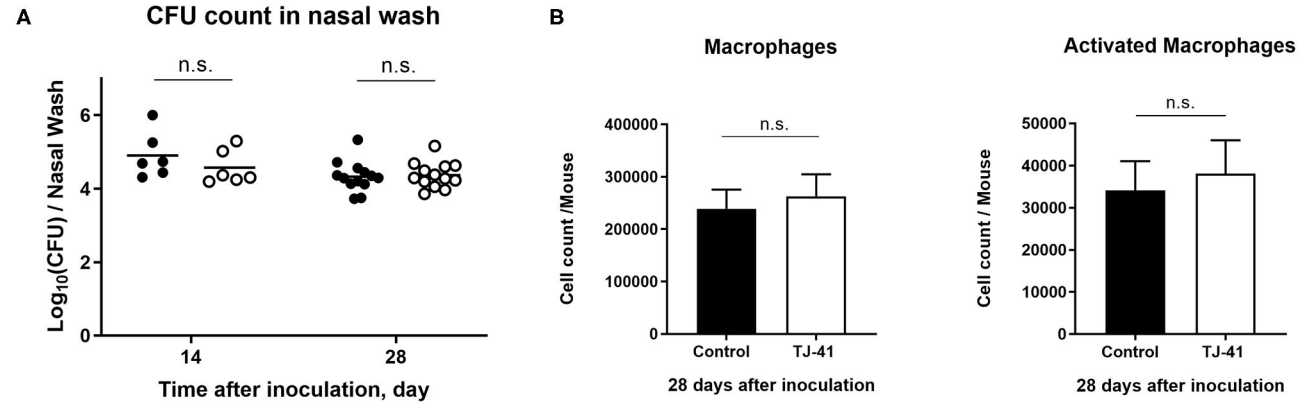

FIGURE 3 | Evaluation of the effect of TJ-41 on IL-17A knockout mice (*Il-17a*$^{-/-}$) during pneumococcal colonization. **(A)** Colonization density in the nasal cavity at 14 and 28 days after inoculation of pneumococcus. Each symbol represents data from one mouse, and the horizontal bars represent values for that group ($n = 3$ mice in each group on day 14 and $n = 7$ in each group on day 28). Black symbols and clear symbols represent the control group and TJ-41-treated group, respectively. Data were pooled from two independent experiments. **(B)** The number of macrophages ($F4/80^+$ cells) and activated macrophages ($CD86^+F4/80^+$ cells) from the nasal tissues on day 28 after inoculation. The bars indicate mean ± standard deviation ($n = 7$ mice in each group). Data were pooled from two independent experiments. n.s., not significant.

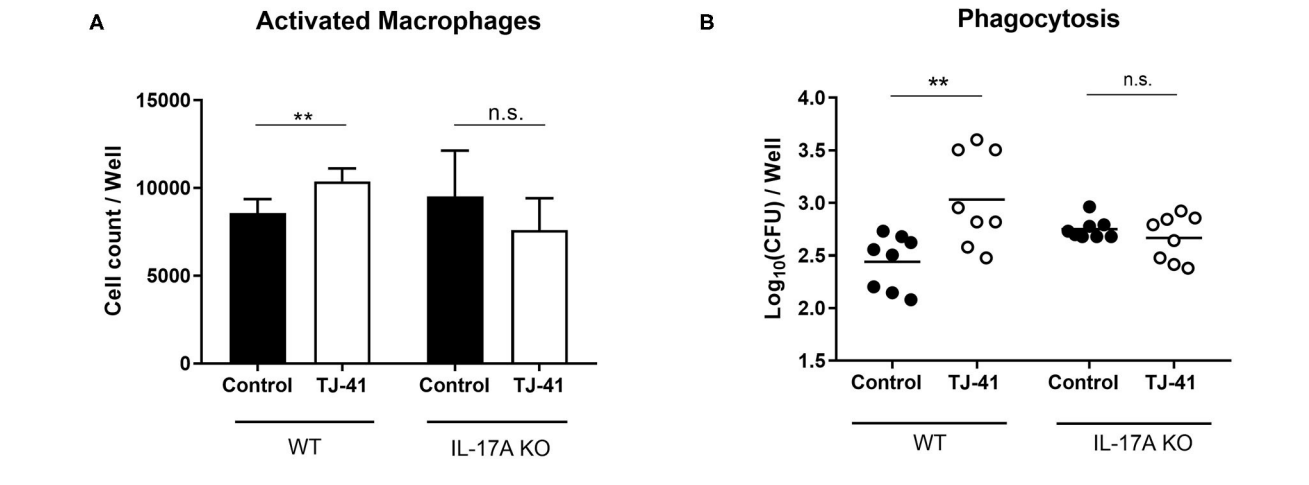

FIGURE 4 | *In vitro* difference in the effect of TJ-41 on macrophages between wild type (WT) and IL-17A KO mice exposed to WCA. Cells were collected from the nasal cavity of either WT or IL-17A KO mice and incubated with WCA for 3 days. **(A)** The difference in $CD86^+F4/80^+$ macrophage count between control and TJ-41-treated cells for WT or IL-17A KO mice. The bars indicate mean ± standard deviation ($n = 5$–6 wells in each group). **(B)** Intracellular bacterial load in adherent cells after pneumococcal exposure, and this represents phagocytic activity. Adherent cells, 75% of which were macrophages, were obtained by removing floating cells. The mean number of adherent cells was 3.0×10^4 cells/well and multiplicity of infection (MOI) was 33. Each symbol represents data from one well, and the horizontal bars represent values for that group. $^{**}P < 0.01$, n.s., not significant. Data are representative of two independent experiments. CFU, colony forming unit.

TJ-41 Stimulates IL-17A mRNA Expression of Nasopharynx-Derived Cells in a Macrophage-Dependent Manner

To determine how TJ-41 acts on the entire immune system during pneumococcal colonization of the nasal cavity, we measured *in vitro* IL-17A mRNA expression in nasal cavity-derived immune cells. There was a significant difference in IL-17A mRNA expression between the groups with or without WCA. A comparison of groups stimulated with WCA showed a significantly higher IL-17A mRNA expression in the TJ-41 group than in the control group (**Figure 5A**). The same procedure was conducted by using nasal cavity-derived cells with macrophage depletion (**Figure 5B**). In the absence of macrophages, enhanced IL-17A expression was not observed in the TJ-41 group treated with WCA, suggesting that TJ-41 accelerates IL-17A production in the nasal cavity via macrophage regulation.

TJ-41 Enhances Macrophage Phagocytosis of Pneumococcal Cells

We evaluated the direct immunomodulatory effects of TJ-41 on macrophages. The results of the phagocytic activity test showed considerable elevation in the intracellular bacterial burden of

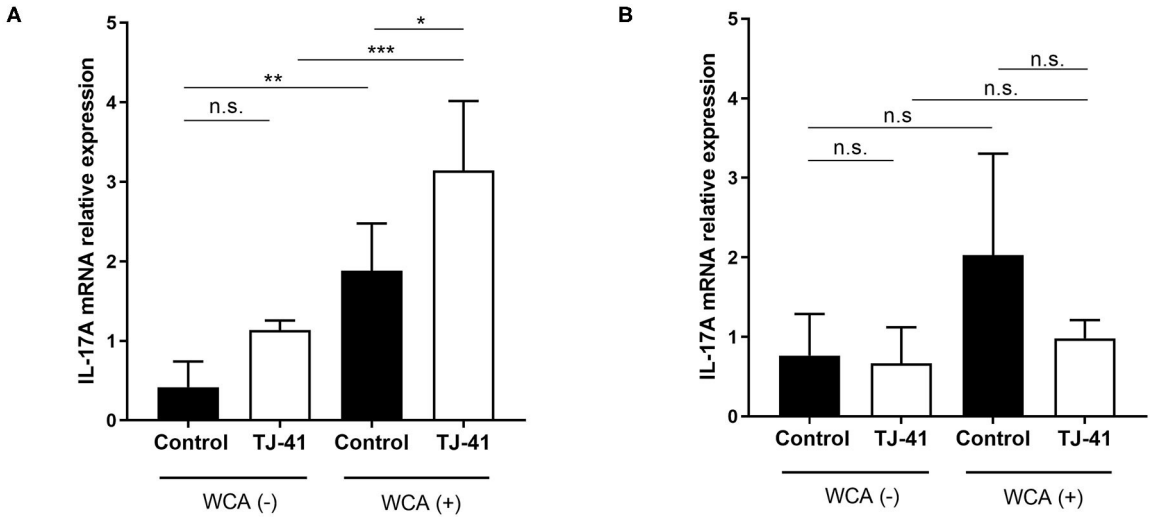

FIGURE 5 | *In vitro* IL-17A messenger RNA (mRNA) expression in immune cells collected from the nasal cavity following pneumococcal antigen exposure and TJ-41 administration. Nasal cavity-derived cells were incubated with or without whole cell antigens (WCA), and each group was administered either TJ-41 or medium only. Relative expression of *IL-17A* mRNA in each group on day 3 was measured for **(A)** normal cells and **(B)** cells without macrophages; treated with clodronate-liposome on day −1. The bars indicate mean ± standard deviation ($n = 4$–7 wells in each group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. n.s., not significant. Data are representative of three independent experiments.

MH-S cells, suggesting pneumococcal uptake in a concentration-dependent manner (**Figure 6A**). We also confirmed that TJ-41 enhanced pneumococcal uptake in adherent cells collected from the nasal cavity (**Figure 6B**). Similar tendencies were observed in the phagocytic activity tests using different cell lines or by inoculation of different serotype pneumococci, suggesting that the phenomenon is not limited to a specific cell line or serotype (**Supplementary Figure 4**).

Antigen-Presenting Capacity and Inhibition of Intracellular Bacterial Growth Are Enhanced by TJ-41

After pneumococcal uptake and the subsequent TJ-41 treatment, MH-S cells were fixed with PFA and co-incubated with T cells to assess the antigen-presenting capacity of macrophages via cytokine production. As shown in **Figure 7A**, the group subjected to pneumococcal uptake and TJ-41 treatment showed a considerable increase in IL-17A production from T cells compared with that in the control group, implicating that brisk antigen presentation was induced by TJ-41. Finally, we evaluated how the bacterial count in the cells was changed by the action of TJ-41 on MH-S. After pneumococcal exposure, MH-S cells were treated with or without TJ-41. As shown in **Figure 7B**, the intracellular bacterial count at 2 h was higher than that at 0 h, suggesting the growth of pneumococci in MH-S cells. A comparison between the two groups at 2 h revealed that the number of bacteria in MH-S cells was significantly reduced in the TJ-41 treated group compared with that in the control group. These results indicate that TJ-41 is effective in inhibiting the growth of pneumococci in MH-S cells.

DISCUSSION

Here, we revealed that TJ-41 treatment promotes the clearance of pneumococcal colonization in a mouse model. Macrophage count and IL-17A production in the nasal cavity were increased in TJ-41-treated mice. Additionally, accelerated pneumococcal clearance was achieved in an IL-17A-dependent manner. Higher IL-17A abundance caused additional macrophage activation and enhanced phagocytosis. Upregulation of IL-17A mRNA expression in the nasal cavity-derived cells by TJ-41 treatment was found to be macrophage dependent. The results of MH-S cell experiments demonstrated the direct effects of TJ-41 on macrophage functions.

Nasopharyngeal colonization of *S. pneumoniae* is the first step toward the development of pneumococcal disease and transmission (Wolter et al., 2014; Weiser et al., 2018). An increase in the load of pneumococci in the nasal cavity has been shown to facilitate pneumococcal transmission in influenza A virus coinfection models (Short et al., 2012). Furthermore, Zafar et al. (2016) reported a relationship between prolonged pneumococcal shedding and high transmission rate in infant monoinfection models. Reducing bacterial burden in the nasal cavity may act as a prevention mechanism against pneumococcal infection and transmission. TJ-41 has the potential to function as a part of a preventive strategy against pneumococcal infections; however, further research is needed to confirm its efficacy in humans.

Macrophages have been shown to be major effector cells in studies on pneumococcal colonization models. Macrophage depletion allows the persistence of pneumococcal cells even in the later phase of colonization (Zhang et al., 2009). Iwanaga et al. reported that macrophage activation via macrolide treatment results in a lower burden of pneumococci in the nasopharynx

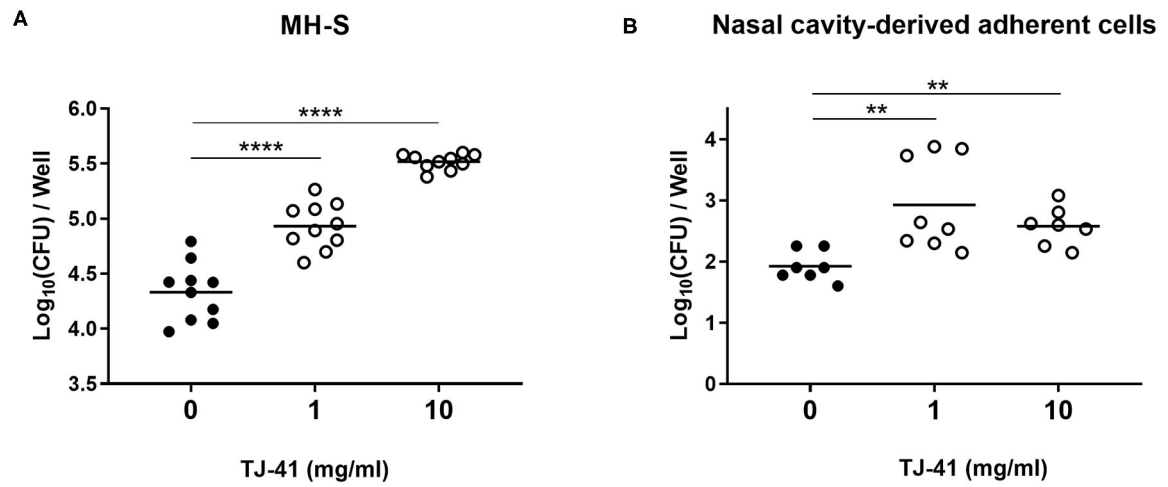

FIGURE 6 | Phagocytic activity of MH-S cells or nasal cavity-derived adherent cells. **(A)** Intracellular bacterial loads of MH-S cells after pneumococcal exposure with or without TJ-41 were measured. Cells were plated at a density of 1×10^5 cells/well. MOI was 10. **(B)** Phagocytic activity of adherent cells derived from the nasal cavity was measured. Mean number of adherent cells was 1.0×10^4 cells/well and MOI was 100. Each symbol represents data from one well, and the horizontal bars represent values for that group. $^{**}P < 0.01$ and $^{****}P < 0.0001$. CFU, colony forming unit.

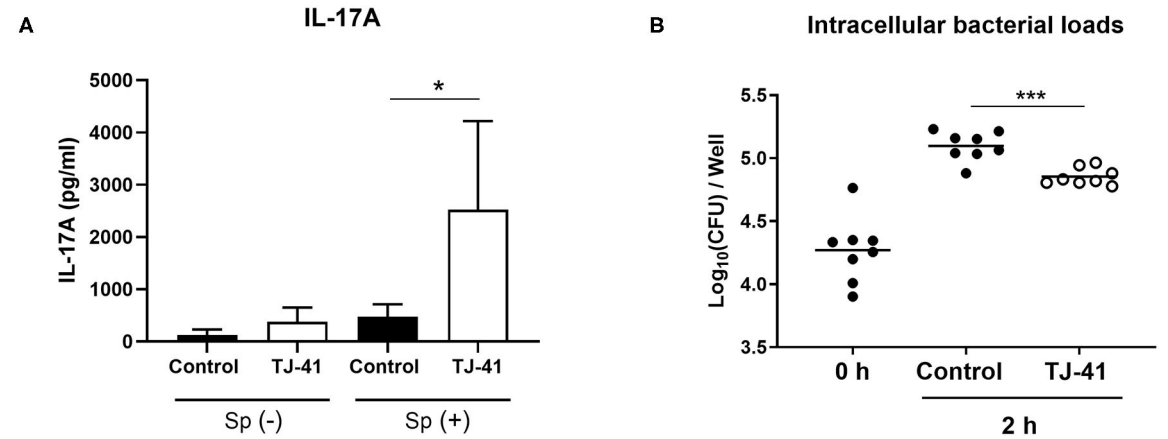

FIGURE 7 | Direct effects of TJ-41 on macrophage antigen presentation to T cells and bacterial growth inhibition *in vitro*. **(A)** Assessment of the antigen-presenting capacity of MH-S cells with TJ-41 treatment. MH-S cells (1×10^5 cells/well) were divided into either pneumococcal exposure (MOI = 10) or medium only group, and incubated with or without TJ-41 (1 mg/mL). Fixation using 2% PFA in PBS was performed and T cells (1×10^6 cells) were added to the fixed MH-S cells under the condition of Th17 polarization. IL-17A concentration in medium on day 3 was measured using ELISA. The bars indicate mean ± standard deviation ($n = 4$ wells in each group). **(B)** Inhibitory effect of TJ-41 on bacterial growth in MH-S cells. After pneumococcal phagocytosis (MH-S: 1×10^5 cells/well, MOI = 10) without any treatment, the cells were incubated for 2 h with TJ-41 (1 mg/mL) or medium only. Intracellular bacterial loads before (0 h) and after (2 h) treatment were enumerated. Each symbol represents data from one well, and the horizontal bars represent values for that group. $^{*}P < 0.05$ and $^{***}P < 0.001$. These data are representative of at least two independent experiments. Sp, *Streptococcus pneumoniae*. CFU, colony forming unit.

(Iwanaga et al., 2015). On the basis of our results, TJ-41 modulates macrophage functions and promotes their phagocytic activity and bacterial growth inhibition. These findings suggest that the direct effects of TJ-41 on macrophages may contribute to a reduction in bacterial load in the nasal cavity to some degree. However, in accordance with the results that no significant bacterial reduction was achieved with TJ-41 treatment in the IL-17A KO mouse model, the direct activation of macrophages cannot achieve clearance by itself. Hence, in addition to macrophage functions, the effect of TJ-41 on IL-17A production should be considered when explaining the background of accelerated pneumococcal clearance.

Of note, IL-17A production in the nasal cavity is considerably upregulated in the later phase of pneumococcal colonization with TJ-41 treatment. Moreover, a similar phenomenon was observed *in vitro*; immune cells derived from the nasal cavity

expressed a higher level of *IL-17A* mRNA in the presence of WCA and TJ-41, and this was found to be macrophage dependent. Furthermore, we demonstrated that macrophages exhibited a substantial increase in the antigen-presentation capacity with pneumococcal exposure and TJ-41 treatment, as evidenced by the subsequent IL-17A production from contacted T cells. Overall, it can be presumed that TJ-41 causes IL-17A upregulation via macrophage activation, especially by promoting antigen presentation. The interaction between innate immunity and IL-17A-mediated immune response is indispensable in the clearance of pneumococcal colonization (van Rossum et al., 2005; Zhang et al., 2009; Davis et al., 2011; Dorrington et al., 2013). However, whether extrinsic activation of the innate immunity provides an additional benefit of acquired immunity has not been validated. Using TJ-41 in the present study, we demonstrated the potential beneficial effect of activating innate immunity on IL-17A-mediated immune response in pneumococcal colonization.

We obtained interesting findings from the experiments using IL-17A KO mice. A significantly higher number of activated macrophages was observed after TJ-41 treatment both *in vivo* and *in vitro*. Contrary to the findings in wild-type model, the difference in the upregulation of macrophages was not observed in the IL-17A KO pneumococcal colonization model. Similarly, TJ-41 treatment did not show additional macrophage activation and enhanced phagocytosis in IL-17A KO mouse-derived nasal cells stimulated with WCA. TJ-41 may help macrophages to lower pneumococcal colonization density by direct modulation. However, a substantial decrease in bacterial load occurred as a result of the activity of IL-17A. A previous study reported that IL-17 acts as a recruitment and survival factor for macrophages (Sergejeva et al., 2005). The presence of IL-17A in the nasal cavity allows macrophages/monocytes to accumulate and clear the pneumococci (Zhang et al., 2009). Furthermore, Wright et al. demonstrated a significant and dose-dependent increase in pneumococcal killing by human alveolar macrophages when exposed to recombinant IL-17A (Wright et al., 2013). In light of these findings, the accelerated pneumococcal clearance of TJ-41 in this study can be considered a result of increased IL-17A production. Relatively higher abundance of IL-17A driven by TJ-41 may cause additive macrophage activation and accumulation. While previous studies validated the findings via exogenous administration of IL-17A, using our TJ-41 treatment model, we succeeded in demonstrating the role of endogenous IL-17A upregulation in the additional activation of macrophage and accelerated pneumococcal clearance.

Our study had some limitations. First, as TJ-41 is an extract of various types of plants, it was difficult to identify its most active components or their combinations. Because TJ-41 has a long-standing history and a variety of clinical applications, its effects and mechanisms can be attributed to one drug. However, further studies should be performed to determine the specific components that act on immunity against pneumococcal colonization, to develop a new treatment. Second, we did not assess whether the reduction in colonization density and IL-17A enhancement by TJ-41 resulted in host protection against invasive infection or pneumococcal transmission. Pneumococcal colonization has been shown to reduce bacterial burden in lung infection by inducing cellular and humoral immunity (Wilson et al., 2015). Enhancement of the IL-17A-mediated immune response by TJ-41 may contribute to additional host defenses against infection, and this should be addressed in future studies.

In summary, we employed a mouse model to reveal the efficacy of TJ-41 against pneumococcal colonization. As a result, we found that direct stimulation of macrophages by TJ-41 may be an inducer of the subsequent IL-17A enhancement and additional activation of effector cells, followed by accelerated clearance of pneumococci. Such findings shed light on a strategy for the prevention of pneumococcal diseases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This animal study was reviewed and approved by Toho University Animal Care and User Committee.

AUTHOR CONTRIBUTIONS

SN conceived the study, performed the procedure, analyzed the data, and wrote the manuscript. SKi conceived the study, advised on all experiments, analyzed the data, and wrote the manuscript. KM assisted with the experimental procedure. CK provided advice on the procedure and analyzed the data. YI, KT, and SKo supervised the whole experiment and proofread the manuscript. All authors approved the submitted paper.

FUNDING

This work was supported by the Japan Society for the Promotion of Science KAKENHI (Grant No. 17K10033 to SKi) and a Grant-in Aid for Private University Research Branding Project from the MEXT.

ACKNOWLEDGMENTS

We thank Tsumura Co., Tokyo, Japan for gifting Hochu-ekki-to extract.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.569158/full#supplementary-material

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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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frontiers in Cellular and Infection Microbiology

REVIEW
published: 23 December 2020
doi: 10.3389/fcimb.2020.613287

Sugar-Coated Killer: Serotype 3 Pneumococcal Disease

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Capsular polysaccharide (CPS), which surrounds the bacteria, is one of the most significant and multifaceted contributors to *Streptococcus pneumoniae* virulence. Capsule prevents entrapment in mucus during colonization, traps water to protect against desiccation, can serve as an energy reserve, and protects the bacterium against complement-mediated opsonization and immune cell phagocytosis. To date, 100 biochemically and serologically distinct capsule types have been identified for *S. pneumoniae*; 20 to 30 of which have well-defined propensity to cause opportunistic human infection. Among these, serotype 3 is perhaps the most problematic as serotype 3 infections are characterized as having severe clinical manifestations including empyema, bacteremia, cardiotoxicity, and meningitis; consequently, with a fatality rate of 30%–47%. Moreover, serotype 3 resists antibody-mediated clearance despite its inclusion in the current 13-valent conjugate vaccine formulation. This review covers the role of capsule in pneumococcal pathogenesis and the importance of serotype 3 on human disease. We discuss how serotype 3 capsule synthesis and presentation on the bacterial surface is distinct from other serotypes, the biochemical and physiological properties of this capsule type that facilitate its ability to cause disease, and why existing vaccines are unable to confer protection. We conclude with discussion of the clonal properties of serotype 3 and how these have changed since introduction of the 13-valent vaccine in 2000.

Keywords: ***Streptococcus pneumoniae*, invasive pneumococcal disease, serotype 3, synthase-dependent pathway, vaccine escape, capsule production, wzy-dependent pathway**

OPEN ACCESS

Edited by:
Yuki Kinjo,
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Reviewed by:
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Susu M. Zughaier,
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Specialty section:
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *05 October 2020*
Accepted: *23 November 2020*
Published: *23 December 2020*

Citation:
Luck JN, Tettelin H and Orihuela CJ (2020) Sugar-Coated Killer: Serotype 3 Pneumococcal Disease. Front. Cell. Infect. Microbiol. 10:613287. doi: 10.3389/fcimb.2020.613287

INTRODUCTION

Streptococcus pneumoniae (*Spn*), also known as the pneumococcus, is a Gram-positive encapsulated bacterium commonly identified by its lancet-shaped diplococcal morphology. An opportunistic inhabitant of the nasopharynx, humans are the only natural host for *Spn*. Problems arise in colonized individuals when pneumococci ascend the Eustachian tubes to the middle ear where they can cause otitis media. Alternatively, when pneumococci are aspirated into the lower respiratory tract and induce pneumonia. Notably, the likelihood of either of these events is starkly increased among very young children, those who are immunocompromised, the elderly, and those who are experiencing or have recently experienced viral infection (Harboe et al., 2009; Klugman et al., 2009; Infante et al., 2015). Critically, and once established in the lower respiratory tract, the pneumococcus can cause bacteremia, i.e. invasive disease. This occurs in ~30% of

hospitalized individuals (Center for Disease Control, 2015). As result *Spn* is a leading cause of bacterial sepsis following pneumonia (Mayr et al., 2014). Bloodborne *S. pneumoniae* are not restricted to the vasculature and able to invade other organs including the central nervous system to cause meningitis and myocardium to cause cardiac complications (Brown et al., 2014; Shenoy et al., 2018; Africano et al., 2020). Not well appreciated, but a critical aspect of pneumococcal disease, is that survivors of severe infection, most often the elderly, often experience considerable sequelae. These include onset of frailty, cognitive declines, loss of independence, increased risk for adverse-cardiac events, and reduced lifespan (Lucas et al., 2016; Brooks and Mias, 2018). In the United States, it is estimated there were 31,400 (9.6 cases per 100,000 individuals) cases of invasive pneumococcal disease in 2018 with 3,480 directly attributable deaths (Centers for Disease Control and Prevention, 2018). Worldwide the number of serious pneumococcal infections is thought to exceed 1–1.5 million per year (World Health Organization, 2007). Thus, this bacterium, which has garnered the alias of "The Old Man's Friend", is a serious cause of morbidity and mortality worldwide and a leading health problem.

ROLE OF CAPSULE

Capsule is a gelatinous external layer produced by bacteria to protect against phagocytosis and other external hazards. In addition to being a common feature of almost all clinical isolates of *Spn*, capsule is a common feature of most extracellular bacterial pathogens (Nelson et al., 2007; Standish and Morona, 2015; Zhensong Wen, 2015). Bacterial capsules are polymers, with most versions being composed of a polysaccharide. Yet, capsule can be made of other molecules such as in *Bacillus anthracis* where it is a polypeptide (Zhensong Wen, 2015). Polysaccharide capsules are oligomers synthesized in sequential manner by a group of enzymes that link monosaccharides and other moieties (e.g., acetyl groups) together, and then assemble them as a repeating chain. In most *Spn*, capsule strands are attached to the bacterial surface via covalent bonds to peptidoglycan. One exception to this is serotype 3 pneumococcal capsule, which instead uses non-covalent interactions with phosphatidylglycerol (Yother, 2011; Cartee et al., 2005; Standish and Morona, 2015; Larson and Yother, 2017). Along such lines and given the extensive genomic variability in the enzymes responsible for construction of the oligosaccharide unit, the biochemical variability that exists between bacterial capsule types including within *Spn* is tremendous. As of 2020, 100 biochemically and serologically distinct versions of *S. pneumoniae* capsule have been identified each of which is encoded by distinct sets of enzymes arranged together in the capsule operon (Ganaie et al., 2020). Importantly, some serotypes, such as serotype 3, have historically been associated with a much higher attack rate and/or morbidity than other serotypes (Harboe et al., 2009; Grabenstein and Musey, 2014). Along such lines, observations by Sandgren et al. as well Brueggemann et al. suggest that the biochemical properties of the capsule directly contribute to virulence, as isogenic capsule switch mutants with the same accessory genome content were shown to have differences in virulence relative to the capsule type that was produced (Brueggemann et al., 2003; Sandgren et al., 2004).

Unencapsulated pneumococci are capable of nasopharyngeal colonization and due to selective pressure from the current vaccines (see below) are thought to be an emerging population. Unencapsulated pneumococci are capable of causing localized infections such as sinusitis, conjunctivitis and keratitis, as well as otitis media (Reed et al., 2005). Importantly, in some instances, unencapsulated isolates have had the capsule cassette replaced with either *aliC*, *aliD*, or *pspK* (Park et al., 2012; Geno et al., 2015). AliC and AliD are oligopeptide binding proteins and their deletion has pleiotropic effects on the bacterium including modulation of surface adhesins (Bradshaw et al., 2018). PspK is a homologue of the pneumococcal adhesin Choline binding protein A (Keller et al., 2013). In other instances where *Spn* is unencapsulated, there are mutations in the capsule operon that preclude capsule synthesis (Park et al., 2012; Geno et al., 2015). As is detailed below the absence of capsule profoundly changes the interactions the bacteria have with its host (Magee and Yother, 2001).

During nasopharyngeal colonization, individual pneumococci must avoid entrapment in mucus (Magee and Yother, 2001; Nelson et al., 2007). It is now recognized that capsule, specifically its electronegativity, acts to electrostatically repel mucus, which is also negatively charged, and thereby avoid entrapment and subsequent expulsion. Nelson et al. showed that isogenic strains carrying capsule with a net negative charge avoided mucous entrapment better than versions carrying neutral-charged capsule or an unencapsulated control (Nelson et al., 2007). Similarly, Li et al. showed that capsule electronegativity influenced the serotype's nasopharyngeal carriage prevalence (Li et al., 2013). A requirement for capsule becomes starkly apparent during pneumonia and invasive disease (Avery and Dubos, 1931). In a mouse model of intraperitoneal challenge and sepsis, the 50% lethal dose of an unencapsulated derivative of a serotype 3 isolate was 5.0×10^7 colony forming units (CFU), as opposed to the 50% lethal dose of its parent wildtype strain of 1 CFU (Watson and Musher, 1990). The reason for this is that unencapsulated pneumococci are exquisitely susceptible to opsonization and phagocytosis by host factors and immune cells, respectively (Oss, 1978; Kim et al., 1999; Wartha et al., 2007). C-reactive protein, components of the alternative complement cascade, ficolin, surfactant, and pre-existing antibodies against conserved host proteins generated as result of past colonization events or infection, individually and in complementary fashion opsonize pneumococci for phagocytosis (Kim et al., 1999; Kraiczy and Wurzner, 2006; Tian et al., 2009; Hyams et al., 2010a; Kjaer et al., 2013). Capsule is inhibitory of phagocytosis as it modulates recognition by the alternative complement pathway (Hyams et al., 2010a; Hyams et al., 2010b). Additionally, capsule prevents the receptors on immune cells from binding to these molecules even though they are bound to the bacterial surface (e.g., Fc receptor with cell wall bound antibody)

(Hyams et al., 2010a; Shenoy and Orihuela, 2016). Thus, the generation of antibody against the capsule itself, is critical for clearance of this pathogen during disease, though the amount of antibody required for clearance varies dependent on the serotype (Alonso De Velasco et al., 1995; Choi et al., 2016).

From the bacterium's perspective, encasement within capsule is not always optimal, as capsule also inhibits interactions between bacterial adhesins and host epithelial cells, a step required for colonization and disease (Moscoso et al., 2006; Bootsma et al., 2007; Sanchez et al., 2011a; Qin et al., 2013). One solution that the bacteria has is phase-variation where the bacterium stochastically and at low frequency switches back and forth between a transparent (low capsule) and opaque (high capsule) phase (Kim and Weiser, 1998; Kim et al., 1999). The transparent version is selected for in the upper airway during colonization, where cell attachment is necessary. Whereas the opaque phase is favored in the lower respiratory tract and bloodstream, during which risk for opsonophagocytosis is greater (Kim and Weiser, 1998; Nelson et al., 2007). It is also now known that pneumococci can shed their capsule and quickly modulate their binding affinity. Using scanning electron microscopy, Hammerschmidt et al. showed that the binding of individual pneumococci to epithelial cells results in the loss of capsule from its surface (Hammerschmidt et al., 2005). One explanation for this are findings by Kietzman et al. which showed that peptidoglycan-bound capsule is released by a hydrolase in response to the bacterium's exposure to cationic antimicrobial peptides (Kietzman et al., 2016). In this instance, capsule shedding serves dual purpose, protecting the bacterium from these positive-charged membrane-damaging products and exposing otherwise capsule-covered bacterial adhesins which in turn allow for intimate interactions with host cells. This concept is in agreement with past findings by our group which showed not only does capsule influence the relative exposure of pneumococcal virulence determinants, but this also occurs in a capsule type dependent manner (Sanchez et al., 2011b). As such, surface proteins must be compatible with the biochemical properties of capsule to be effective. Evidence supporting this includes work by Kelly et al. which showed conversion of a strain from one serotype to the next does not consistently increase or decrease virulence, and instead this was genome dependent (Kelly et al., 1994; Mizrachi Nebenzahl et al., 2004; Bootsma et al., 2007). Thus, the interplay between capsule and other factors on the surface are crucial.

Whereas capsular polysaccharide (CPS) from the K1 serotype of *Porphyromonas gingivalis*, *Neisseria meningitidis*, and numerous other bacterial pathogens have been demonstrated to be pro-inflammatory (d'Empaire et al., 2006; Graveline et al., 2007; Zughaier, 2011), work by Tuomanen et al. found that purified CPS from *Spn* is not inflammatory on its own (Tuomanen et al., 1985). Pneumococcal capsule's poor immunogenicity stood in stark contrast to the robust immune response elicited by purified pneumococcal cell wall, which is known to contain Toll-like receptor (TLR)-1/2 and TLR-2/6 binding tri- and di-acetylated teichoic and lipoteichoic acids, respectively. In fact, *Spn* capsule has been demonstrated to have a general anti-inflammatory role as it hinders the interaction of bacterial components, such as cell wall, with pathogen-associated molecular pattern (PAMP) receptors, such as TLRs. Evidence for this includes work by Kung et al. which showed that encapsulated pneumococci elicited less CXCL8 IL-8, a potent chemokine, from epithelial cells than isogenic unencapsulated mutants (Kung et al., 2014). These differences in capsule immunogenicity between bacterial species are likely the result of each capsule type's distinct biochemical properties and in turn their ability to be recognized by the host (d'Empaire et al., 2006; Graveline et al., 2007; Zughaier, 2011; Kung et al., 2014).

Because capsule is highly abundant and surface exposed, antibody against capsule is typically highly opsonic and protective against invasive disease. Capsule is currently utilized as the principal antigen in multiple present-day vaccines against pathogens such as *Haemophilus influenzae* type B and *Salmonella enterica* serovar Typhi (Centers for Disease Control and Prevention, 2015). CPS is also the primary antigen in the three currently licensed vaccines against *Spn*. These vaccines include purified polysaccharides from the 10 (serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F), 13 (plus serotypes 3, 6A, and 19A), and 23 (plus serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) most virulent serotypes of *Spn*, respectively. The 10- and 13- valent versions composed of capsule conjugated to a protein carrier, the 23-valent being composed of purified CPS alone. Of note, the 10-valent conjugate vaccine is not approved for use in the USA but is extensively used in many other countries.

It is at this point important to note that CPS, not being a protein, is not presented by dendritic cells to CD4+ cells in context of MHC and is therefore a T-cell independent antigen. Key implications of this include that children under 5 fail to develop protective immunity against capsule following immunization (Bonten et al., 2015; Center for Disease Control, 2015). Moreover, immunization with purified polysaccharide elicits only a modest and short-lived, <5 years, protective response among adults (Butler et al., 1999; Center for Disease Control, 2015). It is for this reason that the 10- and 13-valent versions of the pneumococcal vaccine consist of CPS conjugated to a protein carrier. Processing of the protein/polysaccharide by antigen presenting cells now occurs in context of MHC-II presentation resulting in CD4+ T cell involvement, generation of long-lived memory B-cells, and robust immunogenicity in children (Malley et al., 2005; Center for Disease Control, 2015; Shenoy and Orihuela, 2016). The resultant antibody titers against these conjugated capsule types are not only typically sufficient to protect against invasive disease caused by these serotypes but also against nasopharyngeal colonization and thereby confer herd immunity by disrupting transmission (Andrews et al., 2014; Choi et al., 2016). Unfortunately, and as is discussed below, serotype 3 is an important exception as the amount of antibody required for protection is not elicited by the current conjugate vaccine (Choi et al., 2016). Other serotypes that require higher antibody concentrations for efficient opsonization include 1 and 5; albeit this is most likely the result of a combination of factors, not just the biochemical properties of capsule, such as the carriage of virulence determinants that block amplifying complement sensitivity (Hyams et al., 2013; Burton et al., 2017).

Finally, and in contrast to capsule-based vaccines, naturally acquired immunity against *Spn* is the result of antibody generated against the bacterium's surface proteins rather than the capsule (Wilson et al., 2017). For this reason, and given prior exposures as children, healthy adults do not typically develop pneumococcal disease. Ongoing efforts to improve on the existing vaccines are focused on expanding the coverage of the 10- and 13-valent vaccine to include more serotypes, identification of conserved pneumococcal proteins that might be co-administered to provide protection against the serotypes not included in the vaccine, and even development of whole cell pneumococcal vaccines that would generate antibody and a T-cell response against conserved surface proteins (Xu et al., 2015; Pichichero et al., 2016; Pichichero, 2017; Campo et al., 2018; Businesswire, 2020; Feldman and Anderson, 2020).

UNIQUE FEATURES OF SEROTYPE 3 AND ITS IMPORTANCE IN DISEASE

Pneumococcal serotypes were named in numerical fashion based on the order they were identified. Thus, and in general, the lower number serotypes were initially more frequent causes of serious pneumococcal disease. This has changed as result of the conjugate vaccines which have reduced incidence of disease caused by the lower numbered serotypes, with now higher numbered serotypes becoming more prevalent as replacement strains. Clinical isolates producing serotype 3 capsule are distinct from most other pneumococcal isolates as they have a highly mucoid appearance and a wet phenotype when grown on plates (**Figure 1**). In contrast, other serotypes form more discrete colonies. Given their considerable difference in colony appearance, serotype 3 isolates were not initially recognized as being pneumococci (Howard and Perkins, 1901; Watson et al., 1993). In 1901, pathologists William Howard and Roger Perkins published a paper describing their isolation of an unfamiliar streptococcus from the abdomen of a young woman who had died as result of peritonitis (Howard and Perkins, 1901). The investigators observed the emblematic mucoid colonies, describing their appearance as "dewdrops" (Howard and Perkins, 1901). In their study, they recognized a series of others who had isolated bacteria with properties similar to their own and offered the name *Streptococcus mucosus*.

CPS production in pneumococci occurs by one of two mechanisms: Wzy-mediated or synthase-mediated synthesis. So far, Wzy-mediated capsule production has been observed in all but two pneumococcal serotypes; the two exceptions being serotypes 3 and 37 which instead rely on the synthase-mediated pathway (Yother, 2011; Standish and Morona, 2015; Zhensong Wen, 2015). The loci responsible for either mechanism is located at the same region within the *Spn* genome, between the *aliA* and *dexB* genes, except for serotype 37 which operates outside of the canonical CPS locus (Llull et al., 1999; Llull et al., 2001). Within the Wzy-mediated locus are the four highly conserved genes *cpsA*, *B*, *C*, and *D*. Genes encoding the serotype specific glucosyltransferases can be found next along with those encoding the conserved Wzy polymerase and flippase (Yother, 2011; Standish and Morona, 2015; Zhensong Wen, 2015). In Wzy-mediated synthesis, serotype-specific UDP-glycosyltransferases assemble a short polysaccharide chain with an enzyme-specific pattern inside the bacterial cytoplasm. The chain is then transferred to the Wzx flippase which reorients the chain into the periplasm (**Figure 2**). The short polysaccharide is then added on to the previously synthesized chain by the Wzy polymerase. Once the chain has reached a designated length, the polysaccharide is released and bound covalently to the cell wall (Yother, 2011; Standish and Morona, 2015; Zhensong Wen, 2015). Considerable diversity in the UDP-glycosyltransferases encoded within the capsule operon results in Wzy-mediated capsules having multitudes of monosaccharides ordered in specific patterns, the basis of serotypes (Standish and Morona, 2015; Geno et al., 2015). Serotype 3 CPS production utilizes synthase-mediated processes. Though the locus of serotype 3 is organizationally similar to that seen in strains that instead utilize Wzy-mediated synthesis, the CPS locus for synthase models contains multiple mutations and truncations that effectively silence many of the genes (Yother, 2011; Garcia et al., 1997; Standish and Morona, 2015). The only functional genes within a serotype 3 CPS locus are *cps3D*, which encodes UDP-Glc dehydrogenase; and *cps3S*, which encodes for the actual synthase.

Synthase-mediated production of CPS is therefore much simpler than Wzy-mediated synthesis (Yother, 2011; Standish and Morona, 2015; Zhensong Wen, 2015). For example, serotype 3 contains only two monosaccharides, glucuronic acid and glucose, arranged in an alternating pattern, compared to the Wzy-mediated synthesis pathways which typically contain four to six

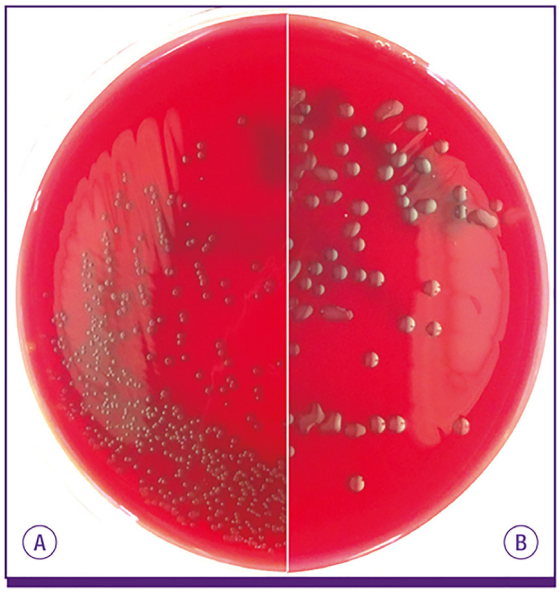

FIGURE 1 | *S. pneumoniae* isolates expressing most capsule types make **(A)** small round colonies similar to doughnuts on blood agar plate, but **(B)** serotype 3 and 37 pneumococci develop characteristically large mucoid colonies. From Song et al. (2013).

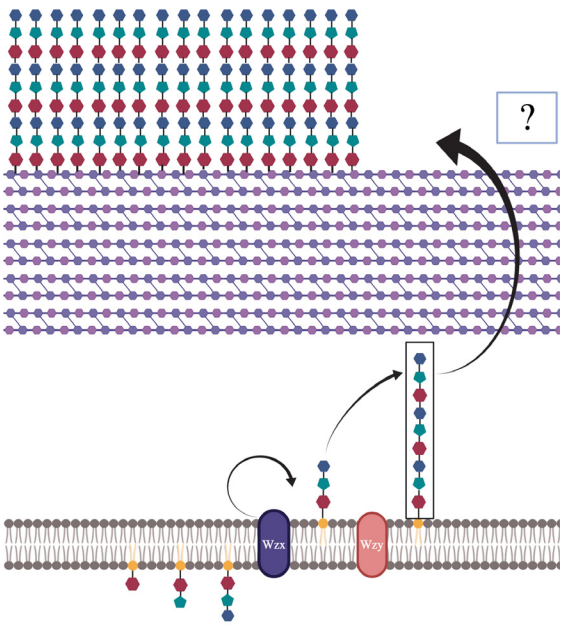

FIGURE 2 | A pictorial explanation of Wzy-mediated CPS synthesis. Repeated patterns of monosaccharides are transported to and added to undecaprenylphosphate (Und-p). The polysaccharide strip is then flipped across the membrane by Wzx flippase and repeat segments are added onto the non-reducing end by Wzy polymerase. Once the desired length is reached, polysaccharide chains are exported from the cell membrane to the peptidoglycan by unknown means, where they bind covalently via glycosidic bond.

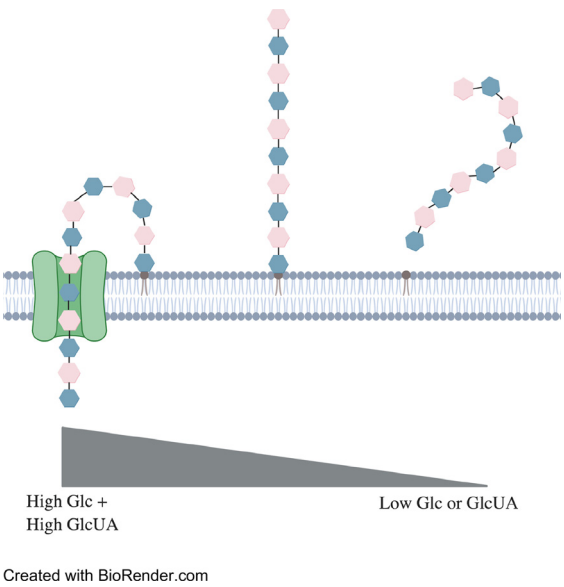

FIGURE 3 | Representation of synthase-mediated CPS production. Synthesis begins when a glucose monosaccharide is transferred to an integral phosphatidylglycerol. Addition of a glucuronic acid is catalyzed by the synthase. This method repeats until concentrations of either sugar runs too low for synthesis to continue. The phosphatidylglycerol then dissociates from the synthase and disperses within the external cell membrane.

monosaccharides per pattern (Standish and Morona, 2015). The sugars of synthase-mediated production are added directly onto the growing (non-reducing) end of the polysaccharide chain which is additionally exported and adhered to the external peptidoglycan via glycosidic bond (**Figure 3**) (Cartee et al., 2000; Forsee et al., 2006; Larson and Yother, 2017). CPS synthesis is continuous in serotype 3 strains and the polysaccharide chains only dissociate from the synthase when component concentrations are running low (Cartee et al., 2000; Ventura et al., 2006). Possible reasons for why capsule production is considerably greater for serotype 3 than other types include fewer steps in synthesis and therefore fewer checkpoints. Alternatively, this method of CPS production may not be as metabolically taxing on the bacterium.

Serotype 3 Is a Major Cause of Human Disease

Even though serotype 3 is included in the both the 13- and 23-valent vaccines against *Spn*, it remains a major causes of serious human disease (Harboe et al., 2009; van Hoek et al., 2012; Grabenstein and Musey, 2014; World Health Organization, 2020). Notably, rates of disease attributable to serotype 3 have not declined since inclusion of serotype 3 in PCV13 (Centers for Disease Control and Prevention, 2015; Slotved et al., 2016; Katoh et al., 2017; Ladhani et al., 2018; Groves et al., 2019; Wijayasri et al., 2019; Goettler et al., 2020). Serotype 3 disease often has severe clinical manifestations: most commonly bacteremia-induced septic shock, meningitis, and pneumonia (Harboe et al., 2009; Silva-Costa et al., 2018). In some countries serotype 3 is also been linked to complicated pneumonia, or empyema, where the bacterium is found in the pleural cavity (Goettler et al., 2020). For all these reasons, serotype 3–influenced invasive disease boasts an ~30% mortality rate. This number climbs to 47% for individuals with multiple comorbidities (Charlson Index of +3), being most often the elderly (Harboe et al., 2009). Notably in a recent study by Africano et al., serotype 3 was independently associated with development of adverse cardiac events in hospitalized individuals (Africano et al., 2020). This was due to the bacterium's propensity for bacteremia, an aspect of disease which confers the bacterium the opportunity to invade the myocardium (Ahl et al., 2013; Brown et al., 2014; Shenoy et al., 2018; Anderson et al., 2018). Thus, and despite exhibiting relatively low carriage rates among the population, serotype 3 associated opportunistic disease is consistently a major cause of IPD within young and old age groups with devastating consequences.

Mechanisms of Vaccine Escape for Serotype 3

In a study by Choi et al., the amount of protection granted by the PCV-13 vaccine versus serotype 3 capsule production was investigated (Choi et al., 2016). Results from this experiment suggest that the profuse production and release of serotype 3 capsule overwhelms the protective capacity of antibody that is elicited by the vaccine. A measured 0.2 µl of serotype 3 culture supernatant containing the respective capsule was sufficient to abolish the antibody-mediated protection provided by the vaccine. In comparison, only 25 µl of supernatant from a serotype

4 isolate was required to reach that same point of neutralization. From these results, it was estimated that approximately eight times more antibody was required to confer protection against serotype 3 invasive pneumococcal disease (Andrews et al., 2014; Choi et al., 2016). In addition to inhibiting interactions of the bacteria with phagocytes, serotype 3 is able to escape from capsular antibody due to the fact that its capsule is not covalently attached to the bacterial surface, allowing it to be released in copious amounts to the extracellular milieu (Cartee et al., 2005; Choi et al., 2016). Capsule antibody therefore instead binds to shed capsule and is neutralized in its capacity to opsonize the bacteria itself. Antibody bound to the capsule on the bacterium's surface would also be eventually released.

Bacterial surface components with negative electric charge can repel the like-charged immune cells (Oss, 1978). This aids the bacterium in avoiding NETs and phagocytosis, as well as complement particles released by phagocytic cells (Oss, 1978; Nelson et al., 2007). Many physical qualities of bacteria have been linked to the determination of cellular surface charge; one being capsule type (Li et al., 2013). With 100 biochemically distinct pneumococcal serotypes in existence, the level of surface charge can vary considerably. Surface charge is typically determined by measuring a cell's zeta potential. Neutral charges exist between −10 and +10 mV. While charges of −30 and +30 mV make up the lower and upper extremes, respectively (Clogston and Patri, 2011). The lower the zeta potential, the higher the surface electronegativity and vice versa. Bacteria with lower surface charges (lower zeta potentials) generally perform better at avoiding phagocytosis and complement deposition than their neutral counterparts (Oss, 1978; Wilson et al., 2001; Li et al., 2013). Among such lines, Li et al. related lower zeta potential to higher rates of carriage in the nasopharynx. In his study, serotype 3 stood as the capsule type with the lowest average zeta potential (Li et al., 2013).

Limitations Imposed by Serotype 3

As indicated, capsule is inhibitory of the adhesin and host ligand interactions that promote bacterial attachment. Studies with a capsule deficient version of serotype 3 strain A66.1 found its unencapsulated derivative to be 10^5-fold more adhesive to host cells (Hammerschmidt et al., 2005). Furthermore, wildtype A66.1 recovered from interaction with HEp-2 cells showcased a decreased capsule expression level similar to that seen in the capsule deficient variant. Both observations support the notion that capsule shedding is necessary for interaction with host epithelia. Capsule has been also shown to be inhibitory of bacterial biofilm formation (Kim and Weiser, 1998; Hammerschmidt et al., 2005; Sanchez et al., 2011a; Qin et al., 2013). Presumably, capsule inhibits initial attachment of bacteria to a surface or the epithelia, it likely also interferes with bacteria to bacteria interactions. Interestingly, *in vitro*, serotype 3 has been found to rely on the formation of unencapsulated small colony variants (SCVs) for the formation of biofilms (Allegrucci and Sauer, 2007; Allegrucci and Sauer, 2008). These unencapsulated mutants have alterations in the genes encoding the capsule operon as result of oxidative stress imposed by the bacteria itself (Allegrucci and Sauer, 2007). These SCVs bind to the abiotic surfaces, and in turn are bound by fully encapsulated *Spn*. While this has not been shown to occur *in vivo*, it demonstrates that type 3 capsule most likely imposes challenges that the bacteria must overcome. Importantly, capsule is one component, along with other polysaccharides, of both *in vitro* and *in vivo* biofilm extracellular matrices (Hall-Stoodley et al., 2008). Thus, and although it is initially inhibitory of biofilm formation, it has an important role in the process.

Capsule, of course, is not the only determining factor of bacterial virulence. Investigations into variations of pneumococcal surface proteins such as PspA and CbpA (PspC) also showcased different levels of virulence and surface exposure when compared using isogenic WU2 (serotype 3) and TIGR4 (serotype 4) strains, respectively (Ren et al., 2003; Georgieva et al., 2018). We have shown, with the pneumococcal serine-rich protein PsrP, that the surface proteins which contain adhesive domains must project these through the capsule in order to function (Shivshankar et al., 2009; Sanchez et al., 2010). Thus serotype 3, being thickly encapsulated, puts in place unique parameters for the surface proteins it relies on for host-pathogen interactions.

Population Genomics of *S. pneumoniae* and Serotype 3 Isolates

More than 20,000 whole genome sequences from isolates of *S. pneumoniae* are currently available in databases. A significant number of genomes were recently contributed by the Global Pneumococcal Sequencing (GPS) project (https://www.pneumogen.net/gps/) (Gladstone et al., 2019). The analysis of 20,027 genomes by Gladstone et al. was performed in the context of issues with current conjugate vaccines, including the concern about the emergence of non-vaccine serotypes. They aimed to study *S. pneumoniae* serotype, antibiotic resistance, and invasiveness in association with the overall genetic background of the isolates. They partitioned lineages into Global Pneumococcal Sequence Clusters (GPSCs) that were then associated with the features of interest listed above, as well as clonal complex (CC) and of course serotype (Gladstone et al., 2019; Lo et al., 2019; Gladstone et al., 2020). A CC groups related bacterial strains through the identification of allele variations or sequence types (STs) within seven highly conserved housekeeping genes (*aroE*, *gki*, *gdh*, *xpt*, *spi*, *ddl*, and *recP*) (Enright et al., 2001). For associations with GPSCs, CCs were defined as STs with single locus variant differences within the GPS dataset.

Serotype 3 has been the focus of recent genome-based studies that used GPSCs as the foundation (at least in part) and focused on the epidemiology and evolution of serotype 3 isolates in the US (Azarian et al., 2018), as well their prevalence in *Spn* carriage and in PCV13 evasion in the UK (Groves et al., 2019; Sheppard et al., 2019). In particular, CC180, an *S. pneumoniae* clone also known as Netherlands^3-31 or PMEN31, is the major complex of serotype 3, containing a majority of clinical isolates and laboratory strains that have been sequenced and analyzed to date (Azarian et al., 2018; Groves et al., 2019). CC180, which is included in GPS lineage GPSC12, can be divided into 3 clades: clade I, which contains subclades Iα and Iβ, and clade II (Azarian et al., 2018; Groves et al., 2019). When the prevalence of isolates from each clade was investigated over a set timeframe, incidences

of Iβ remained relatively unchanged while a switch in preference was observed between clades Iα and II (Azarian et al., 2018; Groves et al., 2019). Clade Iα, the predominant group in terms of isolates showed a slight decrease in population shortly after PCV13 introduction. Meanwhile, the population of clade II expanded, overtaking Clade Iα. By 2014, clade II made up ~41% of CC180-related pneumococcal isolates in the US. Up from ~20% just 4 years earlier in 2010 (Azarian et al., 2018). Interestingly, despite the noticeable increase in Clade II isolates and decrease in Iα isolates, no correlation was found between the introduction of PCV13 and Clade II expansion (Azarian et al., 2018). This suggested other means for clade II selection. Further investigation into the variation of several protein antigens gave insight into this matter. Genes encoding key virulence determinants such as NanA, PspA, and CbpA showed high levels of variability between the three clades. Variations in each of these genes has been shown to impact pneumococcal virulence in a variety of experimental conditions (Camara et al., 1994; Enright et al., 2001; Ren et al., 2003; Shivshankar et al., 2009; Sanchez et al., 2010; Georgieva et al., 2018; Azarian et al., 2018; Gladstone et al., 2019; Lo et al., 2019; Sheppard et al., 2019; Gladstone et al., 2020). Several variations in genes responsible for increased drug resistance, such as Tet32, were also observed within clades Iβ and II, but not within Iα isolates (Azarian et al., 2018; Groves et al., 2019). Azarian et al. hypothesized that these and other untested loci gave clade II the necessary competitive advantage to overtake clade Iα, though this has yet to be tested.

Potential Strategies to Protect Against Serotype 3

The inability of PCV13 to elicit protective antibody against serotype 3 capsule suggests that a new alternative approach is necessary. Along such lines, immunization with conserved pneumococcal proteins has shown to confer protection against invasive disease caused by serotype 3 (Roche et al., 2003). Importantly the selected protein antigens must be conserved across CC180's three distinct clades, be antigenic, be constitutively expressed at high level *in vivo*, and be demonstrated to confer protective immunity (Ginsburg et al., 2012; Moffitt and Malley, 2016). Candidate proteins for this include PspA and pneumolysin among others (Xu et al., 2015; Pichichero et al., 2016). Ideally, the selected proteins should also confer protection against the serotypes not included in PCV13 or other expanded vaccine formulations, thereby conferring protection against all *Spn*. In similar fashion, a whole-cell based vaccine using killed *Spn* that expresses conserved proteins and elicits protective antibody would confer similar serotype-independent protection against *Spn* (Pichichero, 2017; Campo et al., 2018). In both instances, these new vaccines would expand protection against serotypes that, although individually infrequent, together inflict a significant burden of human disease (Butler et al., 1999; Ginsburg et al., 2012; Pichichero et al., 2016; Moffitt and Malley, 2016; Pichichero, 2017; Campo et al., 2018). It is noteworthy that efforts are ongoing to expand on the current conjugate vaccines. A 15-valent conjugate vaccine, now including serotypes 22F and 33F, is currently undergoing phase 3 clinical trials and appears to generate more antibody against serotype 3 than the 13-valent version (Businesswire, 2020). Whether this will be sufficient to reduce overall serotype 3 disease remains to be determined.

CONCLUSION

Throughout this article, we have discussed the vital role of capsule on pneumococcal pathogenesis and how unique feature of serotype 3 provides the bacterium with enhanced virulence and an intrinsic ability to resist vaccine induced antibody (**Figure 4**). Whereas the biochemical properties of serotype 3 indeed influence its interactions with host cells, more simplistically, the copious amount of capsule that are produced by serotype 3 pneumococci overwhelms the 0.35 μg/ml antibody threshold provided by the current conjugate vaccine formulation (Andrews et al., 2014; Choi et al., 2016). Physiological properties of the capsule itself, such as surface electronegativity, and its ability to confer protection against host factors are also an explanation for why disease caused by serotype 3 has such severe disease manifestations (Nelson et al., 2007; Harboe et al., 2009; Li et al., 2013; Grabenstein and Musey, 2014). The bountiful production of serotype 3 capsule both benefits and challenges the bacterium, preventing agglutination by the nasal mucin and phagocytosis by host immune cells while simultaneously deterring adherence to the host epithelium (Allegrucci and Sauer, 2007; Allegrucci and Sauer, 2008). As such, obtaining a general understanding of the

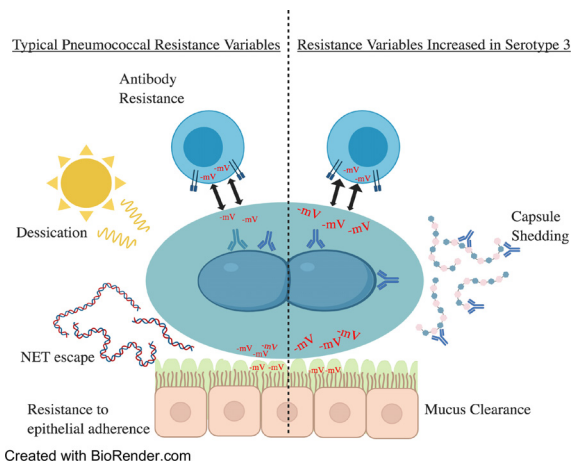

FIGURE 4 | A summary of the topics covered within this review. To the left are virulence factors commonly seen and utilized by pneumococcal serotypes. On the right are virulence factors increased in serotype 3 strains. These include but are not limited to electronegativity and capsule production/shedding. Electronegativity has a variety of effects on virulence as demonstrated above. Higher electronegativity acts as a repellent of immune cells and mucin, allowing for increased escape of immune responses and nasopharyngeal colonization by mucin evasion. Increases in capsular polysaccharide shedding in serotype 3 have been hypothesized to overwhelm the immune responses by sequestering antibodies and minimizing the deposit of antibody on the cell surface.

variability and basis for selection within serotype 3 regarding its complementary virulence determinants becomes an important goal (Azarian et al., 2018; Groves et al., 2019). A new approach seems to be necessary in order to prevent serotype 3 disease. Vaccines that generate antibody against conserved pneumococcal proteins have been tested in the past with reasonable efficacy and may hold the key to a stark decrease in pneumococcal-related incidents (Roche et al., 2003; Ginsburg et al., 2012; Moffitt and Malley, 2016). Moving forward serotype 3 is likely to continue being a major medical problem for some time until a new alternative prophylactic approach is approved.

AUTHOR CONTRIBUTIONS

JL wrote the first draft of the paper. CJO, JL, and HT contributed to the writing, editing, and direction of the paper. All authors contributed to the article and approved the submitted version.

FUNDING

HT and CJO received grant support from NIH grants AI114800 and AI146149.

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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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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 15 February 2021
doi: 10.3389/fcimb.2020.613467

Extracellular Pneumococcal Serine Proteases Affect Nasopharyngeal Colonization

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OPEN ACCESS

Edited by:
Masaya Yamaguchi, Osaka University, Japan

Reviewed by:
Carlos J. Orihuela, University of Alabama at Birmingham, United States
Cornelis Van 'T Veer, Amsterdam University Medical Center, Netherlands

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *02 October 2020*
Accepted: *30 December 2020*
Published: *15 February 2021*

Citation:
Ali MQ, Kohler TP, Burchhardt G, Wüst A, Henck N, Bolsmann R, Voß F and Hammerschmidt S (2021) Extracellular Pneumococcal Serine Proteases Affect Nasopharyngeal Colonization. Front. Cell. Infect. Microbiol. 10:613467. doi: 10.3389/fcimb.2020.613467

Streptococcus pneumoniae has evolved versatile strategies to colonize the nasopharynx of humans. Colonization is facilitated by direct interactions with host cell receptors or *via* binding to components of the extracellular matrix. In addition, pneumococci hijack host-derived extracellular proteases such as the serine protease plasmin(ogen) for ECM and mucus degradation as well as colonization. *S. pneumoniae* expresses strain-dependent up to four serine proteases. In this study, we assessed the role of secreted or cell-bound serine proteases HtrA, PrtA, SFP, and CbpG, in adherence assays and in a mouse colonization model. We hypothesized that the redundancy of serine proteases compensates for the deficiency of a single enzyme. Therefore, double and triple mutants were generated in serotype 19F strain EF3030 and serotype 4 strain TIGR4. Strain EF3030 produces only three serine proteases and lacks the SFP encoding gene. In adherence studies using Detroit-562 epithelial cells, we demonstrated that both TIGR4Δ*cps* and 19F mutants without serine proteases or expressing only CbpG, HtrA, or PrtA have a reduced ability to adhere to Detroit-562 cells. Consistent with these results, we show that the mutants of strain 19F, which preferentially colonizes mice, abrogate nasopharyngeal colonization in CD-1 mice after intranasal infection. The bacterial load in the nasopharynx was monitored for 14 days. Importantly, mutants showed significantly lower bacterial numbers in the nasopharynx two days after infection. Similarly, we detected a significantly reduced pneumococcal colonization on days 3, 7, and 14 post-inoculations. To assess the impact of pneumococcal serine proteases on acute infection, we infected mice intranasally with bioluminescent and invasive TIGR4 or isogenic triple mutants expressing only CbpG, HtrA, PrtA, or SFP. We imaged the acute lung infection in real-time and determined the survival of the mice. The TIGR4*lux* mutant expressing only PrtA showed a significant attenuation and was less virulent in the acute pneumonia model. In conclusion, our results showed that pneumococcal serine proteases contributed

significantly to pneumococcal colonization but played only a minor role in pneumonia and invasive diseases. Because colonization is a prerequisite for invasive diseases and transmission, these enzymes could be promising candidates for the development of antimicrobials to reduce pneumococcal transmission.

Keywords: pneumococci, serine proteases, colonization, adherence, pneumonia

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a Gram-positive, facultative human pathogen, and colonizes asymptomatically and highly successful mucosal epithelial surfaces of the upper respiratory tract (URT) (Kadioglu et al., 2008; Hilleringmann et al., 2015). However, under certain conditions, when the immune system is compromised, pneumococci can disseminate from the nasopharynx into the lung and blood to cause invasive diseases, including pneumonia, meningitis, and sepsis (Song et al., 2013; WHO, 2019). Despite the development of antimicrobial therapies, vaccines, and the use of antibiotics, pneumococcal diseases remain a major threat to humans (WHO, 2019). The burden of the disease continues to be high in both industrialized and developing countries. In 2015, approximately 300,000 children under the age of 5 years died from pneumococcal related disease globally (Wahl et al., 2018). Importantly, pneumococci have to avoid entrapment in the mucus and clearance by the host immune system (Bergmann and Hammerschmidt, 2007; Weiser et al., 2018). Consequently, pneumococci use various strategies to interact with epithelial cell surface receptors. Firstly, bacterial adhesins such as the pneumococcal surface protein C (PspC, also known as CbpA), PavB, PsrP, or other adhesive pneumococcal surface components interact directly with host cell receptors (Pracht et al., 2005; Hammerschmidt, 2006; Orihuela et al., 2009; Kanwal et al., 2017; Weiser et al., 2018). Secondly, binding to host cells is promoted by the interaction between bacterial proteins referred to as microbial surface components recognizing adhesive matrix molecules like enolase or the pneumococcal adherence and virulence factor A and B (PavA, PavB) and extracellular matrix (ECM) components such as fibronectin, vitronectin, thrombospondin-1, and plasminogen (Holmes et al., 2001; Rennemeier et al., 2007; Bergmann et al., 2009; Voss et al., 2012; Kanwal et al., 2017). Thirdly, pneumococci exploit hosts proteolytic activity such as plasmin to degrade mucosal and ECM components, thereby facilitating the tight interaction with host cells (Bergmann and Hammerschmidt, 2007; Bergmann et al., 2013).

Despite this knowledge, the contribution of pneumococcal surface proteins to colonization and dissemination to the lower respiratory tract is still a crucial issue to understand. We, therefore, hypothesized that pneumococcal extracellular serine proteases could also be important for colonization under physiologically relevant *in vivo* conditions.

Pneumococci express different types of proteases. These include the zinc metalloprotease ZmpA (also known as IgA1 protease), which interacts with the host immune system by cleaving IgA into inactive components (Proctor and Manning, 1990), and ZmpB, which is involved in the modification of pneumococcal surface proteins (Novak et al., 2000). Additionally, serine proteases can contribute to pneumococcal virulence by cleaving host proteins, such as immunoglobulins, complement compounds, and proteins of the ECM (Mann et al., 2006; Mirza et al., 2011). Serine proteases possess proteolytic activity due to the presence of the catalytic triad Ser-His-Asp (Hedstrom, 2002; Supuran et al., 2002) and have been found in many organisms (Kochan and Dawid, 2013).

Depending on the serotype, pneumococci can produce, strain-dependent, up to four different serine proteases, namely the high-temperature requirement A (HtrA) protein, the subtilase family protein (SFP), the cell wall-associated serine proteinase A (PrtA), and the choline-binding-protein G (CbpG). A common feature of these proteins is their catalytic domain and that they are secreted or located on the bacterial cell surface. Interestingly, all serine proteases of interest for this study are highly conserved among the different pneumococcal serotypes (Bethe et al., 2001; Desa et al., 2008). Some of them have already been shown to influence pneumococcal pathogenesis (Mann et al., 2006; Mirza et al., 2011; de Stoppelaar et al., 2013).

The best studied serine protease is the HtrA, which is highly conserved in many bacteria and can switch from chaperon function to protease function at high temperatures (Seol et al., 1991; Spiess et al., 1999). The pneumococcal HtrA contains no specific anchoring motif and has multifunctional roles, including facilitation of pneumococcal growth at high temperatures, resistance to oxidative stress and the control of bacteriocin activity (Fan et al., 2010). HtrA has been shown to be immunogenic and protective in mice against invasive pneumococcal diseases (Li et al., 2016). The influence of HtrA on pneumococcal pathogenesis has been addressed in several studies. For example, HtrA is considered to be one of the most important serine proteases in *S. pneumoniae* virulence as it degrades the competent stimulating peptides (CSPs) and has, therefore, an impact on pneumococcal competence (Ibrahim et al., 2004a; Ibrahim et al., 2004b; Cassone et al., 2012). Furthermore, the deficiency of HtrA in *S. pneumoniae* D39 decreased bacterial loads and inflammation in the lung after intranasal challenge (de Stoppelaar et al., 2013).

Two other serine proteases, SFP and PrtA, belong to the subtilisin family. PrtA contains a typical sortase A recognition motif at the C-terminal end, which binds covalently bound to the bacterial peptidoglycan (Bethe et al., 2001; de Stoppelaar et al., 2013). Infection experiments using

S. pneumoniae D39 demonstrated that SFP had only a minor effect on pneumococcal virulence and may facilitate the growth

of the bacteria even after a low dose infection in the lower respiratory tract (de Stoppelaar et al., 2013). However, this was shown in the presence of all other serine proteases produced by D39. Meanwhile, PrtA has been shown to contribute to the pathogenesis of pneumococcal infections in an intraperitoneal mice infection model and a contribution to lung damage in a high dose pneumonia model (de Stoppelaar et al., 2013). In contrast, PrtA does not contribute to bacterial outgrowth in pneumococcal pneumonia (Bethe et al., 2001; de Stoppelaar et al., 2013). Other previous studies showed that PrtA was highly immunogenic and activated IL-17A response, but failed to protect against pneumococcal pneumonia in infected mice (Hsu et al., 2018). Furthermore, PrtA plays an important role in blood invasion (Mahdi et al., 2015).

The fourth pneumococcal serine protease is CbpG, which is a member of the choline-binding protein (CBP) family (Gosink et al., 2000). So far, 13 to 17 pneumococcal proteins have been identified and they share a repetitive choline-binding module (CBM). The repetitive sequences of the CBM attach CBPs non-covalently to phosphorylcholine residues of cell wall anchored teichoic acids (WTA) and membrane-anchored lipoteichoic acids (LTA) of *S. pneumoniae* (Maestro and Sanz, 2016).

Mann and co-workers (Mann et al., 2006), have shown that CbpG exists in two variants in some strains. The secreted form is without choline domain due to a premature stop codon before the choline-binding domain. The other variant is surface-attached (full-length). In addition, it could play an important role in both mucosal colonization and sepsis (Gosink et al., 2000).

So far, the individual role or the synergistic effects of pneumococcal serine proteases on colonization and subsequent dissemination into the lung or blood have not been analyzed systematically. In this sense, we report here the impact of serine proteases on epithelial adherence and nasopharyngeal colonization of *S. pneumoniae* using mutants expressing only a single or no serine protease. To assess the impact of serine proteases, *in vitro* adhesion assays and an *in vivo* murine nasopharyngeal colonization model was applied using the non-invasive serotype 19F strain EF3030 (Junges et al., 2019). In addition, we assessed the role of serine proteases on virulence and dissemination in an acute murine pneumonia model using the invasive TIGR4 strain and isogenic triple serine protease mutants.

Importantly, our findings emphasize that the loss of serine proteases reduces the adherence to human epithelial cells and the nasopharyngeal colonization of mice. However, the loss of function of pneumococcal serine proteases has only moderate effects on pneumococcal virulence as analyzed in our acute murine pneumonia model. Therefore, these proteases are most likely required to facilitate colonization. They can be promising antimicrobial candidates to prevent colonization and transmission of pneumococci.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. pneumoniae strains and mutants used in this study are listed in **Table 1**. The pneumococcal isolate EF3030 serotype 19F originally obtained from otitis media (Andersson et al., 1983; Junges et al., 2019) was kindly provided by Anders P. Håkansson (Lund University, Sweden), while strain TIGR4 was described earlier (Tettelin et al., 2001). Growth of parental pneumococcal strains and isogenic mutants was monitored in a complex Todd-Hewitt medium supplemented with 0.5% yeast extract (THY) (Roth, Germany) and $RPMI_{modi}$, a chemically defined medium described earlier (Schulz et al., 2014). To prepare liquid cultures, strains were thawed from glycerol-stocks and cultured on blood agar plates (Oxoid, Germany), incubated at 37°C under 5% CO_2 atmosphere with the appropriate antibiotics presented in **Table 1** (50 µg/ml spectinomycin, 50 µg/ml kanamycin, 5 µg/ml erythromycin, 8 µg/ml chloramphenicol). Pneumococcal growth was monitored by measuring the optical density at 600nm (OD_{600nm}).

Construction of Pneumococcal Mutants

The plasmids and oligonucleotide primers used in this study are listed in **Tables 2** and **3**. Single, double, and triple *serine protease* gene deletion mutants were generated by insertion-deletion mutagenesis in *S. pneumoniae* 19F EF3030, and in TIGR4 strains (non-encapsulated Δ*cps*, and bioluminescent *lux* strains) (Schulz et al., 2014). Briefly, for the deletion of *cbpG* (*sp_0390* in TIGR4 and *EF3030_01920* in 19F), primer pair, P1421/1422 was designed to amplify the gene, including 500 bp up-and downstream using TIGR4 chromosomal DNA as a template. The PCR product was cloned into plasmid pSP72D cleaved with *Eco*RV, which contains a modified poly-linker region by deletion from the *Xho*I-*Sac*I site of pSP72 (Promega, Germany). The resulting plasmid containing the *cbpG* gene region was used as a template for an inverse PCR with the primer pair P1423/1424 to delete 642 bp of the *cbpG* gene. The primers incorporated a *Hin*dIII and *Bam*HI site for cloning of the *ermB* or *aad9* gene cassette (**Figure S1**) amplified with primers P99/100 for *ermB* and p177/118 for *aad9* to obtain plasmids pAW1100 (pSP72DΔ*cbpG*::Erm^r) and pAW1101 (pSP72DΔ*cbpG*::$Spec^r$). For the deletion of *sfp* in TIGR4 (*sp_*1954), primers P1284/P1285 were used to amplify the *sfp* gene region, including up-and downstream sequences from TIGR4. The PCR product was cloned into the *Eco*RV restriction site of pSP72D. The recombinant plasmid was used as a template for an inverse PCR with primer pair P1286/1287 containing *Bam*HI and *Hin*dIII restriction sites to delete the complete *sfp* gene sequence from the plasmid. After digestion with *Bam*HI and *Hin*dIII, the antibiotic resistance gene cassette *ermB*, *aad9* or *cat* were separately ligated to generate the plasmids pRB1119 (pSP72DΔ*sfp*::Erm^r), pRB1132 (pSP72DΔ*sfp*::$Spec^r$), and pRB1131 (pSP72DΔ*sfp*::Cm^r), which were used to delete *sfp* in TIGR4. The *sfp* gene is not present in strain EF3030 (**Figure S2**). To construct a plasmid for the *htrA* gene deletion, the encoding gene sequence (*sp_2239 in* TIGR4 and *EF3030_11105* in 19F) of the *htrA* gene region was cloned into the *Eco*RV site of pSP72D after PCR amplification with upstream and downstream sequences using primer pair P1061/P1062. For the inverse PCR primers, P1063/1064 containing *Eco*RI restriction sites were used to delete parts of the *htrA* sequence (1104 bp) in the plasmid. The antibiotic *cat* gene

TABLE 1 | Characteristics of the pneumococcal strains used in this study.

Strain no.[1]	Genotype (gene locus tag)	Resistance[2]	Phenotype	Reference
Streptococcus pneumoniae:				
EF3030	19F_EF3030	None	-	(Andersson et al., 1983)
PN762	19F_EF3030Δ*htrA* (*EF3030_11105*)	Cm^r	-	This work
PN763	19F_EF3030Δ*prtA* (*EF3030_03025*)	Erm^r	-	This work
PN769	19F_EF3030Δ*cbpG* (*EF3030_01920*)	$Spec^r$	-	This work
PN768	19F_EF3030Δ*cbpG* (*EF3030_01920*)	Erm^r	-	This work
PN765	19FΔ*htrA*Δ*cbpG* (*EF3030_11105, EF3030_01920*)	Cm^r, $Spec^r$	*prtA+*	This work
PN770	19F_EF3030Δ*prtA*Δ*htrA (EF3030_03025, EF3030_11105*)	Erm^r, Cm^r	*cbpG+*	This work
PN766	19F_EF3030Δ*prtA*Δ*cbpG (EF3030_03025, EF3030_01920*)	Erm^r, $Spec^r$	*htrA+*	This work
PN767	19F_EF3030Δ*htrA*Δ*cbpG*Δ*prtA* (*EF3030_11105, EF3030_01920, EF3030_03025*)	Cm^r, $Spec^r$, Erm^r	*All proteases*	This work
PN259	TIGR4Δ*cps*	Km^r	-	(Saleh et al., 2014)
PN494	TIGR4Δ*cps*Δ*prt*A (*sp_0641*)	Erm^r, Km^r	-	This work
PN488	TIGR4Δ*cps*Δ*htr*A (*sp_2239*)	Erm^r, Km^r	-	This work
PN663	TIGR4Δ*cps*Δ*cbpG* (*sp_0390*)	Km^r, Erm^r	-	This work
PN674	TIGR4Δ*cps*Δ*sfp* (*sp_1954*)	Km^r, $Spec^r$	-	This work
PN681	TIGR4Δ*cps*Δ*htr*AΔ*sfp*	Km^r, Erm^r, Cm^r	*prtA+ cbpG*$^+$	This work
PN530	TIGR4Δ*cps*Δ*prt*AΔ*htr*A	Km^r, Erm^r, Cm^r	*sfp+ cbpG*$^+$	This work
PN682	TIGR4Δ*cps*Δ*prt*AΔ*sfp*	Km^r, Erm^r, Cm^r	*htrA+ cbpG*$^+$	This work
PN692	TIGR4Δ*cps*Δ*htr*AΔ*cbpG*Δ*sfp* (*sp_2239, sp_0390, sp_1954*)	Km^r, Erm^r, Cm^r, $Spec^r$	*prtA+*	This work
PN685	TIGR4Δ*cps*Δ*prt*AΔ*htr*AΔ*sfp* (*spd_0558*, *sp_2239, sp_1954*)	Km^r, Erm^r, Cm^r, $Spec^r$	*cbpG+*	This work
PN693	TIGR4Δ*cps*Δ*prt*AΔ*cbpG*Δ*sfp* (*spd_0558, sp_0390, sp_1954*)	Km^r, Erm^r, Cm^r, $Spec^r$	*htrA+*	This work
PN695	TIGR4Δ*cps*Δ*htr*AΔ*prt*AΔ*cbp*G (*sp_2239, spd_0558, sp_0390*)	Km^r, Erm^r, Cm^r, $Spec^r$	*sfp+*	This work
PN315	TIGR4*lux*	Km^r	–	(Saleh et al., 2014)
PN675	TIGR4*lux*Δ*sfp* (*sp_1954*)	Km^r, $Spec^r$	–	This work
PN665	TIGR4*lux*Δ*cbpG* (*sp_0390*)	Km^r, Erm^r	–	This work
PN489	TIGR4*lux*Δ*htr*A (*sp_2239*)	Erm^r, Km^r	–	This work
PN495	TIGR4*lux*Δ*prt*A (*sp_0641*)	Erm^r, Km^r	–	This work
PN750	TIGR4*lux*Δ*prt*AΔ*cbpG* (*sp_0641, sp_0390*)	Km^r, Erm^R, $Spec^r$	*htrA+ sfp+*	This work
PN743	TIGR4*lux*Δ*sfp*Δ*htr*A (*sp_1954, sp_2239*)	Km^r, $Spec^r$, Cm^r	*cbpG+ prtA+*	This work
PN531	TIGR4*lux*Δ*prt*AΔ*htr*A (*sp_0641*, *sp_2239*)	Km^r, Erm^r, Cm^r	*sfp+ CbpG+*	This work
PN747	TIGR4*lux*Δ*htr*AΔ*cbpG*Δ*sfp* (*sp_2239, sp_0390, sp_1954*)	Km^r, Erm^r, Cm^r, $Spec^r$	*prtA+*	This work
PN686	TIGR4*lux*Δ*htr*AΔ*prtA*Δ*sfp* (*sp_2239, sp_0641, sp_1954*)	Km^r, Erm^r, Cm^r, $Spec^r$	*cbpG+*	This work
PN752	TIGR4*lux*Δ*prt*AΔ*cbpG*Δ*sfp* (*sp_0641, sp_0390, sp_1954*)	Km^r, Erm^r, Cm^r, $Spec^r$	*htrA+*	This work
PN760	TIGR4*lux*Δ*htr*AΔ*prt*AΔ*cbp*G (*sp_2239*, *sp_0641*, *sp_0390*)	Km^r, Erm^r, Cm^r, $Spec^r$	*sfp+*	This work

[1]*Numbering is the stock list of the Department of Molecular Genetics and Infection Biology, University of Greifswald.*
[2]*Erm: Erythromycin, Km: Kanamycin, Cm: Chloramphenicol, Amp: Ampicillin, R: resistance.*

TABLE 2 | Plasmids used in this study.

Plasmid	Vector name	Properties	Resistance[2]	Reference
SP72		Cloning vector	Ap^r	Promega
pSP72D		Cloning vector for PCR products (derivative of pSP72)	Ap^r	This work
pGB1019	pUC18Δ*prtA*::Erm^r	pUC18 vector with *sp_0641* (*prtA*) gene partial deleted replaced with Erm^r resistance gene cassette, (4861 bp.)	Ap^r, Erm^r	This work
pAW1101	pSP72DΔ*cbpG*::$Spec^r$	pSP72D vector with *sp_0390* (*cbpG*) gene partial deleted replaced with $Spec^r$ resistance gene cassette, (4375 bp.)	Ap^r, $Spec^r$	This work
pAW1100	pSP72DΔ*cbpG*::Erm^r	pSP72D vector with *sp_0390* (*cbpG*) gene partial deleted replaced with Erm^r resistance gene cassette, (4313 bp.)	Ap^r, Erm^r	This work
pNM991	pSP72DΔ*htrA*::Cm^r	pSP72D vector with *sp_2239* (*htrA*) gene partial deleted replaced with Cm^r resistance gene cassette, (4923 bp.)	Ap^r, Cm^r	This work
pRB1131	pSP72DΔ*sfp*::Cm^r	pSP72D vector with *sp_1954* (*sfp*) gene partial deleted replaced with Cm^r resistance gene cassette, (4926 bp.)	Ap^r, Cm^r	This work
pRB1132	pSP72DΔ*sfp*::$Spec^r$	pSP72D vector with *sp_1954* (*sfp*) gene partial deleted replaced with $Spec^r$ resistance gene cassette, (5029 bp.)	Ap^r, $Spec^r$	This work
pRB1119	pSP72DΔ*sfp*::Erm^r	pSP72D vector with *sp_1954* (*sfp*) gene partial deleted replaced with $Spec^r$ resistance gene cassette, (4968 bp.)	Ap^r, Erm^r	This work

TABLE 3 | Primer used in this study.

Primer intended use	Primer/restriction enzyme	Sequence (5'-3')[3]
Primers used for the amplification of antibiotic resistance genes:		
erythromycin (*ermB*)	Erm^r (*ermB*)_99 (*EcoRI*)	5'-CCCGGGGAAATTTTGATATCGATGGATCCGAATTCGACG GTTCGTGTTCGTGCTG-3'
	Erm^r (*ermB*)_100 (*EcoRI*)	5'-CCCGGGGAAATTTTGATATCGATAAGCTTGAATTCCCGT AGG CGCTAGGGACCTC -3'
Spectinomycin (*aad9*)	Spec^r (*aad9*) _118 (*HindIII*)	5'-AAAAGCTTGCTAGCAATTAGAATGAATATTTCCC-3'
	Spec^r (*aad9*) _117	5'-GTACAGGATCCGAATTCATCGATTTTCGTTCGTGAATAC-3
Chloramphenicol (*cat*)	BM19 Cm^r (*cat*)_158 (*EcoRI*)	5'-GCGCGAATTCGAAAATTTGTTTGATTTTTAATGG -3'
	BM18 Cm^r (*cat*)_159 (*SacI*)	5'-ATATGAGCTCGGGTTCCGAGGCTCAACGTCAA -3'
Chloramphenicol (*cat*)	BM19 Cm^r (*cat*)_180 (*BamHI*)	5'-GCGCGGATCCGAAAAT TTGTTTGATTTTTAATGG-3'
	BM19 Cm^r (*cat*)_181 (*HindIII*)	5'-GCGCAAGCTTGGGTTCCGAGGCTCAACGTCAA-3'
Primers used for insertion-deletion mutagenesis		
Amplification of *cbpG* (*sp_0390)* 5´ and 3´ flanking region	cbpG_1421 (*SacI*)	5'-GCGCGAGCTCGAAGGTGG TAGATTTCTTGATTC-3'
	cbpG_1422 (*SacI*)	5'-GCGCGAGCTCGTAATACA CCATCTTGACC-3'
Inverse PCR of *cbpG* (*sp_0390)* 5´ and 3´ flanking region (pSP72D vector)	cbpG_1423 (*BamHI*)	5'-GCGCGGATCCGTGAGCCG CTGTAATTAACAC-3'
	cbpG_1424 (*HindIII*)	5'-GCGCAAGCTTGGTAAGAT GCTTACAGATTG-3'
Mutations analyse of upstream region *cbpG* (*sp_0390*)	cbpG_1467	5'-GAATGGCTGAACTTAGTAT C-3'
Amplification of *sfp* (*sp_1954)* 5´ and 3´ flanking region	sfp_1284 (*SacI*)	5'-GCGCGAGCTCGGAGCAGTGTTACAAAATTC-3'
	sfp_1285 (*SacI*)	5'-GCGCGAGCTCGTTGTGGTAACCTGTTTGC-3'
Inverse PCR of *sfp* (*sp_1954)* 5´ and 3´ flanking region (pSP72D vector)	sfp_1286 (*BamHI*)	5'-GCGCGGATCCCGCTAGTCTGAGTGTGAG-3'
	sfp_1287 (*HindIII*)	5'-GCGCAAGCTTGATCAGCCCTATAATTATATG-3'
Mutations analyse of upstream region *sfp* (*sp_1954*)	sfp_1416	5'-GAACCTAATATTGGTTCAATAG-3'
Amplification of *htrA* (*sp_2239*) 5´ and 3´ flanking region	htrA_1061 (*BamHI*)	5'-CGCGCGGATCCAGTCAATTTTCTATTTATG-3'
	htrA_1062 (*PstI*)	5'-CTCACTGCAGAAGAGCTTCTAATTTCC-3'
Inverse PCR of *htrA* (*sp_2239*) 5´ and 3´ flanking region (pSP72D vector)	htrA_1063 (*EcoRI*)	5'-CATGCGGAATTCGCTAATGACGATAACGAC-3'
	htrA_1064 (*EcoRI*)	5'-GCGCGAATTCCTTAACAAGAGTTCAGGTG-3'
Mutations analyse of upstream and downstream region *htrA* (*sp_2239*)	htrA_1088	5'-CCAGCTTTGCTATTATATTG-3'
	htrA_1089	5'-ACAGCCTTATTTCAGGCTG-3'
Amplification of *prtA* (*sp_0641*) 5´ flanking region	prtA_1073 (*BamHI*)	5'-GCACGGATCCTTAAGCCTTACTCTTAGCG-3'
	prtA_1074 (*SacI*)	GCGCGAGCTCATAAACTTTAACTTTGCTAGC
Amplification of *prtA* (*sp_0641*) 3´ flanking region	prtA_1075 (*SacI*)	GCGAGAGCTCGTTTATGTACTGAGATTAGATAG-3'
	prtA_1076 (*SalI*)	5'-GCGAGTCGACCACTTTCAGAATAAGGAGCCTG

[3]*The primers were synthesized by Eurofins MWG Operon, Germany. The restriction site used for cloning are underlined.*

cassette was amplified using primers P158/159, digested with *Eco*RI and cloned into the digested plasmid resulting in pNM991 (pSP72DΔ*htrA*::Cm^r) (**Figure S3**). Due to the large size of *prtA* (*sp_0641* and *EF3030_03025* in 19F), only 500 bp upstream and downstream of the *prtA* gene were amplified with primers P1073/1074 (containing *Bam*HI/*Sac*I restriction sites) for the 5'-region and P1075/1076 (containing *Sac*I/*Sal*I restriction sites) for the 3'-region. The resulting PCR fragments were cloned into the *Bam*HI/*Sal*I digested vector pUC18. The recombinant plasmid was cleaved with *Ecl*136II for the insertion of the *ermB* antibiotic resistance gene cassette resulting in plasmid pGB1019 (pUC18Δ*prtA*::Erm^r) (**Figure S4**). *S. pneumoniae* were transformed with the recombinant plasmids to delete the serine protease genes by homologous recombination as described (Hammerschmidt et al., 1997). The recombinant pneumococcal strains lacking one serine protease gene were selected on blood agar plates (Oxoid) with an appropriate antibiotic and confirmed by PCR. Finally, the recombinant plasmids pAW1100, pAW1101, pRB1131, pRB1132, pRB1119, pNM991, and pGB1019, were used to transform pneumococci to generate double and triple serine protease deficient mutants.

Pneumococcal Adherence Assays and Immunofluorescence Microscopy

Pneumococcal adherence to human nasopharyngeal epithelial Detroit-562 cells (ATCC CCL-138) was conducted as described (Bergmann et al., 2009). Briefly, epithelial cells were seeded (2×10^5 cells per well) in 24-well tissue culture plates (Greiner Bio-One, Germany) in RPMI-1640 (HyClone^{TM}, Germany) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco, Germany), 2 mM glutamine, 1 mM sodium pyruvate, 1% HEPES (Sigma, Germany) and incubated for 24 h at 37°C and 5% CO_2. The confluent monolayer (75–80% confluency) was washed three times with infection medium (cell culture medium containing 1% heat-inactivated FBS) and infected with the indicated 19F EF3030 and TIGR4Δ*cps* wild-type or mutant pneumococci using a multiplicity of infection (MOI) of 50 pneumococci per epithelial cell. Prior to infection, pneumococci were grown in THY to mid-log phase (OD_{600} of 0.35–0.4) and, after centrifugation, resuspended in phosphate-buffered saline (PBS, pH 7.4) and infection medium (RPMI-1640, 1% heat-inactivated FBS) at a ratio of 1:10. The infection was carried out for the indicated time points at 37°C, and 5%

CO_2 and non-adherent pneumococci were removed in three washing steps with RPMI-1640. Pneumococcal adherence was quantified by plating the attached and internalized (less than 0.1% of host cell-associated bacteria) pneumococci (Bergmann et al., 2009) on blood agar plates. The bacteria were counted using a colony counter (Bern University of Applied Sciences). Immunofluorescence microscopy was performed to visualize pneumococcal adherence to host cells. Detroit-562 cells were seeded on glass coverslips (diameter 12 mm) in wells of 24-well tissue culture plates and infected with pneumococci as described above. Staining host cell-attached pneumococci were performed as described (Jensch et al., 2010; Hess et al., 2017). In brief, infected host cells were treated after three washing steps with infection medium and fixed overnight at 4°C with 4% paraformaldehyde in PBS. Infected host cells were incubated with PBS/10% FBS for 3 h at room temperature to block unspecific antibody binding. Host cell-bound pneumococci were stained using a polyclonal anti-pneumococci IgG (1:1000) followed by anti-mouse Alexa-Fluor® 488-coupled secondary antibody (green) (abcam, Germany). The actin cytoskeleton was stained with Phalloidin-iFlour®-594 conjugate (red) (abcam, Germany). Image acquisition was performed with a fluorescence microscope (Zeiss Axio-Observer.Z), imaging software (Zen 2.6, Zeiss, Germany). Each bar in the images represents 20 mM. All experiments were performed with three replicate wells tested for each experimental setup.

Mouse Model of Colonization and Pneumonia

The influence of serine proteases on nasopharyngeal colonization and pneumonia was analyzed *in vivo* by applying two different mouse infection models, namely the nasopharyngeal colonization model and the acute pneumonia model. For the mouse colonization model, strain 19F_EF3030 was used, which colonizes the nasopharynx of mice while being mostly noninvasive (Briles et al., 1992; Junges et al., 2019). TIGR4 is a clinical isolate and causes severe pneumonia and invasive diseases in mice (van Ginkel et al., 2003). Female CD-1 outbred mice (age, 8–10 weeks) were purchased from Charles River, Sulzfeld, Germany. Mice were anesthesized intraperitoneally with ketamine (Ketanest S; Pfizer Pharma, Karlsruhe, Germany) and xylazine (Rompun®; Provet AG, Lyssach, Germany). Afterward, mice were intranasally challenged with 20 µl PBS/1% FBS containing 1×10^7 bacteria of 19F_EF3030 (wild-type) or isogenic *serine protease* mutants in the colonization model. Nasopharyngeal washes (NP) and bronchoalveolar lavages (BAL) were collected at time points 2, 3, 7, and 14 days post-infection and CFU determined by plating as described previously (Cohen et al., 2012; Schulz et al., 2014). The acute pneumonia model was conducted by infecting mice intranasally with 20 µl PBS/1%FBS containing 9×10^7 bacteria of bioluminescent TIGR4*lux* or isogenic triple *serine protease* mutants as described (Saleh et al., 2014). Infected mice were imaged and monitored using the IVIS® Spectrum Imaging System (Caliper Life Sciences) to determine the dissemination of the bioluminescent pneumococci in the mice as described (Saleh et al., 2013; Saleh et al., 2014). Besides, the bioluminescent intensity was quantified as the total photon emission using LivingImage® 4.1 software package (Caliper Life Sciences). The CFU of the infection dose was confirmed by plating serial dilutions on blood agar plates.

Ethics Statement

All animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (National Research Council, USA), the guidelines of the ethics committee at The University of Greifswald and the German regulations of the Society for Laboratory Animal Science (GVSOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). All experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg–Vorpommern (LALLFV M-V, Rostock, Germany) and the LALLFV M-V ethical board (LALLF M-V permit no. 7221.3-1-056/16). All efforts were made to minimize suffering, ensure the highest ethical standard and adhere to the 3R principle (reduction, refinement and replacement).

Statistical Analysis

Statistical significance between different groups was calculated using a one-way ANOVA (Kruskal-wallis test) followed by Dunnett's post-test for the mouse colonization model and bioluminescence measurements in the acute pneumonia model. Unpaired two-tailed Students t-test (Mann-Whitney test) was performed to analyze the difference between two groups. Two-way ANOVA analysis was used with the optical density for growth behavior. Kaplan-Meier survival curves of mice were compared by the log-rank (Mantel-Cox) test. A p-value of <0.05 was considered statically significant. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad, Software, La Jolla, CA, USA).

RESULTS

Bioinformatics Analysis and Genomic Organization of Serine Protease Genes

We first analyzed pneumococcal serine protease encoding genes *in silico*. Gene and amino acid sequences were extracted from the NCBI online tool database for homolog analysis search by Clustal Omega (source data are available in supporting information). The genomic sequence of TIGR4 (ATCC BAA-334) was used as a reference sequence (Tettelin et al., 2001). Our bioinformatic analyses has been performed to provide insight into the genome organization of genes encoding serine proteases. The best known pneumococcal protein with serine proteases activity is the chaperon HtrA encoded by *sp_2239* in TIGR4 and *EF3030_11105* in 19F (protein accession numbers AAK76286.1 and QBF69928.1). HtrA consists of 393 amino acids (aa) that form a molecular weight of 42 kDa without a specific anchoring motif exhibiting two domains, the serine protease catalytic domain (residues 96–277) and the C-terminal PDZ domain (residues 289–375) (postsynaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens 1 protein) (de Stoppelaar et al., 2013). The modular organization of the

HtrA protein is shown in **Figure 1**. The genomic organization of the *htrA* gene region is shown in **Figure S3A**. PrtA *sp_0641* (AAK74791.1) in TIGR4 and *EF3030_03025* (QBF68585.1) in 19F is a cell wall-anchored serine protease A belonging to the subtilisin-like proteases and consists of 2140 aa, thus having a molecular weight of 240 kDa. The genomic region of the *prtA* gene (*sp_0641*, 6423 nt) is shown in **Figure S4A**. PrtA exhibits an N-terminal signal peptide and a C-terminal LPKTG anchoring motif. The peptidase domain spanning the aa residues 223–764 contains the catalytic triad, and a DUF (the domain of the unknown function 1034) of 140 aa is localized between residues 795–934. The protein model of PrtA is illustrated in **Figure 1**. Another serine protease encoding gene in pneumococci is *sfp* (*sp_1954* in TIGR4 (ABC75782.1), which encodes a subtilase family protein. The TIGR4 *sfp* gene encodes a protein of 467 aa containing a hydrophobic N-terminal signal peptide sequence followed by the catalytic domain spanning aa 167–461 (**Figure 1**), with a molecular weight of 52 kDa and without a sortase A anchoring motif. *In silico* comparative analyses of the *sfp* gene in TIGR4 *sp_1954 sfp* (467 aa) with the complete genome of 19F_EF3030 (CP035897.1 - NCBI) strains were performed using the Clustal Omega database to check whether the *sfp* gene is located in a different locus in 19F. Remarkable, *in silico*, confirmed that the *sfp* gene (subtilase family protein) and six upstream and three downstream genes are not present in the genome of the 19F strain EF3030 (**Figure S2A**). Therefore, we could generate a full *serine proteases* deficient mutant in 19F EF3030 by deleting all the other three serine proteases (CbpG, HtrA, PrtA) genes. CbpG *sp_0390* (AAK74556.1) in TIGR4 and *EF3030_01920* (QBF69943.1) in 19F, a protein composed of 285 aa, belongs to the class of pneumococcal choline-binding proteins that are non-covalently associated with the phosphorylcholine residues of teichoic acids *via* their choline-binding module (CBM). In CbpG, the CBM consists only of three choline-binding repeats (CBR) (Maestro and Sanz, 2016). In serotype 19F strain EF3030, the CBM also consists of only 3 repeats; thus, CbpG is most likely not attached to the cell surface, because it is hypothesized that at least 4 CBRs are needed for proper attachment (Yother and White, 1994). In addition to the CBM, CbpG has a functional domain spanning amino acid residues 14–197, encoding for a trypsin type domain. The *in silico* analysis by signalP software tool (Nielsen, 2017) showed that CbpG had no recognizable signal peptide for protein secretion (**Figure 1**). The *sp_0389* gene located upstream of the *cbpG* locus encodes a hypothetical protein. In contrast, the downstream located gene *sp_0391* encodes the choline-binding protein F (Molina et al., 2009) (**Figure S1A**).

Impact of Pneumococcal Serine Proteases on Bacterial Fitness and Growth Behavior

To evaluate the effect of gene knockouts of different serine proteases on pneumococcal fitness and growth, we have investigated *S. pneumoniae* serotype 19F strain EF3030 and serotype 4 strain TIGR4 and their isogenic mutant strains under two different culture conditions. Pneumococcal growth was monitored in chemically defined $RPMI_{modi}$ or complex THY

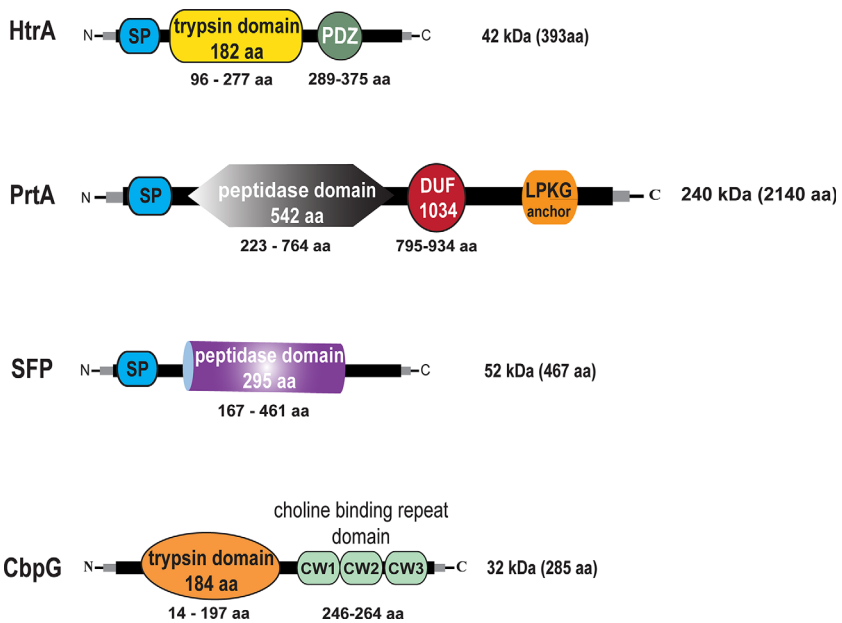

FIGURE 1 | Schematic models and *in silico* analysis of pneumococcal serine proteases. High-temperature requirement A belongs to the family of trypsin-like proteases (enzymatic domain shown in yellow). PrtA (pneumococcal protease A) is a cell wall-associated serine protease (enzymatic domain in gray), which belongs to the subtilisin-like proteases with an N-terminal signal peptide (SP) and a C-terminal LPKTG sortase A anchoring motif. Subtilase family protein with the peptidase domain (purple) but lacking an anchoring motif. Choline binding protein G (enzymatic domain orange), which contains in the C-terminal part a short choline-binding module consisting of three CW-repeats.

medium. Furthermore, the generation times of the wild-type and mutants were calculated in all the growth curves. In THY, the 19F wild-type strain and the *serine protease* mutants showed a comparable growth pattern, except for the mutant with CbpG+ as the only functional serine protease, which started to lyse shortly after reaching the stationary phase (**Figure 2A**). Statistically significant differences were only monitored in the late stationary phase for the CbpG+ mutants at time points 8, 9, and 10 h compared to the wild-type. In $RPMI_{modi,}$ the 19F wild-type strain and the mutant expressing only PrtA+ had a similar growth behavior. However, 19F mutants with only one functional serine protease (CbpG+ or HtrA+) showed a

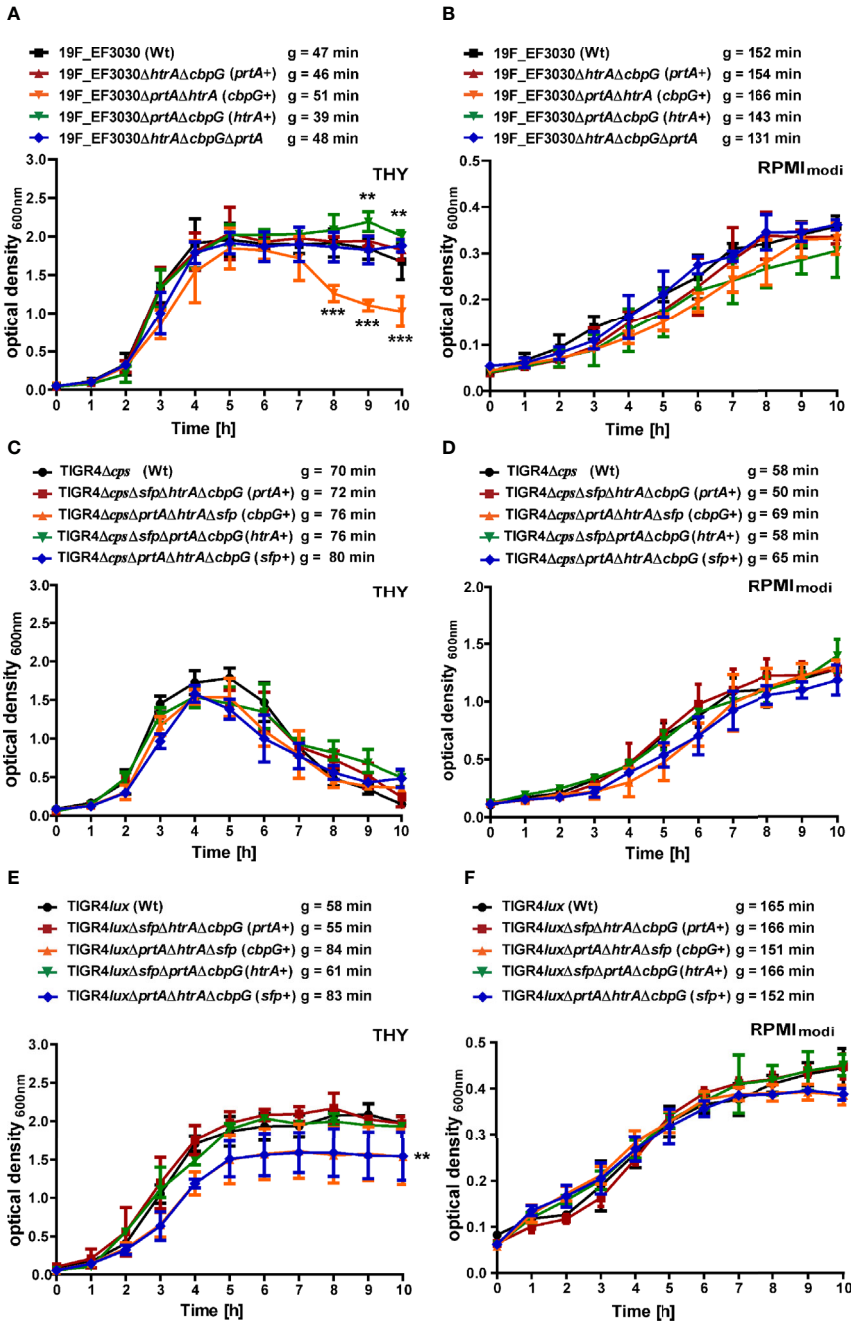

FIGURE 2 | Growth behavior of serine protease deficient pneumococci. Wild-type and isogenic mutants were cultured at 37°C and 5% CO_2 in THY and chemically defined medium ($RPMI_{modi}$). Pneumococcal growth was monitored at OD_{600}. The mean of four individual growth experiments is shown for 19F_EF3030 **(A, B)**, TIGR4Δ*cps* **(C, D)**, and TIGR4*lux* **(E, F)**. Error bars represent SD (n = 4). The symbol "g" indicates the generation time, calculated from four biological replicates. The data were statistically analyzed using a two-way ANOVA analysis $^*P < 0.05$.

delayed lag phase. Nevertheless, they reached a similar optical density compared to the wild-type (**Figure 2B**). No significant differences in the growth of the wild-type and isogenic mutants were observed in $RPMI_{modi}$. Compared to 19F, the TIGR4Δ*cps* strain and its isogenic serine protease mutants showed similar growth behavior in complex and chemically-defined medium. However, TIGR4 and the corresponding mutants started to lyse immediately after reaching the stationary growth phase in the THY medium (**Figures 2C, D**).

However, the encapsulated TIGR4*lux* and its isogenic *serine protease* mutants exhibited an extended stationary growth in THY medium. The mutants expressing CbpG+ or SFP+ showed similar generation times compared to the parental strain, but the overall growth was significantly reduced. The mutants entered the stationary phase already at a lower optical density (**Figure 2E**). TIGR4*lux* mutants cultured in $RPMI_{modi}$ medium showed no significant differences compared to the parental strain (**Figure 2F**).

We have further investigated growth of 19F single isogenic mutants in TYH and $RPMI_{modi}$ medium. No growth differences were observed when compared to the wild-type 19F (**Figure S5**). To determine whether the deletion of serine proteases impacts nutrient acquisition strain 19F and TIGR4 and their the isogenic mutants expressing no serine protease (19F) or only SFP were grown in CDM (Mickelson, 1964; Leonard et al., 1970) supplemented with 2% casein hydrolysate (Härtel et al., 2011). No significant growth differences were observed between the mutants and the corresponding parental strains (**Figure S5**). Growth rates are listed in **Table S.1**.

Serine Proteases Deficiency Reduced *S. pneumoniae* 19F and TIGR4Δ*cps* Adherence to Nasopharyngeal Host Cells

The initial step of pneumococcal infection is the specific adherence to host epithelial cells of the upper respiratory tract leading to colonization (Weiser et al., 2018). We investigated extracellular serine proteases role in adherence to host epithelial cells using the human nasopharyngeal epithelial cell line Detroit-562. Capsule expression is known to have a negative effect on adherence (Kim and Weiser, 1998; Hammerschmidt et al., 2005), and the non-encapsulated TIGR4Δ*cps* shows significantly higher *in vitro* adherence to epithelial cells than the encapsulated TIGR4 strain (Bootsma et al., 2007). Therefore, the impact of serine proteases on pneumococcal adherence was studied by infecting Detroit-562 with TIGR4Δ*cps* (serotype 4) and 19F EF3030. Detroit-562 cells were infected with wild-type or isogenic *serine protease* mutants with an MOI 50 for 4 h. For 19F, double *serine protease* mutants expressing only one functional serine protease (CbpG+, PrtA+ or SFP+) or a mutant lacking all serine proteases revealed a significant reduction of 19F adherence to Detroit-562 cells in comparison to the parental strain ($P < 0.05$, and $P < 0.01$) (**Figures 3A, C**). The TIGR4Δ*cps* triple serine protease mutants with only a single functional protease showed a significant reduction of adherence compared to the parental strain ($P < 0.05$), with the exception of the PrtA positive triple knockout (**Figures 3B, C**). Thus, the results suggest a substantial role of extracellular serine proteases in adherence to epithelial cells. Therefore, our adherence data suggest that serine proteases contribute to interacting with the host respiratory epithelial cells.

Extracellular Serine Proteases Are Involved in Pneumococcal Colonization

We showed that loss of functional serine proteases leads to reduced adherence to human nasopharyngeal epithelial cells. We assumed therefore that *in vivo* nasopharyngeal colonization is reduced in the absence of serine proteases. Hence, the impact of pneumococcal serine proteases on nasopharyngeal colonization has been assessed in a murine colonization model. We intranasally infected female CD-1 mice (7 mice/group) with 10^7 CFUs of either 19F EF3030 (wild-type) or isogenic *serine protease* mutants. Pneumococci were recovered 2, 3, 7, and 14 days post-infection from the nasopharyngeal cavity and lungs. In comparison to the isogenic wild-type, the deficiency in serine proteases resulted in a significant reduction ($P < 0.01$, and $P < 0.001$) of pneumococcal CFU in the nasopharyngeal cavity two days post-infection. Ten-fold less mutant pneumococci were determined after 2, 3, 7, and 14 days post-infection (**Figure 4A**).

Furthermore, on day three post-infection the bacterial load of 19F_EF3030Δ*prtA*Δ*cbpG* expressing only HtrA+ was significantly reduced in bronchoalveolar lavages. Although there was a trend to lower CFU in the lower respiratory tract, the other mutants did not show significant differences compared to the parental wild-type 19F (**Figure 4B**). In general, mice colonized with mutants deficient for serine proteases eliminated the bacteria faster on days 7 and 14 from the lower respiratory tract in comparison to the 19F wild-type. These data confirm the low invasive potential of strain 19F in the lung host compartment. Taken together, the results of the *in vitro* adherence study and the experimental mouse colonization model indicate that pneumococcal adherence to the nasopharynx is strongly affected by the loss of different serine proteases.

An Acute Pneumonia Model Indicates Only a Moderate Effect of Serine Proteases on *S. pneumoniae* TIGR4 Virulence

We further investigated the role of serine protease deficiency on pneumococcal virulence using an acute pneumonia model in mice. Because reduced colonization also leads to lower infection severity of the lungs, we hypothesized that the development of acute lung infection will be prevented in the absence of serine proteases. We infected 8–10-week old female CD-1 outbred mice (14 mice/group) intranasally with 9×10^7 bioluminescent TIGR4*lux* or corresponding isogenic triple serine protease knockout strains expressing only one out of four functional serine protease. We monitored the influence of serine proteases on pneumococcal dissemination into the lungs and transcytosis of the respiratory epithelial barrier into the bloodstream *in vivo* using the IVIS® Spectrum bioimaging system. Mice infected with the parental TIGR4*lux* strain showed the first weak signs of pneumonia in the lung after 24 h.

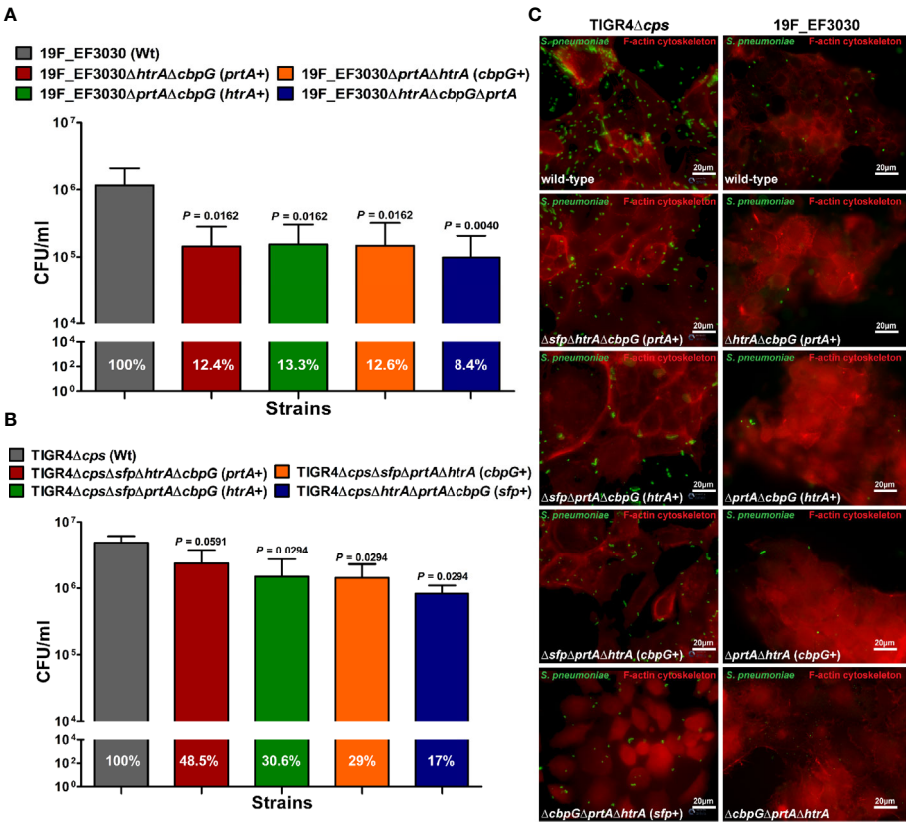

FIGURE 3 | Impact of pneumococcal serine proteases on pneumococcal adherence to host epithelial cells. Human Detroit-562 cells were infected for 4 h with an MOI of 50 of *S. pneumoniae* EF3030, TIGR4Δ*cps* wild-type or mutant strain. **(A, B)** Colony-forming units were determined post-infection by plating host cell adherent pneumococci on blood agar plates. Results were presented as the mean ± SD for at least four independent experiments performed in triplicates. *n.s.* $^{*}P < 0.05$, and $^{**}P \leq 0.01$ relative to the parental 19F pneumococcal strain. **(C)** Immunofluorescence microscopy of pneumococci attached to Detroit-562 cells after 4 h infection. Adherent pneumococci were stained with anti-pneumococcal antiserum followed by secondary Alexa-488 conjugated anti-IgG antibody (green). The epithelial F-actin was stained with Phalloidin-iFlour-594 conjugate (red).

On the contrary, the lung infection of mice infected with triple mutants, lacking the expression of three out of four serine proteases, started earliest 40 h post-infection (**Figures 5A, B**). Besides, the overall bioluminescent intensity was significantly decreased for all mice infected with mutants expressing only PrtA+, CbpG+ or SFP+ (**Figures 5C, D**). However, the bioluminescent flux of mice infected with the mutant expressing HtrA+ showed no significant difference compared to the wild-type (**Figure 5D**). The survival of mice infected with triple *serine protease* mutants expressing only one functional protease (CbpG+, SFP+, or HtrA+) showed, except for the PrtA+ mutant, no differences and are comparable to the parental strain TIGR4*lux* (**Figures 5E, F**). The triple mutant TIGR4 PrtA+ was significantly attenuated (*p-value* 0.0414). For example, only one mouse out of fourteen developed severe pneumonia 64 h post-infection as visualized by bioimaging (**Figure 5B**). Indeed, our results of the real-time bioimaging with the PrtA+ expressing TIGR4 mutant showing the lowest bioluminescence correlates with the survival time of the mice (**Figures 5C, F**). Taken together, the loss of serine proteases did not dramatically affect the virulence of TIGR4*lux* in the acute mouse pneumonia model. The only exception was the PrtA positive TIGR4 triple knockout, which was significantly attenuated, pointing to an important role of PrtA in the acute pneumonia model.

DISCUSSION

Serine proteases secreted by pneumococci or bound to the cell surface play a pivotal role in the pathogenesis of this human pathogen. These proteases have critical pathological and physiological functions including enzyme modification and cleavage of host immune proteins, which affect colonization and evasion of the host defense (Supuran et al., 2002; Bergmann and Hammerschmidt, 2007; Janoff et al., 2014). This has been intriguingly shown for zinc-metalloproteases (Novak et al., 2000; Govindarajan et al., 2012). Other studies have also assessed the impact of serine proteases on pneumococcal virulence using different experimental infection models (**Table 4**). In these studies the pneumococcal mutants were deficient for only one of the serine proteases (Gosink et al., 2000; Bethe et al., 2001; Ibrahim et al., 2004a; Mann et al., 2006; Mirza et al., 2011;

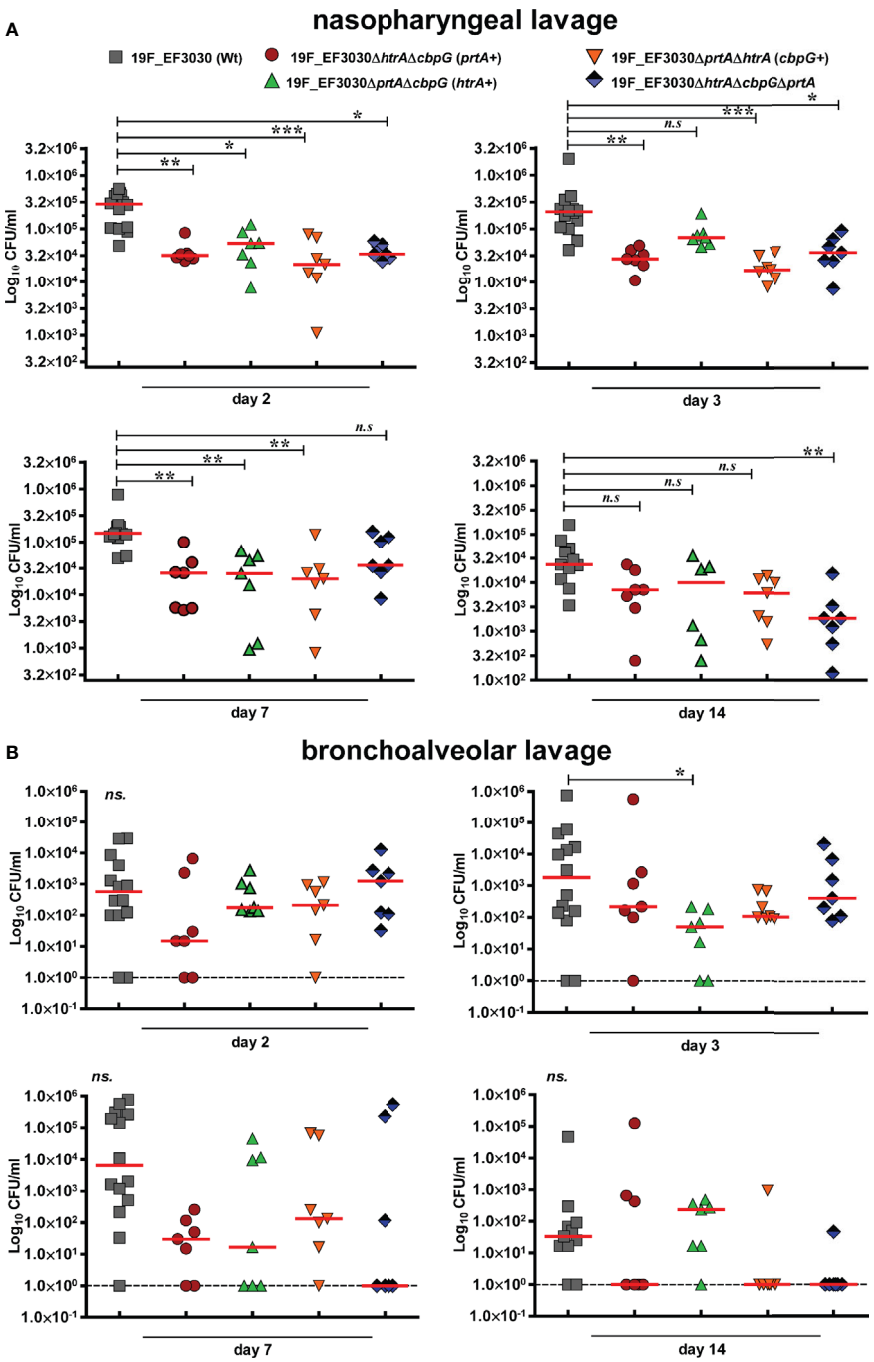

FIGURE 4 | Nasopharyngeal colonization in a murine infection model. Eight to ten-week-old female CD-1 outbred mice (n = 7) were infected intranasally with a CFU of 1×10^7 pneumococci of serotype 19F (EF3030) or isogenic *serine protease* mutants: 19F_EF3030Δ*htrA*Δ*cbpG* (*prtA*+), 19F_EF3030Δ*prtA*Δ*cbpG* (*htrA*+), 19F_EF3030Δ*htrA*Δ*prtA* (*cbpG*+), or 19F_EF3030Δ*htrA*Δ*prtA*Δ*cbpG*. Mice were sacrificed at day 2, 3, 7, or 14 post-infections, and pneumococci recovered by a nasopharyngeal **(A)** or bronchoalveolar lavage **(B)** and plated on blood agar plates for quantification. Results are shown as scatter plots, where each dot represents one individual mouse. The data were statistically analyzed using a Kruskal-Wallis test $^*p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$. The dashed line represents the limit of detection.

de Stoppelaar et al., 2013; Mahdi et al., 2015; Hsu et al., 2018). Hence, a potential redundancy of their mode of action could not be finally excluded. However, the lack of a functional HtrA attenuated *S. pneumoniae* D39 in the acute mouse pneumonia model and leads to lower bacterial burden in the lung, blood and organs. This indicated the crucial role for virulence, while the lack of PrtA had only a minor effect (de Stoppelaar et al., 2013). Similar, our studies with TIGR4 demonstrated that only mutants

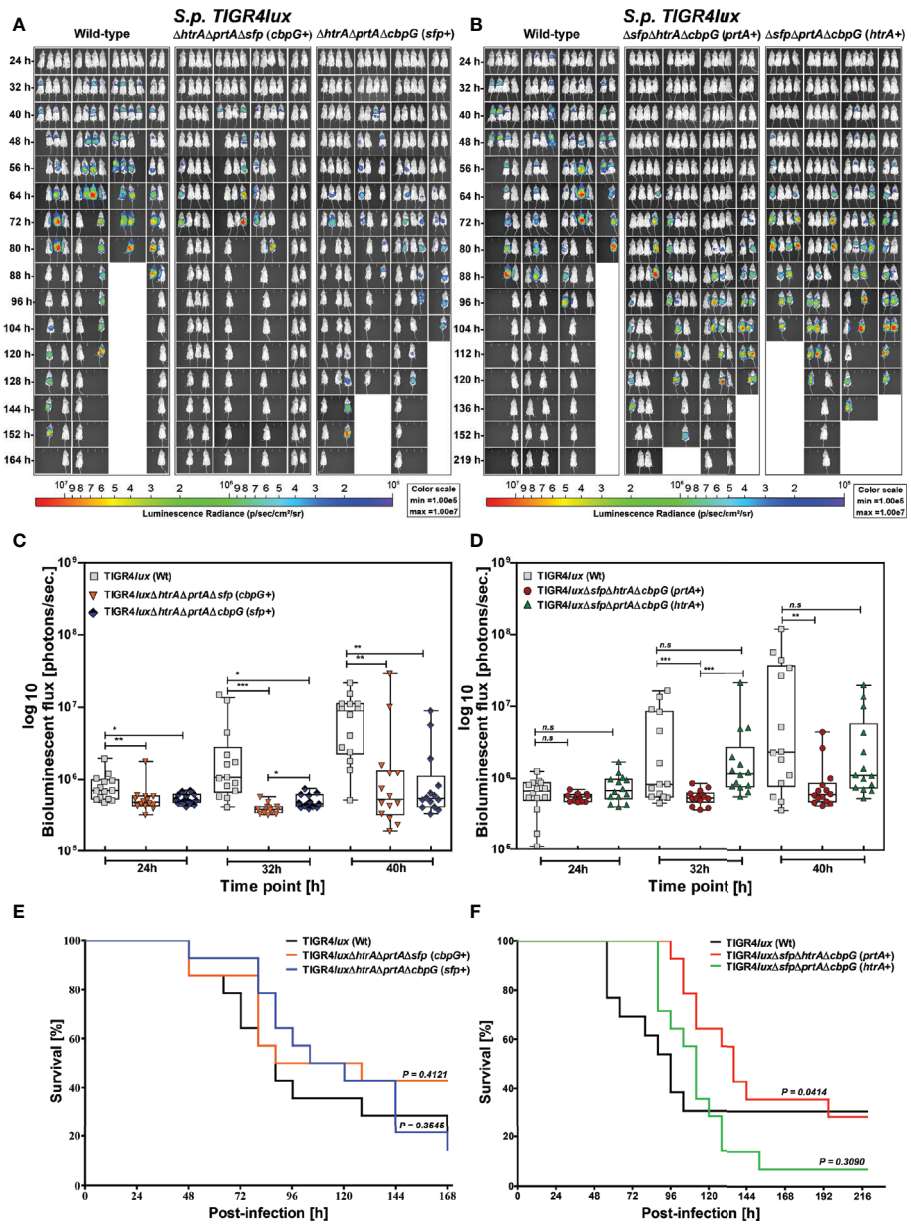

FIGURE 5 | Acute infections in an experimental pneumonia model. Eight to ten-week-old female CD-1 mice (n = 14) were intranasally infected with *S. pneumoniae* TIGR4*lux* wild-type or isogenic triple *serine protease* mutants using an infection dose of 9×10^7 CFU. **(A, B)** The course of infection was monitored in real-time using the IVIS®-Spectrum *in vivo* bioimaging system. **(C, D)** The multiplication and dissemination of bioluminescent pneumococci in infected mice were quantified at indicated time points by measuring the luminescence intensity (photons/second). Data are shown as a Box-Whisker graph showing the values for each mouse. Kruskal-Wallis test was used for statistical analysis. **(E, F)** Kaplan-Meier survival curves of mice infected with *S. pneumoniae* TIGR4*lux* or *serine protease* deficient mutants. The log-rank (Mantel-Cox) test was used for statistical analysis. $^*p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$.

expressing HtrA showed a similar bioluminescence of the infected lungs post-intranasal infection, suggesting similar bacterial burden in the lungs (**Figures 5C, D**). Recently, the important role of the chaperone/protease HtrA in bacto-viral co-infections was shown. HtrA was highly expressed under influenza A virus induced inflammation and protects against opsonophagocytosis and mediates resistance against oxidative damage (Sender et al., 2020). Thus, HtrA of pneumococci but also of other bacterial species is an indispensable virulence factor (**Table 4**).

However, the role of pneumococcal serine proteases including HtrA on colonization is still unknown. To investigate the influence of pneumococcal serine proteases on the host-pathogen interaction in the upper respiratory tract, we have studied the effect on adherence and colonization in the genetic background of triple protease deletion mutants. For adherence

TABLE 4 | Bacterial serine proteases and their role in pathogenicity.

Serine Proteases	Bacterial Species	Associated Disease	Role in Pathogenesis	Host-Targets	References
PrtA	*Streptococcus pneumoniae*	CAP[1], sepsis, meningitis	killing by apolactoferrin colonization adherence, pneumonia	cleaves human apolactoferrin	(Bethe et al., 2001; Mirza et al., 2011), **this study**
CbpG	*Streptococcus pneumoniae*	CAP, sepsis, meningitis	adherence, virulence factor adherence, colonization	cleaves human fibronectin	(Gosink et al., 2000; Mann et al., 2006), **this study**
SFP	*Streptococcus pneumoniae*	CAP, sepsis, meningitis	facilitates bacterial growth, adherence, colonization	unknown function	(de Stoppelaar et al., 2013), this study
CspA (SFP homolog)	*Streptococcus agalactiae*	CAP, sepsis, and meningitis	virulence factor, resistance to opsonophagocytosis	cleaves human fibronectin, inactivates chemokines	(Harris et al., 2003; Bryan and Shelver, 2009)
HtrA chaperone/ protease	*Streptococcus pneumoniae*	CAP, sepsis, meningitis	chaperone, heat-shock protein, protease, virulence factor, competence pathways, growth advantage in influenza A virus co-infections adherence, colonization	quality control of secreted proteins	(Ibrahim et al., 2004b; Sebert et al., 2005; Cassone et al., 2012; de Stoppelaar et al., 2013; Kochan and Dawid, 2013; Sender et al., 2020), **this study**
	Streptococcus pyogenes	purulent diseases of the pharynx and skin	processing of extracellular virulence factors and haemolytic activity	cleavage of complement factor C5a	(Wexler et al., 1985; Lyon and Caparon, 2004)
	Streptococcus mutans	dental caries	colonization	biofilm formation	(Biswas and Biswas, 2005)
	Campylobacter jejuni	Campylo-bacteriosis, Guillian Barré syndrome	bacterial adhesion, transmigration and invasion	cleavage of E –cadherin, apoptosis, and immune responses	(Boehm et al., 2013; Boehm et al., 2018)
	Helicobacter pylori	gastritis, ulcers symptoms	bacterial transmigration, activation of type IV secretion	cleavage of occludin, claudin-8, E-cadherin and fibronectin	(Hoy et al., 2010; Schmidt et al., 2016; Tegtmeyer et al., 2017)

[1]*community-acquired pneumonia.*

and nasopharyngeal colonization studies, we have used the serotype 19F (strain EF3030) (Junges et al., 2019), while the invasive TIGR4 strain was used in the acute pneumonia model. The *S. pneumoniae* serotype 19F strain EF3030 described as the causative agent for otitis media has already been shown to be an efficient colonizer in murine model systems (Joloba et al., 2001; Blevins et al., 2014). Our results showed that adherence of *S. pneumoniae* 19F to host epithelial cells is affected in a mutant deficient for two or three serine proteases produced by wild-type 19F and TIGR4Δ*cps*. This finding is in accordance with other studies showing that already the deletion of HtrA or homologs in other species than pneumococci leads to decreased bacterial adhesion to epithelial cells (Brondsted et al., 2005; Frees et al., 2013). The underlying molecular mechanisms in pneumococci are so far not fully understood. However, HtrA homologs in other bacterial species were involved in processing of adhesins and thus in the activity of adhesins (Backert et al., 2018). Furthermore, CbpG is proposed to be a multifunctional protein cleaving ECM proteins and is involved in adherence as indicated by a reduced adherence of a TIGR4 *cbpG*-mutant to nasopharyngeal epithelial cells (Gosink et al., 2000; Mann et al., 2006). Thus, each serine protease might contribute to pneumococcal adherence and the expression of a single serine proteases is probably not sufficient to reach adherence comparable to the wild-type. However, the molecular mechanisms might differ and have to be explored in further studies. Because of the altered adherence of the mutants under *in vitro* infections, we hypothesized that the loss of serine proteases would also have an impact on pneumococcal colonization of the nasopharynx. Hence, the experimental nasopharyngeal colonization model was used to assess whether the diminished adhesion of the 19F mutants to human epithelial cells correlates with the inability of serine protease deficient mutants to colonize the murine nasopharynx. Indeed, we monitored a dramatic decrease in the bacterial loads of the serine protease mutants in the nasopharyngeal colonization model compared to the isogenic parental strain 19F. Thus, these data strongly suggest that serine proteases are indispensable for colonization. Interestingly this effect is more pronounced in the nasopharynx compared to the bronchoalveolar space, which is not surprising considering the low capacity of 19F to cause lung infections in the mouse pneumonia model (Marks et al., 2013; Junges et al., 2019). In addition, the double mutant expressing HtrA shows 14 days post-infection a similar behavior in the bronchoalveolar lavage compared to the wild-type 19F, despite early time points show a significant reduction in colonization.

When using TIGR4 in the acute pneumonia model our data show that the deficiency of three serine proteases did not impair the full virulence of TIGR4 in mice. However, the quantification of bioluminescence as well as the monitoring of mouse survival suggest that the triple knockouts have a slightly reduced capacity to cause pneumonia and, in consequence, invasive disease. The only exception is the mutant with a functional PrtA, because this

mutant shows a significant attenuation in the acute pneumonia model. This is an interesting finding because the PrtA positive mutant is deficient in HtrA. The protein HtrA was shown earlier to be a major virulence factor in pneumococcal pneumonia caused by *S. pneumoniae* D39 in C57BL/6 mice, while SFP and PrtA played no major role (de Stoppelaar et al., 2013). It has to be mentioned that C57BL/6 are more resistant to pneumococcal infections compared to CD-1 mice (Gingles et al., 2001) used in our study and that, despite being able to cause severe pneumonia in mice, D39 and TIGR4 differ in their genomic content and show different regulatory processes (Saleh et al., 2014; Schulz et al., 2014). The importance of HtrA is furthermore evident in our pneumonia model because the HtrA expressing triple *serine protease* mutant shows, according to the bioluminescence data, a similar multiplication in the lung compared to the parental TIGR4 strain. Mutants lacking HtrA show significantly lower bioluminescence compared to the isogenic parental strain TIGR4. In addition, the mouse survival rates confirm the importance of HtrA for full virulence of *S. pneumoniae* TIGR4.

Therefore, the most interesting questions for further studies are the substrate-specificity of the serine proteases and how do these proteases mechanistically contribute to colonization or adhesion. The PDZ domain of HtrA is important for protein-protein interactions and is also important for the interaction with the protease domain and hence, for the proteolytic activity of HtrA (Fan et al., 2010; Fan et al., 2011). Therefore, the underlying mechanism is likely related to the HtrA enzymatic activity function, which can be activated by the interaction with host matrix components to modify host proteins during colonization. The role of CbpG for pneumococcal adherence and binding to human cells has already been shown in previous studies (Gosink et al., 2000; Mann et al., 2006), which may also apply to the other serine proteases. CbpG was shown to degrade the host protein fibronectin and casein (Mann et al., 2006).

The cleavage or degradation of host proteins is not limited to the activity of CbpG. The cell wall anchored PrtA is involved in the cleavage of host proteins like fibrinogen or collagen in order to penetrate tissues or escape from the immune system (Frolet et al., 2010). Besides, PrtA cleaves the host protein apolactoferrin to the even more bactericidal lactoferricin facilitating killing of pneumococci (Mirza et al., 2011). Therefore, it has been suggested that the presence of PrtA in the host may reduce pneumococcal load during systemic infection in the mouse model (de Stoppelaar et al., 2013) due to the bactericidal effect of apolactoferrin (Mirza et al., 2011).

Another study showed that the deficiency of PrtA in *S. pneumoniae* D39 reduced virulence in a sepsis mouse model after intraperitoneal infection (Bethe et al., 2001). Considering our colonization data with 19F and the pneumonia data with TIGR4, we hypothesize that PrtA contributes to colonization but not to lung infections. A finding that confirms earlier data proposing that some of the sortase anchored pneumococcal proteins including PrtA have adhesive functions (Frolet et al., 2010; de Stoppelaar et al., 2013). However, to decipher the individual impact of PrtA or other serine proteases on adherence and colonization or even pneumonia single knockout strains and *in trans* complemented mutants have to be tested in adherence, colonization but also in biofilm assays. Additionally, a structure-function analysis is needed, which requires the structural analysis of serine proteases. So far, the complete structure of HtrA of *Campylobacter jejuni* is solved, while for pneumococci only the PDZ domain is reported (Fan et al., 2010; Fan et al., 2011; Zarzecka et al., 2020).

SFP has previously been shown to play only a minor role in pneumococcal virulence of strain D39 (de Stoppelaar et al., 2013). CspA, a serine protease from *S. agalactiae* (group B streptococci), is highly homologous to SFP and has been shown to inactivate chemokines (Bryan and Shelver, 2009). Importantly, *in silico* results indicated that the *sfp* gene is not present in serotype 19F strain EF3030. Furthermore, SFP is shortened in TIGR4 due to a premature stop codon. Our data confirm that SFP is probably not crucial for *S. pneumoniae* virulence, while its role in colonization is still elusive.

We postulate that extracellular serine proteases influence pneumococcal adherence to mucosal cells. Colonization is therefore also affected either by their proteolytic activity or their adhesive function. On the one hand, it is likely that serine proteases contribute to host-pathogen interactions by degrading host proteins, facilitating binding to host cells or even dissemination in the host. On the other hand, it cannot be excluded that serine proteases are also involved in the cleavage and release of other pneumococcal surface proteins, which might be a strategy to evade the host immune system and facilitate adhesion to host cells by demasking important receptors. A direct adhesive activity has already been proposed for CbpG (Mann et al., 2006). This adhesion function may also be possible for the other serine proteases but must be further investigated in future studies.

In conclusion, we highlight here that the deficiency of serine proteases impairs significantly nasopharyngeal colonization. Therefore, serine proteases have the potential to facilitate pneumococcal colonization and binding to their host targets.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg–Vorpommern (LALLFV M-V, Rostock, Germany) and the LALLFV M-V ethical board (LALLF M-V permit no. 7221.3-1-056/16).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MA and SH. Experiments performed by MA, TK, FV. Mutants constructed

by MA, NH, AW, RB, and GB. Editorial advice: SH, TK, and GB. Data analyzed and wrote the manuscript: MA, and revision SH. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the German Academic Exchange Service (DAAD) as a grant scholarship and part of the PhD thesis of MA, Funding programme/-ID: Research Grants - Doctoral Programmes in Germany, 2017/18 (57299294), ST33. The work was further supported by the Bundesministerium für Bildung und Forschung (BMBF- Zwanzig20 -InfectControl 2020 – Project VacoME – FKZ 03ZZ0816A to SH) and the Federal Excellence Initiative of Mecklenburg Western Pomerania and European Social Fund (ESF) Grant KoInfekt (ESF_14-BM-A55-0001_16). The funders had no role in study design, data collection, analysis, decision to publish, or manuscript preparation. We acknowledge support for the Article Processing Charge from the DFG (German Research Foundation, 393148499) and the Open Access Publication Fund of the University of Greifswald.

ACKNOWLEDGMENTS

We would like to acknowledge Professor Anders Håkansson (Lund University, Sweden) for providing *S. pneumoniae* 19F strain EF3030. We thank Birgit Rietow (University of Greifswald) for technical assistance. We also thank Prof. Juan A. Hermoso (Spanish National Research Council, Madrid) for kind help in CbpG protein analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.613467/full#supplementary-material

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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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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 11 March 2021
doi: 10.3389/fcimb.2021.613195

Novel Virulence Role of Pneumococcal NanA in Host Inflammation and Cell Death Through the Activation of Inflammasome and the Caspase Pathway

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *01 October 2020*
Accepted: *05 February 2021*
Published: *11 March 2021*

Citation:
Tseng Y-W, Chang C-C and Chang Y-C (2021) Novel Virulence Role of Pneumococcal NanA in Host Inflammation and Cell Death Through the Activation of Inflammasome and the Caspase Pathway. Front. Cell. Infect. Microbiol. 11:613195. doi: 10.3389/fcimb.2021.613195

Streptococcus pneumoniae is one of most deadly Gram-positive bacterium that causes significant mortality and morbidity worldwide. Intense inflammation and cytotoxicity is a hallmark of invasive pneumococcal disease. Pneumococcal NanA has been shown to exaggerate the production of inflammatory cytokines via unmasking of inhibitory Siglec-5 from its sialyl *cis*-ligands. To further investigate the mechanistic role of NanA and Siglec-5 in pneumococccal diseases, we systemically analyzed genes and signaling pathways differentially regulated in macrophages infected with wild type and NanA-deficient pneumococcus. We found that NanA-mediated desialylation impairs the Siglec-5-TLR-2 interaction and reduces the recruitment of phosphatase SHP-1 to Siglec-5. This dysregulated crosstalk between TLR-2 and inhibitory Siglec-5 exaggerated multiple inflammatory and death signaling pathways and consequently caused excessive inflammation and cytotoxicity in the infected macrophage. Collectively, our results reveal a novel virulence role of NanA in pneumococcal pathogenesis and suggest that targeting NanA activity may ameliorate the pneumococcus-mediated inflammation and cytotoxicity in severe invasive pneumococcal diseases.

Keywords: ***Streptococcus pneumoniae*, sialidase, NanA, inflammasome, caspase**

INTRODUCTION

Streptococcus pneumoniae (SPN, pneumococcus) is a common colonizer of the human upper respiratory tract (URT) with an carriage rate of 20-50% and 8-30% in healthy children and adults, respectively (Melegaro et al., 2004; Regev-Yochay et al., 2004; Mccullers, 2006). Invasive pneumococcal diseases such as pneumonia, bacteremia, and meningitis occur when the bacteria spread from the nasopharynx to the lungs, blood, and brain. Over 14.5 million invasive

pneumococcal diseases are recorded annually, with a case fatality rate of 11% for children under the age of 5 years and 10-25% for the elderly (Black et al., 2010; Spijkerman et al., 2011; Musher and Thorner, 2014). This bacterium is responsible for over a million deaths annually (Kadioglu et al., 2008; O'brien et al., 2009).

The host recognizes pneumococci and orchestrates immune responses via multiple pattern recognition receptors (PRRs), including the membrane-bound Toll-like receptors (TLRs) and the cytosolic nucleotide-binding oligomerization domain-like receptors (NLRs). Upon activation, these PRRs induce the expression of proinflammatory cytokines at the levels of transcription and post-translational proteolytic processing (Koedel et al., 2003; Malley et al., 2003; Knapp et al., 2004; Opitz et al., 2004; Mcneela et al., 2010; Davis et al., 2011; Witzenrath et al., 2011). Intense inflammation and cytotoxicity is hallmarks of pneumococcal diseases, which contribute to the clearance of bacteria, however excessive activation of the same immune responses is often detrimental to the host (Dockrell et al., 2003; Musher et al., 2004; Corrales-Medina and Musher, 2011).

Siglecs are sialic acid-binding immunoglobin (Ig)-like lectins which broadly express throughout the immune system. Most Siglecs have a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and are considered to play an inhibitory role in the immune system via recruiting the SH2 domain containing protein tyrosine phosphatase (SHP)-1 and SHP-2 (Crocker et al., 2007; Pillai et al., 2012). In static state, Siglecs are 'masked' by the *cis*-sialyl ligands expressing on the same cell to initiate an ITIM-mediated suppressive signal, which limits immune cell activation and maintains immunological homeostasis. Removal of sialic acids from the cell surface has been shown to enhance the inflammatory response of monocytes to LPS stimulation (Stamatos et al., 2010). Sialic acid mimetic treatment, which reduces sialic acid expression and subsequently abrogates the interaction between Siglecs and its *cis*-sialyl ligands, drastically lowered the activation threshold of dendritic cells upon TLR engagement (Bull et al., 2017). In contrast, administration of sialidase inhibitor protected mice from polymicrobial sepsis and LPS-induced endotoxemia (Chen et al., 2011; Chen et al., 2014). These observations suggest that the content of surface sialic acids plays a crucial role in controlling immune cell activation.

A broad and direct interaction between Siglec and TLR was identified where Siglec negatively regulates TLR activation (Chen et al., 2014). Mammalian neuraminidase-1 (Neu-1), which translocated to the cell surface upon LPS stimulation, has been shown to disrupt the interaction between Siglecs and TLR-4 and restore the TLR-4 function (Amith et al., 2010; Abdulkhalek et al., 2011; Chen et al., 2014). In addition, we found that bacterial sialidase, NanA, causes exacerbated host inflammation through releasing Siglec-mediated immunosuppression (Chang et al., 2012), although the mechanism by which NanA exerts this immunomodulatory effect is not fully understood. In this study, we demonstrated that NanA-mediated desialylation impairs the Siglec-5-TLR-2 interaction and reduces the recruitment of phosphatase SHP-1 to Siglec-5. This dysregulated crosstalk between TLR-2 and inhibitory Siglec-5 provokes the activation of PRR-related signaling molecules, inflammasomes, and caspases, which consequently results in the excessive inflammation and cytotoxicity of infected host cells.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies used in this study were listed in **Table 1**. Inhibitors Ac-YVAD-cmk and MCC950 were from Sigma and Z-IETD-FMK was from Enzo Life Sciences. Biotin-conjugated *Erythrina cristagalli* lectin (ECA) and peanut agglutinin (PNA) were from Vector Laboratories. The Annexin V/7-AAD apoptosis kit was from BioLegend.

Bacterial Strains and Cell Culture

Streptococcus pneumoniae (SPN) serotype 2 strain D39 (NCTC 7466), isogenic Δ*nanA* mutant, and *nanA*-complemented strains used in this study have been previously described (Chang et al., 2012). SPN was cultured in static liquid Todd-Hewitt broth (Acumedia) containing 2% yeast extract (Acumedia) at 37°C with 5% CO_2 to mid-log phase for experiments. THP-1 cells (ATCC TIB-202), Siglec-5 overexpressing THP-1 cells (Sig-5/THP-1 cells, from Dr. Angata Takashi, Academia Sinica), and Siglec-5 knockdown THP-1 cells (Chang et al., 2012) were maintained in RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 2.5 g/L glucose, and 0.05 mM 2-mercaptoethanol. For some experiments, THP-1 cells were differentiated into macrophages with 25 ng/ml PMA for 24 h, followed by resting for 48 h in fresh RPMI 1640 medium before being used for experiments. Human primary monocytes were isolated from healthy donors (with use and procedures approved by the National Taiwan University IRB 201911067RINC) using MagniSort™ Human CD14 Positive Selection Kit (Thermo) and were differentiated to macrophages by culturing in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 0.05 mM 2-mercaptoethanol, and 10 ng/ml M-CSF for 6 days. The *SIGLEC5/14* genotype of each donor was characterized by genomic PCR as previously described (Yamanaka et al., 2009), and macrophages derived from individuals with *SIGLEC14*-null genotype were used in this study.

NanoString Gene Expression Analysis

THP-1 cells were infected with pneumococcus at a multiplicity of infection (MOI) of 10 for 6 h. RNA from infected cells was extracted by RNeasy Mini kit (Qiagen), followed by quantification for gene expression analysis using nCounter® human Immunology v2 panel (NanoString Technologies) through the service provided by Cold Spring Biotech, Taiwan. The generated gene expression data sets were analyzed by nSolver™ analysis software (NanoString Technologies). Background values were corrected from raw data and normalized using 15 housekeeping genes (ABCF1, ALAS1, EEF1G, G6PD, GAPDH, GUSB, HPRT1, OAZ1, POLR1B, POLR2A, PPIA, RPL19, SHDA, TBP, and

TABLE 1 | Antibodies and primers used in this study.

Antibody	Source	Application
β-actin	Sigma #A5441	WB
ASC	Santa Cruz Biotechnology #sc-271054	WB
Caspase-1	Cell Signaling Technology #3866	WB
Caspase-8	Cell Signaling Technology #9496	WB
Flotillin-1	BD Biosciences #610821	WB
Gasdermin D	Cell Signaling Technology #93709	WB
IKKβ	Cell Signaling Technology #8943	WB
IL-1β	Cell Signaling Technology #12242	WB
LC3A/B	Epitomics #2057-1	WB
p38α MAPK	Cell Signaling Technology #9217	WB
p44/p42 MAPK (Erk1/2)	Cell Signaling Technology #9107	WB
Siglec-5	BioLegend #352002	IP
Siglec-5/14	R&D Systems #AF1072	WB
SHP-1	Santa Cruz Biotechnology #sc-7289	WB
SHP-2	Santa Cruz Biotechnology #sc-280	WB
SOCS3	Origene #TA503055	WB
TLR-2	BioLegend #309702	IP
TLR-2	Cell Signaling Technology #12276S	WB
phospho-AMPKα (T172)	Cell Signaling Technology #4188	WB
phospho-Akt (S473)	Cell Signaling Technology #9271	WB
phospho-(c)Jun (S63)	Cell Signaling Technology #2361	WB
phospho-IKKα β (S176/S180)	Cell Signaling Technology #2697	WB
phospho-JNK (T183/Y185; T221/Y223)	Merck #07-175	WB
phospho-Lck (Y505)	Cell Signaling Technology #2751	WB
phospho-Lyn (Y507)	Cell Signaling Technology #2731	WB
phospho-MEK1 (S218/222)/ MEK2 (S222/226)	Merck #05-747	WB
phospho-MKK3 (S189)/MKK6 (S207)	Cell Signaling Technology #9236	WB
phospho-MKK7/SKK4 (T275)	Merck #36-013	WB
phospho-NF-κB p65(S529)	Epitomics #2884-1	WB
phospho-p38α MAPK (T180/ Y182)	Cell Signaling Technology #4511	WB
phospho-p44/42 MAPK (Erk1/ 2)(T202/Y204)	Cell Signaling Technology #4370	WB
phospho-p70 S6 Kinase (T389)	Merck #MABS82	WB
phospho-PDK1 (S241)	Cell Signaling Technology #3438	WB
phospho-PKCγ (T655)	Merck #07-879	WB
phospho-PKR (T446)	Merck #07-532	WB
phospho-PLCγ1 (Y783)	Cell Signaling Technology #2128	WB
phospho-Shc (Y317)	Cell Signaling Technology #2431	WB
phospho-SHP2 (Y542)	Epitomics #2184-1	WB
phospho-Syk (Y323)	Merck #07-915	WB
phospho-Syk (Y525/526)	Cell Signaling Technology #2710	WB
phospho-Src (Y416)	Merck #05-677	WB
phospho-Src (Y527)	Cell Signaling Technology #2015	WB
IRDye® 800CW Donkey anti-Rabbit IgG (H + L)	LI-COR, Cat#926-32213	WB
IRDye® 680RD Donkey anti-Mouse IgG (H + L)	LI-COR, Cat#926-68072	WB
IRDye® 680RD Donkey anti-Goat IgG (H + L)	LI-COR, Cat#926-68074	WB

IP, immunoprecipation; WB, western blot.

TUBB). Differential expressed genes (WT/Δ*nanA*>1 or <1) were further analyzed using the **d**atabase for **a**nnotation, **v**isualization and **i**ntegrated **d**iscovery (DAVID, https://david.ncifcrf.gov/).

RNA Isolation, RT-PCR, and qRT-PCR

Total cellular RNA was extracted using NucleoZOL reagent (Macherey-Nagel) and transcribed to cDNA by PrimeScript RT reagent (TaKaRa) according to the manufacturer's instructions. The resulted cDNA was amplified by quantitative RT-PCR using iQ™ SYBR® Green Supermix (Bio-rad) on the CFX96 Touch™ real-time detection System (Bio-rad). Primers used for experiments were as follows: HPRT-1, CAAGCTTGCTGGTGAAAAGGAC, GTCAAGGGCATATCCTACAACAAA; IL-1β, AAATACCT GTGGCCTTGGGC, TTTGGGATCTACACTCTCCAGCT; IL-8, ATAAAGACATACTCCAAACCTTTCCAC, AAGCTTTACAAT AATTTCTGTGTTGGC; TNF-α, CCCAGGGACCTCTCTCT AATCA, GCTTGAGGGTTTGCTACAACATG.

Cytokine Detection

Released IL-1β, IL-8, and TNF-α in culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (all from Invitrogen).

Immunofluorescence Microscopy

THP-1 cells were seeded on poly-L-lysine coated coverslip in the presence of PMA at a final concentration of 25 ng/ml overnight. The differentiated THP-1 cells were infected with CFSE (carboxyfluorescein succinimidyl ester, BioLegend)-labeled pneumococcus at an MOI of 10 at 37°C for 1 h. Infected cells were fixed with fix solution (2% paraformaldehyde/PBS), permeabilized with 0.5% Triton X-100/fix solution, blocked with 3% BSA/PBS, and stained with biotin-conjugated ECA and Alexa Fluor 568-conjugated streptavidin (Thermo). Stained cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole, Thermo) and visualized under fluorescent microscope (EVOS cell imaging system, Thermo).

Isolation of Lipid Raft Fractions

THP-1 cells were infected with pneumococcus at an MOI of 10 for 1 h at 37 °C, rinsed with PBS, lysed with ice-cold 1% Brij-58/ TNE buffer (25 mM Tris pH7.5, 150 mM NaCl and 5 mM EDTA) containing protease inhibitor cocktail (Roche), and kept on ice for at least 30 min. The raft-containing supernatants were collected by centrifuging at 300g at 4 °C for 5 min, gently mixed with an equal volume of 80% sucrose/TNE buffer, and centrifuged through a 5-30% continuous sucrose gradient in a SW41 Ti rotor (Beckman Coulter) at 40,000 rpm for 18 h at 4°C. Fourteen 0.7 ml fractions were sequentially collected from the top to bottom.

Western Blotting and Co-Immunoprecipitation

THP-1 cells were infected with pneumococcus at an MOI of 10, 30, and 100 for 1 and 3 h. Infected THP-1 cells were lysed in 1% NP-40 lysis buffer containing Halt™ protease and phosphatase inhibitor cocktail (Thermo) and centrifuged at maximal speed to collect cell lysates. Proteins released into the culture supernatants were precipitated with 25% trichloroacetic acid (TCA) at -80 °C overnight and pelleted by centrifugation. The resulted samples were resuspended with SDS-PAGE sampling buffer, separated on SDS-PAGE gels, transferred to PVDF membranes, detected with

indicated primary antibodies and IRDye® 800CW- or 680RD-conjugated secondary antibodies (Li-Cor), and visualized and quantified with a Li-Cor Odyssey scanner and software. For Co-immunoprecipitation, cells were lysed with 1% NP-40 lysis buffer with Halt™ protease and phosphatase inhibitor cocktail. Cell lysates were incubated with indicated antibodies plus protein A/G mix magnetic beads (Millipore) at 4 °C overnight. The immunoprecipitates were washed extensively and resuspended with SDS-PAGE sampling buffer for western blot analysis. Densitometry of various analyte proteins and their respective loading controls from the same blot was performed using Image J 1.53 (NIH) software.

Micro-Western Array (MWA)

THP-1 cells and human primary macrophages were infected with pneumococcus at an MOI of 5 at 37 °C for the indicated times. Lysates from infected cells were collected and subjected to Micro-Western Array analysis as previously described (Ciaccio et al., 2010). The resulted images were scanned by the Odyssey Infrared Imaging System (Li-Cor), quantified with Image Studio V5.2 software (Li-Cor), and normalized against β-actin.

ASC Oligomerization Assay

THP-1 cells were infected with pneumococcus at an MOI of 30 and 100 for 1 and 3 h. The infected cells were resuspended in buffer A (20 mM Hepes-KOH, pH7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 320 mM sucrose) containing protease inhibitor cocktail, sheared by passing through 27G needles 10 times, and centrifuged at 1800 rpm for 8 min to remove intact cells and nuclei. The collected supernatant was mixed with an equal volume of CHAPS buffer (20 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.1% CHAPS) and centrifuged at 5000 rpm for 8 min. The resulted pellet was cross-linked with dextran sulfate sodium (Thermo, final concentration of 2 mM) for 30 min at room temperature and resuspended with SDS-PAGE sampling buffer for western blot analysis.

WST-1 Assay

Cell viability was measured in triplicates by a colorimetric WST-1 kit (TaKaRa) according to manufacturer's instructions. Briefly, THP-1 cells were cultured in phenol red-free RMPI medium containing 10% FBS and infected with pneumococcus at an MOI of 3, 10, and 30 for 3 h. The infected cells were incubated with WST-1 PreMix (10% of total volume) for 3 h at 37 °C. The absorbance of the samples was determined at 450 nm.

Cell Death Analysis

THP-1 cells were infected with pneumococcus at an MOI of 30 for 1 h and 3 h, stained with APC-conjugated Annexin V and 7-ADD viability staining solution (BioLegend) for 15 min at room temperature in the dark, and immediately analyzed by FACS Calibur flow cytometer (BD).

Apoptotic Nuclei Determination by Flow Cytometry

The percentage of apoptotic nuclei was measured by propidium iodine (PI) staining as previously described (Nicoletti et al., 1991). THP-1 cells were infected with pneumococcus at an MOI of 30 and 100 for 4.5 h, followed by fixation with 70% cold ethanol at -20 °C overnight. The fixed cells were stained with PI solution (PBS containing 20 μg/ml PI (Sigma-Aldrich), 400 μg/ml RNase A (Sigma-Aldrich), and 1% Triton X-100) for 30 min at room temperature in the dark. The PI fluorescence of individual nuclei was measured by FACS Calibur and analyzed with DNA analysis ModFit LT™ software (Verity Software House).

Statistics

All statistical tests were performed using GraphPad Prism version 8 software (GraphPad Software, Inc.). Differences were determined using the two-tailed t test, one-way ANOVA, or two-way ANOVA tests as indicated in the legend. A p-value <0.05 was considered statistically significant for all tests.

RESULTS

Differentially Expressed Genes in Pneumococcus-Infected THP-1 Monocytes

To achieve a comprehensive understanding of inflammation-related genes modulated by NanA and Siglec-5 in response to pneumococcal infection, we infected Siglec-5 overexpressing THP-1 cells (Sig-5/THP-1) with wild-type (WT) SPN or isogenic sialidase deficient mutant (Δ*nanA*) and profiled the expression of 579 immune-related genes using the NanoString nCounter Human Immunology Panel. The complete list of the differentially expressed genes (DEGs, defined by WT/Δ*nanA* >1 or <1) between WT SPN- and Δ*nanA*-infected Sig-5/THP-1 cells was shown in **Table S1**. A total of 100 top differentially expressed genes, including 50 upregulated genes and 50 downregulated genes, were shown in **Figure 1A**. We found that NanA significantly upregulates the expression of proinflammatory and chemoattractant genes like IL1β, IL1RN, IL23A, IL8, TNF, CXCL1, CXCL2, CXCL10, CXCL11, CCL4, and CCL20 and genes involved in NF-κB signaling pathway, such as NFKB1, NFKB2, NFKB1A, NFKB1Z, and RELB. In contrast, surface receptors involved in pathogen recognition and antigen presentation, such as FCGR1A/B, HLA-DRA, CLEC5A, ITGA6, MR1, and CCR2, were downregulated by NanA. To identify the biological pathways targeted by pneumococcal NanA, we conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for all the DEGs identified from WT SPN- and Δ*nanA*-infected Sig-5/THP-1 cells. KEGG pathway analysis revealed that genes upregulated by NanA are highly enriched in pathways related to TLR, NF-κB, and cytokine/cytokine receptor (**Figure 1B** and **Table 2**). On the other hand, NanA-downregulated genes seemed to be involved in host responses related to viral infections, but these genes showed sporadic distribution and were not enriched in a given pathway (**Figure 1C** and **Table 3**).

Overexpression of Siglec receptors in myeloid cells has been shown to play both a positive and a negative role in regulating the immune responses upon various stimulations (Ohta et al., 2010;

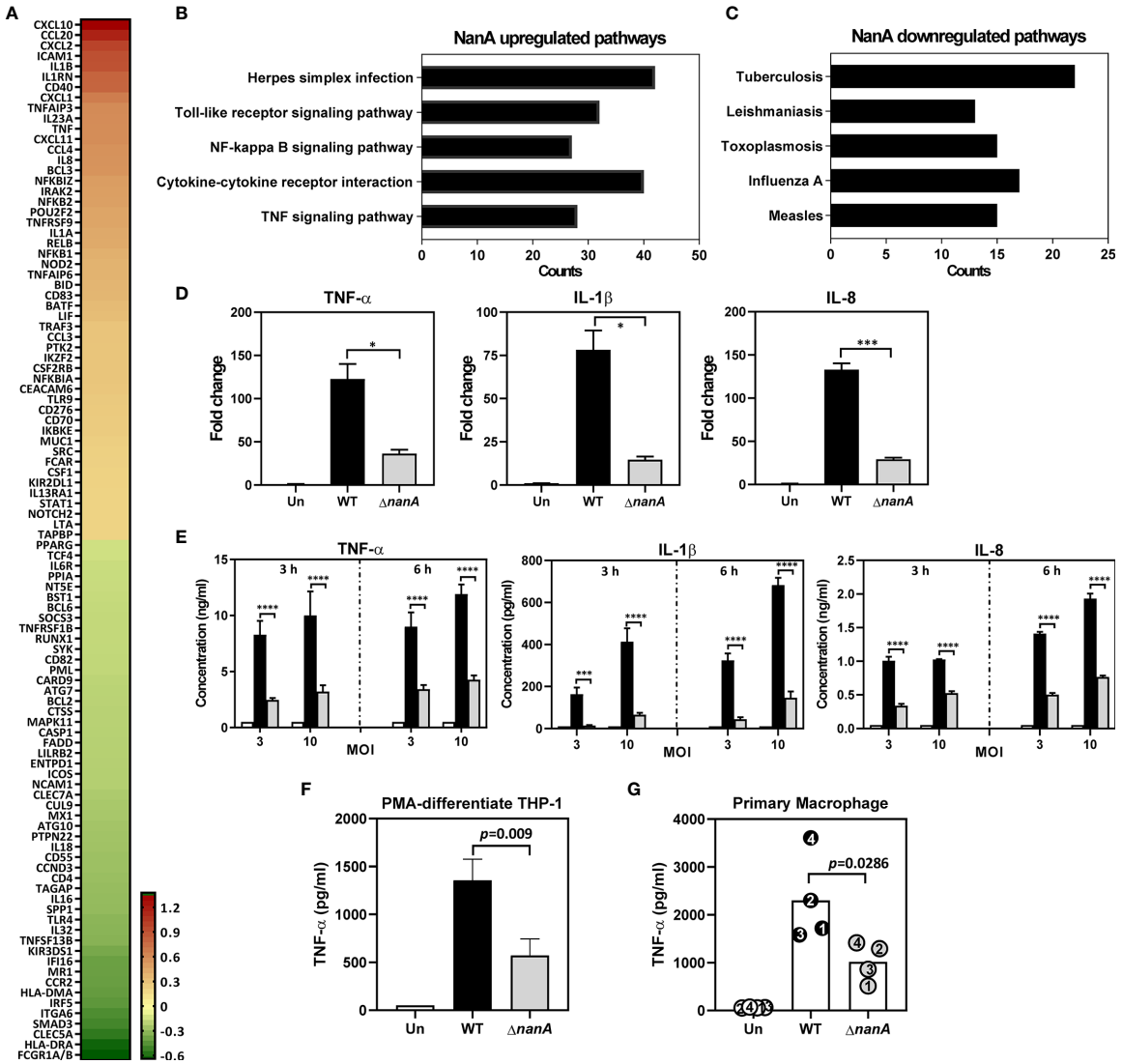

FIGURE 1 | Major gene expression profile of THP-1 monocytes upon pneumococcal infection. **(A)** Heatmap of top 50 genes differentially expressed in WT SPN- and Δ*nanA*-infected Sig-5/THP-1 cells. Fold changes combined from two independent Nanostring experiments were calculated as the ratio of WT/ Δ*nanA*, followed by log2 transformation. A heatmap was created by Prism software. Top 5 pathways identified by KEGG pathway enrichment analysis of the **(B)** 220 NanA-upregulated genes and the **(C)**128 NanA-downregulated genes. **(D)** Parental THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 10 for 3 h and the transcript levels of TNF-α, IL-1β, and IL-8 in the infected cells were analyzed by RT-qPCR analysis. Representative data from three independent experiments was shown as mean±SD. **(E)** Parental THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 3 and 10 for 3 h and 6 h, and the released TNF-α, IL-1β, and IL-8 in the culture supernatant were measured by ELISA. The data presented as mean±SD was representative of three independent experiments, each performed in triplicate. **(F)** TNF-α concentration in supernatants was measured from PMA-differentiated THP-1 macrophages 3 h post pneumococcal infection (MOI of 10). Data represented mean±SD were pooled from two independent experiments, each performed in triplicate. **(G)** Primary human macrophages were infected with WT SPN and Δ*nanA* at an MOI of 10 for 3 h, and TNF-α concentration in supernatants was measured by ELISA. Each dot represented a different donor. Statistical analysis was performed using Student's *t* test **(D–G)**. ****$P < 0.0001$; ***$P < 0.001$; *$P < 0.05$.

Higuchi et al., 2016; Li et al., 2019). To ascertain that the profound changes observed in Sig-5/THP-1 cells are not attributed from Siglec receptor overexpression, we used quantitative reverse transcription-PCR (qRT-PCR) to examine the expression of NanA-upregulated inflammatory mediators in parental THP-1 cells which express only low levels of endogenous Siglec-5. In line with what has been shown in Sig-5/THP-1, NanA also remarkably increased the expression of inflammatory genes TNF-α, IL-1β, and IL-8 in pneumococcus-infected parental THP-1 cells (**Figure 1D**). In accordance with the gene expression data, a 3-fold increase in TNF-α, IL-1β, and IL-8 protein levels was found in the WT SPN-infected THP-1 cells compared to the Δ*nanA* -infected cells (**Figure 1E**). Similar findings were also observed for TNF-α production in the PMA-

TABLE 2 | KEGG pathway analysis of genes upregulated by NanA.

KEGG ID	Description	Counts	P value (Benjamini)	Genes
hsa05168	Herpes simplex infection	42	7.61E-28	TRAF1, TRAF2, CCL2, TNF, TBK1, C3, C5, TLR2, TLR3, NFKBIA, NFKB1, CCL5, CD74, TLR9, CFP, CASP3, MYD88, TAP2, TICAM1, TAP1, IL1B, HLA-DOB, CHUK, LTA, IFNGR1, TRAF3, RELA, HLA-A, HLA-C, TNFRSF14, HLA-B, STAT1, IFNAR1, STAT2, IKBKE, IFNAR2, C1QBP, IKBKG, IL12A, JAK1, IRF3, IKBKB
hsa04620	Toll-like receptor signaling pathway	32	6.57E-25	CCL3, TNF, TBK1, TOLLIP, TLR1, TLR2, TLR3, NFKBIA, NFKB1, CXCL11, CCL5, CCL4, CXCL10, TLR9, IRAK4, MYD88, TICAM1, IL1B, CHUK, TRAF3, RELA, CD40, STAT1, IFNAR1, IKBKE, IFNAR2, CD86, CD80, IKBKG, IL12A, IRF3, IKBKB
hsa04064	NF-kappa B signaling pathway	27	2.92E-21	TRAF1, TRAF2, TNF, PTGS2, NFKBIA, NFKB1, NFKB2, CCL4, BTK, IRAK4, MYD88, TICAM1, IL1B, LTA, CHUK, TRAF3, ICAM1, BCL10, LTBR, RELA, RELB, TNFRSF13C, CD40, IKBKG, TNFAIP3, IKBKB, PLAU
hsa04060	Cytokine-cytokine receptor interaction	40	7.30E-21	CXCL1, CCL3, CCL2, TNF, IL6ST, CSF1, CXCL2, CXCR2, CD70, CXCR3, CXCL11, CCL5, CCL4, TGFB1, CXCL10, LIF, CCL22, IL23A, CCL20, CXCR4, CSF2RB, IL1B, IL13RA1, XCR1, LTA, IFNGR1, IL1A, IL18R1, LTBR, TGFBR2, TNFRSF13C, TNFRSF14, CD40, IL11RA, IFNAR1, IFNAR2, TNFRSF9, CCR7, CX3CR1, IL12A
hsa04668	TNF signaling pathway	28	6.57E-20	CXCL1, TRAF1, TRAF2, TNF, CCL2, PTGS2, CSF1, CXCL2, NFKBIA, NFKB1, CCL5, CXCL10, LIF, NOD2, CASP3, CCL20, BCL3, IL1B, LTA, CHUK, TRAF3, ICAM1, IL18R1, CEBPB, RELA, IKBKG, IKBKB, TNFAIP3

TABLE 3 | KEGG pathway analysis of genes downregulated by NanA.

KEGG ID	Description	Counts	P value (Benjamini)	Genes
hsa05152	Tuberculosis	22	3.61E-14	IRAK1, CARD9, IL18, TLR4, FADD, MALT1, ITGB2, MAPK11, CTSS, HLA-DMA, MAPK1, IL10RA, BCL2, MAPK14, CASP8, FCER1G, JAK2, FCGR2A, CLEC7A, TRAF6, SYK, HLA-DRA
hsa05140	Leishmaniasis	13	2.54E-10	PTPN6, IRAK1, ITGB2, MAPK11, TLR4, ITGA4, HLA-DMA, MAPK1, MAPK14, JAK2, FCGR2A, TRAF6, HLA-DRA
hsa05145	Toxoplasmosis	15	3.79E-10	IRAK1, SOCS1, MAPK11, TLR4, HLA-DMA, TYK2, MAPK1, ITGA6, IL10RA, MAPK14, BCL2, CASP8, JAK2, TRAF6, HLA-DRA
hsa05164	Influenza A	17	2.56E-09	IFIH1, SOCS3, IL18, PML, TLR4, MAPK11, HLA-DMA, TYK2, MAPK1, TNFRSF10C, TNFSF10, IRF7, MAPK14, JAK2, MX1, CASP1, HLA-DRA
hsa05162	Measles	15	4.82E-09	TYK2, IRAK1, TNFRSF10C, IFIH1, TNFSF10, CCND3, STAT5A, IRF7, STAT5B, TP53, TLR4, JAK2, IL2RG, TRAF6, MX1

differentiated THP-1 macrophages (**Figure 1F**) and human primary macrophages (**Figure 1G**). Together, these results highlight a critical role of pneumococcal NanA in regulating the inflammatory responses in both THP-1 macrophages and human primary macrophages.

NanA Dysregulates the Interaction of Siglec-5 With TLR2 and SHP-1

TLR-2 is the major surface PRR responsible for recognizing a wide range of Gram-positive bacterial cell wall constituents, including peptidoglycans, lipopeptides, and lipoteichoic acids, to initiate host antibacterial responses (Yoshimura et al., 1999; Koedel et al., 2003; Schröder et al., 2003; Basset et al., 2013). TLR agonist stimulation has been shown to trigger lateral mobilization of TLR and Siglec receptors into a specialized cholesterol-enriched lipid raft domain, a crucial platform organizing surface receptors and related intracellular signaling molecules (Triantafilou et al., 2002; Munro, 2003; Triantafilou et al., 2006; Sezgin et al., 2017). It has been recently reported that the lectin activity (i.e. the sialic acid-binding activity) of Siglec is required for its raft translocation in response to TLR ligand stimulation (Ando et al., 2015). To examine whether the surface sialylation levels was reduced in the bacterial contact sites, we infected THP-1 cells with WT SPN and Δ*nanA* mutant and stained the infected cells with *Erythrina cristagalli* lectin (ECA) which preferentially recognizes uncapped glycans without terminal sialic acids. We found that the ECA signals clearly increase on the THP-1 surface where WT SPN contacts. In contrast, Δ*nanA*-infected cells showed weak or no ECA signals (**Figure 2A**). Given that NanA reduces the surface sialylation of infected cells and binding of Siglec to its sialyl ligands is necessary for its raft localization, we then investigated whether NanA has any effect on the translocation of TLR-2 and Siglec-5 into lipid rafts upon pneumococcal infection. Consistent with the previous report that a substantial amount of TLR-2 is raft-resident without agonist stimulation (Snodgrass et al., 2013), TLR-2 was detected in the raft fraction 5, indicated by the raft marker flotillin-1, isolated from unstimulated THP-1 cells (**Figure 2B**). Upon pneumococcal infection, a considerate amount of Siglec-5 and TLR-2 were recruited to the raft fractions 4 and 5, and a significantly reduced raft translocation of Siglec-5 was observed in the WT SPN-infected cells (**Figure 2B**).

To determine whether the interaction between Siglec-5 and TLR-2 was sensitive to NanA-mediated desialylation, we infected Sig-5/THP-1 cells with WT SPN or Δ*nanA* mutant and examined the sialylation level of immunoprecipitated TLR-2 with peanut agglutinin (PNA) which detects the galactosyl-β1,3-*N*-acetylgalactosamine structure that normally appears after removal of the terminal sialic acids. PNA blotting demonstrated that TLR-2 precipitated from WT SPN-infected cells was less sialylated than TLR-2 from Δ*nanA*-infected cells

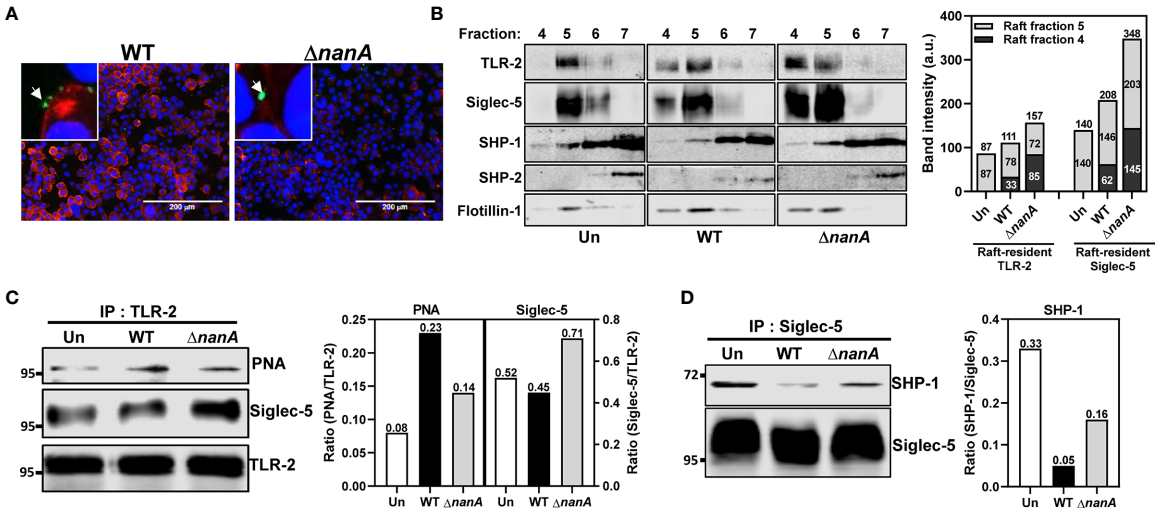

FIGURE 2 | NanA dysregulated the interaction of Siglec-5 with TLR2 and SHP-1. **(A)** Surface desialylation analysis of WT SPN- and Δ*nanA*-infected THP-1 cells. THP-1 cells were infected with CFSE-labeled pneumococcus (green) at an MOI of 30 for 1 h followed by staining with biotin-conjugated ECA and Alexa Fluor 568-conjugated streptavidin (red). DNA was stained with DAPI (blue). **(B)** The distribution of TLR-2 and Siglec-5 in the lipid rafts. Sig-5/THP-1 cells were uninfected (Un) or infected with WT SPN and Δ*nanA* at MOI of 10 for 1 h. Cell lysates collected from infected cells were separated by sucrose-gradient ultracentrifugation and immunoblotting with Ab recognizing TLR2, Siglec-5, SHP-1, SHP-2, and flotillin-1. The summed band intensities of TLR-2 and Siglec-5 in the raft fractions 4 and 5 were quantified by Image J. **(C)** Sig-5/THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 10 for 1 h. The sialylation level and Siglec-5-interaction of TLR-2 were analyzed by immunoprecipitating cell lysates with anti-TLR-2 Ab, followed by probing with PNA and anti-Siglec-5 Ab, respectively. Band intensities of PNA signal and co-precipitated Siglec-5 were quantified by Image J and normalized to TLR-2 values. **(D)** Sig-5/THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 10 for 40 min and the SHP-1 recruitment to Siglec-5 was examined by immunoprecipitating cell lysates with anti-Siglec-5 Ab, followed by probing with anti-SHP-1 Ab. Band intensities of co-precipitated SHP-1 were measured by Image J and normalized Siglec-5 values. The data shown in **Figure 2** were representative of two independent experiments.

(**Figure 2C**). In addition, co-precipitation of Siglec-5 by anti-TLR-2 antibodies was remarkably reduced in WT SPN-infected cells compared to Δ*nanA*-infected cells (**Figure 2C**). Siglecs are known to negatively regulate immune responses via recruiting SHP phosphatases to suppress tyrosine kinase-dependent signals (Crocker et al., 2007; Pillai et al., 2012). Reduced recruitment of SHP-1 to Siglec-5 was observed in WT SPN-infected but not Δ*nanA*-infected THP-1 cells (**Figure 2D**). These results indicate that the sialyl-sugar residues of TLR-2 are important for its interaction with Siglec-5 and this sialic acid-dependent interaction is sensitive to NanA-mediated desialylation.

NanA Enhances the Activation of Multiple Inflammation-Related Signaling Molecules

Pneumococcus was known to activate multiple TLRs and NLRs to induce a range of inflammatory responses (Koedel et al., 2003; Malley et al., 2003; Knapp et al., 2004; Opitz et al., 2004; Mcneela et al., 2010; Davis et al., 2011; Witzenrath et al., 2011). To further investigate the mechanistic role of NanA in PRR signaling pathways, we used micro-western array (MWA) and regular western blot analysis to examine the phosphorylation level of numerous downstream signaling proteins of PRRs. As shown in **Figures 3A, B**, the phosphorylation level of signaling proteins belonging to the NF-κB and MAPK pathways, such as IKK, NF-κB(p65), MKK7/SKK4, p38 MAPK, Erk1/2, and JNK, was markedly more upregulated in WT SPN-infected cells than in Δ*nanA*-infected cells (**Figures 3A, B**).

In addition, several proteins acting more upstream in the PRR signaling cascade, such as AMPKα, Akt, Shc, and Syk, showed higher phosphorylation levels in WT SPN-infected cells (**Figures 3A, B**). AMPKα has been shown to regulate multiple inflammatory pathways, including NK-κB, JNK, and NLRP3 inflammasome (Moon et al., 2015; Gaber et al., 2017; Silwal et al., 2018). Akt plays a critical role in the induction of the transcriptional activity of NF-κB (Kane et al., 1999; Ozes et al., 1999). Shc is a key adaptor protein known to activate the MAPK pathway in response to various stimulation (Ravichandran, 2001). Syk has been shown to phosphorylate MyD88 and ASC to regulate IL-1β-driven inflammation and NLRP3 inflammasome-mediated caspase-1 activation, respectively (Lin et al., 2015; Gurung et al., 2017; Feng et al., 2018). These observations suggest that NanA may target the upstream regulators of the PRR signaling pathway to exaggerate inflammation upon pneumococcal infection.

On the other hand, phosphorylation of Src at tyrosine 527 (Y527) negatively regulates its kinase activity by locking Src in a closed conformation (Roskoski, 2005; Byeon et al., 2012). Phosphorylation of SHP-2 at tyrosine 542 (Y542) is required for its phosphatase activity, which negatively regulates TLR-induced immune responses (An et al., 2006). Elevated phosphorylation of Src and SHP-2 at Y527 and Y542, respectively, were more pronounced in Δ*nanA*-infected cells (**Figure 3A**), which suggests that negative regulators in the PRR signaling pathway may be repressed in the presence of NanA upon pneumococcal infection. Notably, a similar phosphorylation profile of the

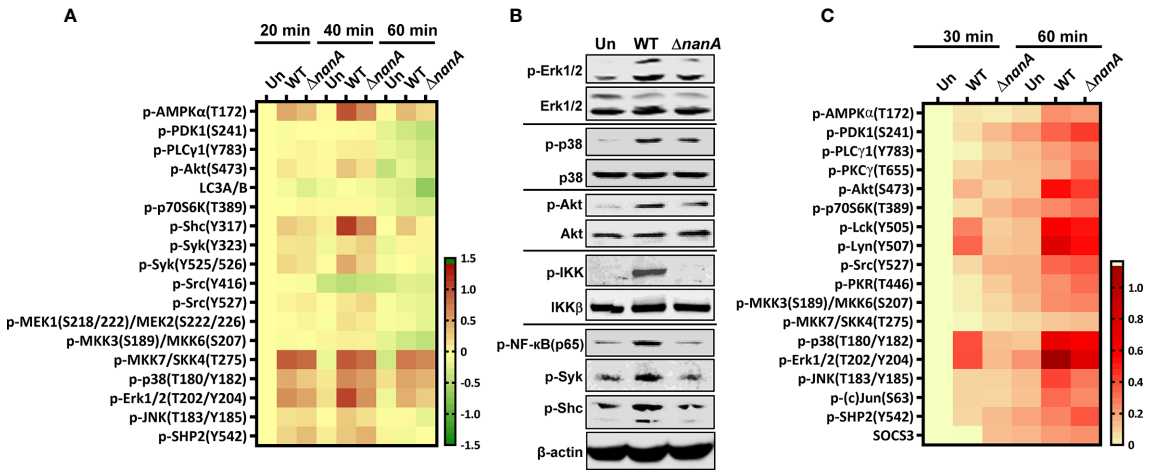

FIGURE 3 | Inflammation-related signaling molecules modulated by NanA upon pneumococcal infection. **(A)** THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 5 for 20, 40, and 60 min. The cell lysates were collected and subjected to Micro-Western analysis (MWA). Image density was quantified by Image Studio software and normalized to β-actin. Fold change was calculated as the ratio of each normalized net intensity to the net intensity of the uninfected control, followed by log2 transformation. The heatmap was created by Prism software. **(B)** Western blot verification of the phosphorylation of signaling molecules revealed by MWA. Cytoplasmic proteins were extracted from WT SPN- and Δ*nanA*-infected THP-1 cells and subjected to western blotting for detection of the phosphorylated Erk1/2, p38, Akt, IKK, NF-κB p65, Syk, and Shc. **(C)** Human primary macrophages were infected with WT SPN and Δ*nanA* at an MOI of 5 for 30 and 60 min, and the activation of inflammation-related signaling molecules was examined in by MWA as described in **(A)**.

TLR-related kinases and phosphatases was observed in WT SPN- and Δ*nanA*-infected primary human macrophages, which more closely resembles the phenotype of healthy cells *in vivo* (**Figure 3C**). Taken together, our data suggest that kinases and phosphatases may be differentially regulated by NanA to broadly exaggerate inflammatory signals emanating from PRRs.

NanA-Mediated Caspase-1 and NLRP3 Inflammasome Activation Causes the Excessive IL-1β Production

In addition to the proinflammatory cytokines which secrete immediately following their transcription and translation, we noted that NanA strongly promotes the production of IL-1β which requires additional proteolytic maturation steps (**Figure 1**). The canonical cleavage and process of pro-IL-1β to mature IL-1β is catalyzed by caspase-1 (Franchi et al., 2009); thus, we tested whether NanA promotes caspase-1 activation upon pneumococcal infection. THP-1 cells were infected with WT SPN and Δ*nanA*, and the culture supernatant was collected from infected cells, TCA-precipitated, and probed with anti-caspase-1 antibodies. In accordance with the excessive IL-1β production in WT SPN-infected THP-1 cells, elevated caspase-1 activation, as indicated by the increased amount of cleaved p20 subunits of caspase-1, was observed in WT SPN-infected THP-1 cells compared to Δ*nanA*-infected cells (**Figure 4A**). Given that assembly of the multimeric protein complex known as inflammasome, which comprises members of the NLR family, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and pro-caspase-1, is a prerequisite step for caspase-1 cleavage (Franchi et al., 2009), we then examined the role of NanA in ASC oligomerization, a hallmark of inflammasome activation. As shown in **Figure 4B**, greater levels of ASC oligomerization were induced in WT SPN-infected THP-1 cells than in Δ*nanA*-infected cells. These findings indicate that NanA positively regulates the formation of inflammasome complexes and subsequent caspase-1 activation.

To verify the role of NanA on caspase-1 and IL-1β maturation, we analyzed the levels of cleaved IL-1β p17 fragments in culture supernatants collected from infected THP-1 cells by western blot. As shown in **Figure 4C**, higher levels of cleaved caspase-1 p20 and IL-1β p17 were detected in THP-1 cells infected with SPN WT, although complementation of the Δ*nanA* mutant with NanA expressed on a plasmid only moderately increased the production of active caspase-1 and IL-1β. This partial complementation phenotype may be attributed to different NanA levels expressed from the plasmid in *trans* or driven by its native promoter. To understand whether the production of IL-1β is dependent on the activation of canonical NLRP3-ASC-caspase-1 inflammasome, we tested the effect of specific inhibitors targeting caspase-1 (Ac-YVAD-cmk) and NLRP-3 inflammasome (MCC950). Enhanced IL-1β release seen in WT SPN-infected cells was drastically reduced in the presence of caspase-1 and NLRP3 inflammasome inhibitors (**Figure 4D**), which supports the critical role of NLRP3 inflammasomes in NanA-augmented IL-1β secretion. However, small amounts of IL-1β were still detectable in the WT SPN-infected THP-1 cells in the presence of NLRP3 inflammasome or caspase-1 inhibitors, indicating that an alternative mechanism for IL-1β maturation may exist. Given that caspase-8 has been identified as an alternative protease to mediate atypical pro-IL-1β processing (Maelfait et al., 2008; Gringhuis et al., 2012), we thought to verify the role of caspase-8 in NanA-augmented IL-1β

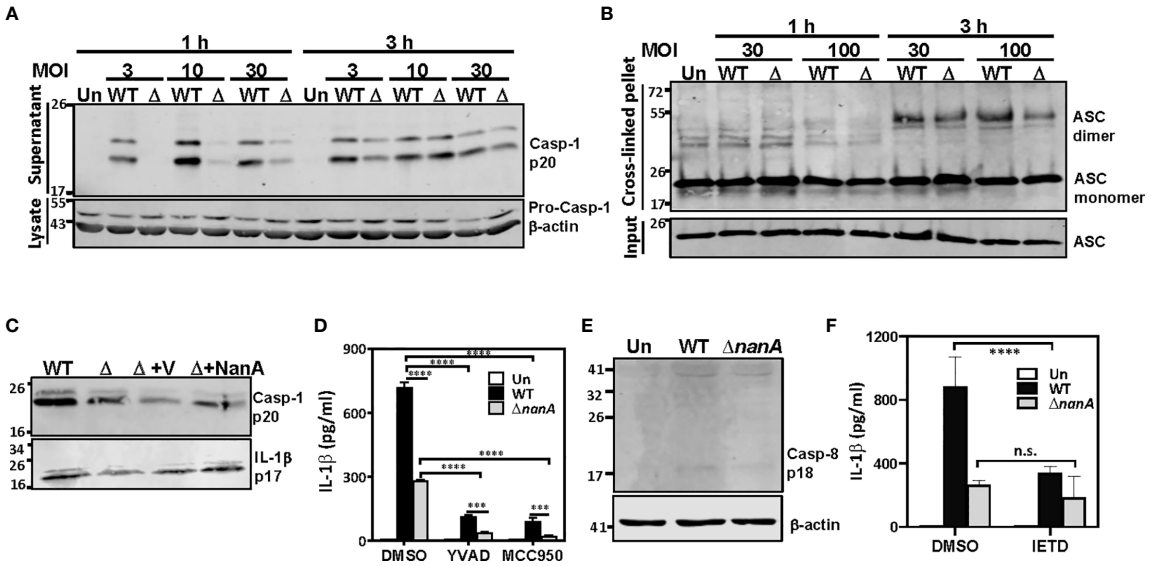

FIGURE 4 | NanA-mediated caspase-1 and NLRP3 inflammasome activation causes excessive IL-1β production. **(A)** THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 3, 10 and 30 for 1 and 3 h, and the supernatant and cell lysates were analyzed by western blotting with anti-caspase-1 Ab. **(B)** NanA promotes ASC oligomerization. THP-1 cells were infected with WT SPN or Δ*nanA* mutants at an MOI of 30 and 100 for 1 h and 3 h. The cell lysates and DSS cross-linked pellets prepared from infected cells were subjected to western blotting to analyze ASC oligomerization. **(C)** THP-1 cells were infected with WT SPN, Δ*nanA*, and NanA-complemented strains at an MOI of 10, and the supernatant was collected 1 h and 8 h post-infection and analyzed by western blotting with anti-caspase-1 and anti-IL-1β Ab, respectively. **(D)** THP-1 cells were treated with caspase-1 (Ac-YVAD-cmk) and NLRP3 (MCC950) inhibitors at 20 μM and 10 μM, respectively, for 1 h before infection with WT SPN or Δ*nanA* mutants at an MOI of 10 for 3 h. Culture supernatants were collected and quantified for IL-1β by ELISA. The data presented as mean±SD is representative of three independent experiments performed with biological triplicates. **(E)** THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 30 for 8 h, and the cell lysate was analyzed by immunoblotting with anti-caspase-8 Ab. **(F)** THP-1 cells were treated with caspase-8 inhibitor (Z-IETD-FMK) at 20 μM for 1 h before infection with WT SPN or Δ*nanA* mutants at an MOI of 10 for 3 h. Culture supernatants were collected and quantified for IL-1β by ELISA. The data presented as mean±SD is representative of three independent experiments performed with biological triplicates. Statistical analysis was performed using two-way ANOVA **(D, E)**. *****P* < 0.0001; ****P* < 0.001; n.s., not significant.

production. A small amount of cleaved caspase-8 p18 fragments was detected in WT SPN-infected THP-1 cells (**Figure 4E**), and caspase-8 inhibitors (Z-IETD-FMK) moderately reduced the excessive IL-1β secretion seen in WT SPN-infected THP-1 cells (**Figure 4F**). These data indicate that the canonical NLRP3-caspase-1 inflammasome is responsible for most of the NanA-mediated excessive IL-1β production upon pneumococcal infection. The noncanonical caspase-8 may also contribute to the NanA-mediated excessive IL-1β production, although further experiments will be required to delineate the role of caspase-8 in IL-1β production upon pneumococcal infection.

Multiple Death Pathways Are Involved in NanA-Exacerbated Cell Death Upon Pneumococcal Infection

Bacterial infection often elicits substantial inflammation and cell death in the host. To examine whether NanA also promotes the cell death of pneumococcus-infected cells, we first used a WST-1-based cell cytotoxicity assay to measure the overall viability of THP-1 cells infected with WT SPN or Δ*nanA* mutants. As shown in **Figure 5A**, pneumococcus induced pronounced cell death in THP-1 cells within 3 h in a dose-dependent manner, and more reduced viability was observed in WT SPN-infected cells than in Δ*nanA*-infected cells at all tested MOI. Both pyroptosis and apoptosis have been reported to contribute to pneumococcus-infected cell death (Aliprantis et al., 1999; Srivastava et al., 2005; Bewley et al., 2014; Kim et al., 2015). Pyroptosis is a form of programmed cell death driven by the activation of inflammatory caspases. Characteristic features of pyroptosis include rapid plasma-membrane rupture, release of cytosolic contents, and DNA fragmentation (Bergsbaken et al., 2009; Miao et al., 2011). Caspase-1 has been shown to trigger pyroptosis by proteolytic cleavage of gasdermin D (GSDMD) to generate a N-terminal fragment (GSDMD-N) that forms membrane pores and ultimately causes cell lysis (Shi et al., 2015). Since we have shown that NanA increases caspase-1 activation, the generation of GSDMD-N was further examined in pneumococcus-infected cells. In accordance with the increased caspase-1 activity in WT SPN-infected THP-1 cells shown in **Figure 4A**, higher levels of GSDMD-N were detected in the WT SPN-infected cells than in Δ*nanA*-infected cells at all tested MOIs (**Figure 5B**). The membrane permeability which was determined by the penetration of membrane impermeable dye 7-AAD into cells was also drastically increased in the WT SPN-infected THP-1 cells (**Figure 5C**). In addition, THP-1 cells challenged with WT SPN resulted in higher numbers of cells in the sub-G1 phase

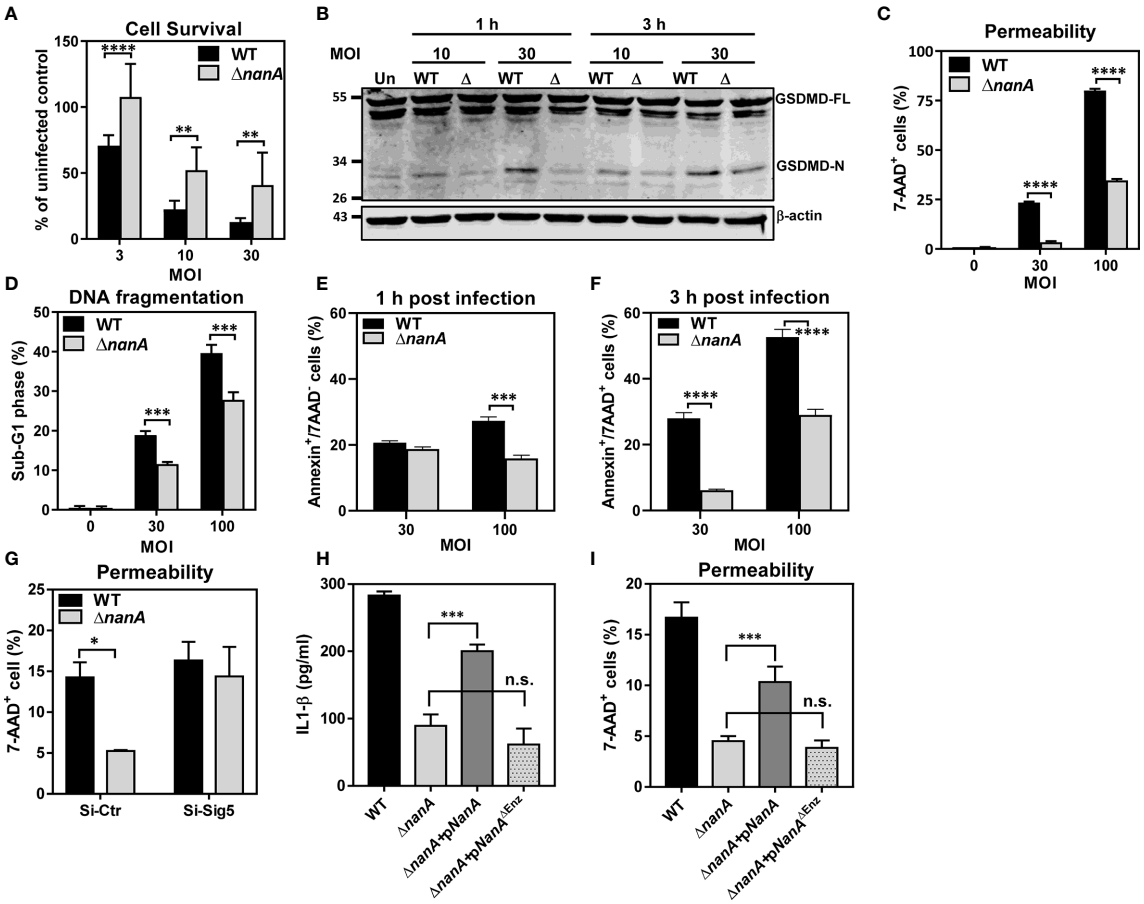

FIGURE 5 | Multiple death pathways contributed to NanA-exacerbated cell death upon infection **(A)** THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 3, 10, and 30 for 3 h. The infected cells were further cultured with premix WST-1 reagent for 3 h. The absorbance was determined at 450 nm and the background values were subtracted. Results were presented as percentages of the uninfected control. Data represented mean±SD pooled from three independent experiments, each performed in triplicate. **(B)** THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 10 and 30 for 1 and 3 h, and cell lysates were harvested for western blotting with antibodies for GSDMD and β-actin. **(C)** Flow cytometric analysis of 7-AAD uptake. THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 30 and 100 for 3 h, and the infected cells were stained with 7-AAD to analyze the membrane integrity of the infected cells. **(D)** THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 30 and 100 for 4.5 h. The DNA content of infected cells was revealed by flow cytometric analysis of propidium iodide-stained nuclei. Flow cytometric analysis of cell apoptosis. THP-1 cells were infected with WT SPN and Δ*nanA* at an MOI of 30 for 1h **(E)** and 3 h **(F)**, and the infected cells were stained with Annexin V apoptosis detection kit with 7-AAD. **(G)** Siglec-5 knockdown (Si-Sig5) and control knockdown (Si-Ctr) THP-1 cells were infected with WT SPN and Δ*nanA* at MOI of 30 for 3 h, and the infected cells were stained with 7-AAD to analyze the membrane integrity of the infected cells. IL-1β concentration in supernatants **(H)** and cell pyroptosis **(I)** were analyzed 3 h post-infection in THP-1 cells challenged with WT SPN, Δ*nanA*, or the Δ*nanA* complemented with NanA or enzymatically inactive NanA ($NanA^{\Delta Enz}$) expression plasmids. The data shown were representative of two independent experiments **(B–I)**. Statistical analysis was performed using Student's *t* test **(A, C–I)**. $^{****}P < 0.0001$; $^{***}P < 0.001$; $^{**}P < 0.01$; n.s., not significant.

which is indicative of DNA fragmentation, a key feature of apoptosis and pyroptosis (**Figure 5D**). To further examine whether NanA enhances the apoptosis of pneumococcus-infected THP-1 cells, we stained the infected cells with Annexin V/7-AAD apoptosis detection reagents. As shown in **Figures 5E, F**, there were more early (Annexin V^+/7-AAD^-) and late apoptotic cells (Annexin V^+/7-AAD^+) detected in WT SPN-infected cells than in Δ*nanA*-infected cells. Our data suggest that NanA-exacerbated cell death was mainly caused by the inflammatory caspase-mediated pyroptosis, while a minority of cell death was possibly mediated by apoptosis.

To further determine the relevance of Siglec-5 in NanA-potentiated pyroptosis, the membrane integrity was determined in the control (Si-Ctr) and Siglec-5 knockdown (Si-Sig5) THP-1 cells following SPN WT and Δ*nanA* mutants challenge. As shown in **Figure 5G**, pronounced cell pyroptosis was observed in WT-infected Si-Ctr THP-1 cells compared to Δ*nanA*-infected cells, while both WT and Δ*nanA* mutant induced pronounced pyroptosis in Si-Sig5 THP-1 cells. In addition, complementation of the Δ*nanA* mutant with the NanA enzyme expressed on a plasmid vector partially restored the released IL-1β (**Figure 5H**) and cell pyroptosis (**Figure 5I**). In contrast, complementation of

the Δ*nanA* mutant with an enzymatically inactive version of NanA ($NanA^{\Delta Enz}$) had no effect. Our findings suggested that NanA-mediated surface desialylation possibly abrogates the Siglec-5-TLR-2 interaction to release the Siglec-5-mediated inhibitory signals, which was attributed to the elevated IL-1β release and cell pyroptosis in pneumococcus-infected cells.

DISCUSSION

Upon infection, immune cells sense the environment through their PRRs and integrate this external information through intracellular signaling molecules to launch robust inflammatory and antimicrobial responses to defend against microbial infection (Janeway and Medzhitov, 2002). In general, a fine-balanced immune response which is sufficient to eliminate pathogens but not too overactive so as to cause widespread host tissue damage is achieved through an intricate interaction between activating and inhibitory receptors (Lee and Kim, 2007; Zak and Aderem, 2009). In this study, we demonstrated that NanA, a virulence factor expressed by all pneumococcal isolates, caused extensive surface desialylation of the infected cells, which in turn impairs the sialic acid-dependent interaction between Siglec-5-TLR-2 and subsequent SHP-1 phosphatase recruitment. Thus, this NanA-dysregulated crosstalk between TLR-2 and inhibitory Siglec-5 exaggerated multiple inflammatory and death signaling pathways and caused excessive inflammation and cytotoxicity in pneumococcus-infected macrophages.

Recognition of bacterial components by the innate immune system is essential for the host to defend against invading pathogens. Many surface and cytosolic PRRs have been shown to recognize numerous pneumococcal components, such as peptidoglycans, teichoic acids, genomic DNAs, and pneumolysins, to initiate a protective innate immune response. Although this mechanism ensures the activation of immune cells upon pneumococcal infection, it may risk affecting the host with overwhelming inflammation when the cell activation goes uncontrolled. Siglecs are membrane-bound lectins that recognize the sialic acid-containing structures. Most of the Siglecs contain cytosolic ITIM- or ITIM-like motifs and are considered to play a negative role in cell activation via associating with tyrosine phosphatases to dephosphorylate key kinases or signaling proteins responsible for cell activation (Crocker et al., 2007; Pillai et al., 2012). A broad and direct interaction between TLR and Siglec was identified where Siglec negatively regulates TLR activation in response to TLR agonist stimulation (Chen et al., 2014). In addition, binding of CD14, a co-receptor for TLR-4, to Siglec-3 also downregulated the LPS-mediated TLR-4 activation (Ishida et al., 2014). These observations indicate that the Siglec-interacting property of TLRs may add an extra assurance to prevent over-activation of TLRs in response to ligand stimulation.

Dimerization and translocation of TLR-2 to lipid rafts, a specialized membrane microdomain organizing surface receptors and intracellular signaling molecules, was required for its activation and signal transduction (Triantafilou et al., 2006; Ruysschaert and Lonez, 2015). Although a comparable amount of TLR-2 was recruited to the lipid rafts upon WT SPN and Δ*nanA* stimulation, reduced translocation of Siglec-5 to the lipid rafts was clearly observed in the WT SPN-infected cells. This discoordinated surface distribution of TLR-2 and Siglec-5 was further evidenced by the reduced co-immunoprecipitation of Siglec-5 by anti-TLR-2 antibodies (**Figure 2**). This decoupled Siglec-5/TLR-2 interaction contributes, at least in part, to the exaggerated activation of multiple inflammation-related signaling molecules and subsequent excessive inflammation in pneumococcus-infected THP-1 cells (**Figures 1–3**).

The primary role of the inflammatory cytokines and chemotactic chemokines released upon microbial infection is to drive the maturation, homing, and activation of immune cells, which often attributes to enhanced microbicidal activities (Commins et al., 2010). Paradoxically, all identified pneumococcal isolates express NanA, which has been shown to exaggerate inflammatory responses upon infection (Chen et al., 2011; Chang et al., 2012). There are several possible explanations for this discrepancy. First, NanA may have an indispensable function in pneumococcal physiology and pathogenesis, which is evident by its critical role in nutrient acquisition, biofilm formation, and host colonization (Tong et al., 2002; Manco et al., 2006; Parker et al., 2009; Uchiyama et al., 2009). Second, inflammation normally occurs to alarm and boost the host immune responses to eradicate invading pathogens. However, the same response may cause undesired collateral tissue damage, which in turn facilitates bacterial dissemination (Chen et al., 2011; Chang et al., 2012). Here, we identified a new virulent role of NanA in exaggerating the cell death of immune cells as a means to subvert host antimicrobial responses (**Figure 5**). Several mechanisms have been suggested for pneumococcus-induced cell death, mostly through the activation of inflammasomes and caspase cascades (Aliprantis et al., 1999; Srivastava et al., 2005; Bewley et al., 2014; Gonzalez-Juarbe et al., 2015; Kim et al., 2015). In this study, we found that NanA-mediated desialylation enhances ASC oligomerization, caspase-1 activation, and GSDMD proteolytic cleavage in infected THP-1 cells (**Figures 4A, B**, and **5B**). In line with these observations, enhanced pyroptosis was observed in the infected THP-1 cells in the presence of NanA (**Figure 5**). Siglec-mediated modulation of inflammasome activation has also been reported in NK cells where human neonatal pathogen group B *Streptococcus* (GBS) suppresses NLRP3 inflammasome activation and prevents subsequent pyroptotic cell death through engaging ITIM-containing Siglec-7 in NK cells (Fong et al., 2018). In contrast, GBS triggered NLRP3 inflammasome activation in THP-1 cells overexpressing ITAM-coupling Siglec-14 whereas the same bacteria inhibited NLRP3 inflammasome activation when THP-1 cells expressed ITIM-containing Siglec-5 (Tsai et al., 2020). These observations suggest that bacterial species may manipulate inflammasome activation through engaging Siglecs (Fong et al., 2018; Tsai et al., 2020) or releasing Siglec-restricted inhibition (our studies), although further studies are required for a better understanding of how Siglec acts on the NLRP3-caspase-IL-1β axis.

In conclusion, our results reveal a novel virulence role of NanA in pneumococcal pathogenesis and suggest that targeting NanA activity may ameliorate the pneumococcus-mediated inflammation and cytotoxicity in severe invasive pneumococcal diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: GEO repository with accession number GSE161269 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161269).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Taiwan University IRB 201911067RINC. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-WT, C-CC, and Y-CC conceived and designed the experiments. Y-WT and C-CC performed the experiments and analyzed the data. Y-WT and Y-CC wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by grants from Taiwan MOST 106-2320-B-002-013-MY3 and 109-2320-B-002-058 and National Taiwan University NTU-CC-110L890502 to Y-CC.

ACKNOWLEDGMENTS

We thank the service provided by the Flow Cytometric Analyzing and Sorting Core of the First Core Laboratory, National Taiwan University, College of Medicine and the support from Micro-Western Array core facility of NHRI (Taiwan). We thank Dr. Li-Chun Hsu (National Taiwan University) for helpful discussion and material sharing and Dr. Takashi Angata (Academia Sinica) for sharing the Siglec-5-expressing THP-1 cells.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.613195/full#supplementary-material

Supplementary Table 1 | Differentially expressed genes between WT SPN- and Δ*nan*A mutant-infected Sig-5/THP-1 cells.

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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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frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 16 March 2021
doi: 10.3389/fcimb.2021.615959

The Role of Neutrophils and Neutrophil Elastase in Pneumococcal Pneumonia

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Streptococcus pneumoniae, also known as pneumococcus, is a Gram-positive diplococcus and a major human pathogen. This bacterium is a leading cause of bacterial pneumonia, otitis media, meningitis, and septicemia, and is a major cause of morbidity and mortality worldwide. To date, studies on *S. pneumoniae* have mainly focused on the role of its virulence factors including toxins, cell surface proteins, and capsules. However, accumulating evidence indicates that in addition to these studies, knowledge of host factors and host-pathogen interactions is essential for understanding the pathogenesis of pneumococcal diseases. Recent studies have demonstrated that neutrophil accumulation, which is generally considered to play a critical role in host defense during bacterial infections, can significantly contribute to lung injury and immune subversion, leading to pneumococcal invasion of the bloodstream. Here, we review bacterial and host factors, focusing on the role of neutrophils and their elastase, which contribute to the progression of pneumococcal pneumonia.

Keywords: innate immunity, neutrophil, pneumonia, pneumolysin, neutrophil elastase, *Streptococcus pneumoniae*, virulence factor

OPEN ACCESS

Edited by:
Victor Nizet,
University of California, San Diego, United States

Reviewed by:
Elaine I. Tuomanen,
St. Jude Children's Research Hospital, United States
Paul Race,
University of Bristol, United Kingdom

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *10 October 2020*
Accepted: *01 March 2021*
Published: *16 March 2021*

Citation:
Domon H and Terao Y (2021) The Role of Neutrophils and Neutrophil Elastase in Pneumococcal Pneumonia. Front. Cell. Infect. Microbiol. 11:615959. doi: 10.3389/fcimb.2021.615959

INTRODUCTION

Pneumonia is a common and serious infectious disease and has been a significant cause of morbidity and mortality worldwide, accounting for approximately three million deaths annually. The World Health Organization (WHO) placed lower respiratory infections as the fourth most common cause of death in 2016. Among a number of infectious agents, *Streptococcus pneumoniae*, also known as pneumococcus, is the most common cause of pneumonia in all age groups. In addition to localized infections such as pneumonia and otitis media, pneumococcus may cause invasive diseases, including meningitis and septicemia. Furthermore, an increase in antimicrobial resistance among pneumococci has raised concerns about the effectiveness of empiric antimicrobial therapy for pneumococcal pneumonia (Feldman, 2004; Ferrara, 2005; Nagai et al., 2019).

S. pneumoniae is a Gram-positive diplococcus that colonizes the mucosal surfaces of the human nasopharynx. Nasopharyngeal aerosolization of *S. pneumoniae* is considered to be the primary mode of population transmission. The molecular interaction of pneumococcal virulence factors and host proteins with respect to nasopharyngeal colonization has been thoroughly reviewed elsewhere (Kadioglu et al., 2008; Weiser et al., 2018). It has been reported that 18–92% of children are carriers of *S. pneumoniae* (Le

Polain de Waroux et al., 2014); thus, they are considered the main reservoirs and transmission vectors of pneumonia (Smith et al., 2019). The aspiration of nasopharyngeal secretions leads to the invasion and propagation of *S. pneumoniae* in the lung parenchyma at the alveolar level, which leads to pulmonary infection (Liu et al., 2015). It has been reported that bacterial virulence factors directly damage human tissues or cause malfunctioning of the human immune system, resulting in an excessive inflammatory response. This excessive or inappropriate host inflammatory response is considered to result in the clinical syndrome of pneumonia. In this review, we discuss the bacterial and host factors that contribute to the progression of pneumococcal pneumonia, specifically focusing on the role of neutrophils and their elastase.

RECOGNITION OF *S. PNEUMONIAE* BY THE INNATE IMMUNE SYSTEM OF THE HOST

Upon pneumococcal colonization or infection, the respiratory epithelium controls the bacterium through antimicrobial peptides, such as LL-37 and defensins (Bals and Hiemstra, 2004). However, *S. pneumoniae* can survive by removing its capsule from the surface (also see section 4) (Kietzman et al., 2016), which allows the organism to adhere to and invade the epithelium (Hammerschmidt et al., 2005). Pneumococcal interactions with other innate immune molecules, such as complements and surfactant protein-D, have been reviewed elsewhere (Kadioglu and Andrew, 2004). Following invasion of epithelial cells, the host innate immune system, which includes respiratory epithelial cells, alveolar macrophages, and dendritic cells, recognizes invading *S. pneumoniae* using pattern recognition receptors (PRRs) (Hartl et al., 2018). Different classes of PRRs include toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I-like receptors, and C-type lectin receptors (Takeuchi and Akira, 2010). These receptors are activated by conserved microbial molecules and bacterial virulence factors. Among the various TLRs, TLR2 recognizes several components of the pneumococcal cell wall, such as lipoteichoic acid, lipoproteins, and peptidoglycan (Yoshimura et al., 1999; Tomlinson et al., 2014), whereas TLR9 recognizes pneumococcal genomic DNA (Mogensen et al., 2006). Although TLR4 is known for its ability to detect lipopolysaccharide (LPS) from Gram-negative bacteria, it has been suggested that TLR4 might additionally recognize pneumolysin (Ply) (Malley et al., 2003), a pneumococcal pore-forming toxin. Additionally, we have demonstrated that pneumococcal cytosolic components, such as the chaperone protein DnaK, elongation factor Tu, and glyceraldehyde-3-phosphate dehydrogenase induce the production of proinflammatory cytokines *via* TLR4 (Nagai et al., 2018). NOD2 recognizes lysosome-digested peptidoglycan fragments of phagocytized *S. pneumoniae* (Davis et al., 2011). Additionally, Ply activates the NLRP3 inflammasome and promotes proinflammatory cytokine secretion by dendritic cells (McNeela et al., 2010). Intracellular signaling cascades triggered by PRRs lead to the transcriptional activation of inflammatory mediators, such as proinflammatory cytokines and chemokines. These mediators stimulate neighboring immune and non-immune cells, activate the acute-phase response, and recruit neutrophils (Koppe et al., 2012) (**Figure 1**).

NEUTROPHIL-MEDIATED KILLING OF *S. PNEUMONIAE*

When infectious agents invade the respiratory tract, immune cells and epithelial cells secrete chemokines and cytokines, as described above, promoting neutrophil migration into the lung through the pulmonary capillary walls (Maas et al., 2018). Neutrophils phagocytose and kill infectious agents with the help of reactive oxygen species, antimicrobial proteins, and serine proteases (Teng et al., 2017). An *in vitro* study demonstrated that neutrophils degrade phagocytized *S. pneumoniae via* serine proteases such as neutrophil elastase (NE) and cathepsin G (CG), which are stored in azurophilic granules (Standish and Weiser, 2009). NE- and CG-

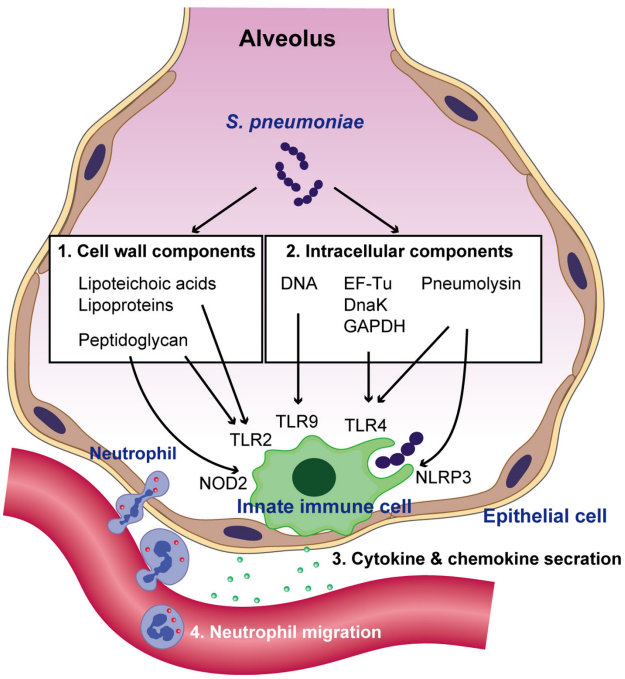

FIGURE 1 | Recognition of *S. pneumoniae* by the innate immune system and neutrophil migration. Pneumococcal cell wall components such as lipoteichoic acids, lipoproteins, and peptidoglycan are recognized by TLR2. Following bacterial uptake by phagocytes and their degradation in phagosomes, pneumococcal peptidoglycan is recognized by NOD2. Pattern recognition receptors (PRRs) also sense pneumococcal intracellular molecules such as pneumolysin, genomic DNA, the chaperone protein DnaK, elongation factor Tu (EF-Tu), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). DNA is recognized by TLR9 within endosomes, whereas pneumolysin, DnaK, EF-Tu, and GAPDH are recognized by TLR4. Additionally, pneumolysin activates the NLRP3 inflammasome. The activation of PRR signaling leads to the transcriptional activation of cytokines and chemokines, which subsequently augments neutrophil migration.

deficient mice exhibit impaired antibacterial defense against *S. pneumoniae* and decrease murine survival without affecting neutrophil recruitment (Hahn et al., 2011). Furthermore, neutrophil depletion results in profound defects in the clearance of *S. pneumoniae* in a murine model of pneumonia (Garvy and Harmsen, 1996). This converging evidence indicates that phagocytic function and phagolysosomal degradation of bacteria by neutrophils are crucial strategies for controlling pneumococcal infection.

In addition to their phagocytic function, previous studies have reported that neutrophils release chromatin DNA decorated with granule-derived antimicrobial peptides and enzymes, including NE, CG, α-defensins, and myeloperoxidase (Brinkmann et al., 2004; Papayannopoulos, 2018). These chromatin structures are termed neutrophil extracellular traps (NETs), which degrade virulence factors and kill multiple microbial genera (Brinkmann et al., 2004). The trapping of microbes by NETs may provide several benefits, including reducing the spread of infection by concentrating host antimicrobial agents at infection sites. In bacterial pneumonia, animal and human studies have indicated that NETs are increased in alveolar spaces (Lefrançais et al., 2018; Mikacenic et al., 2018). Furthermore, an *in vitro* study demonstrated that NETs exhibit significant antibacterial activity against *S. pneumoniae* (Mori et al., 2012). These findings suggest the functional importance of NETs in pneumococcal pneumonia. However, higher concentrations of NETs have been reported to be associated with reduced hazards of clinical stability and increased mortality in pneumonia (Ebrahimi et al., 2018). In this context, excess NETs released by activated neutrophils have been implicated in promoting tissue damage, including sepsis (Czaikoski et al., 2016), and lung injury (Narasaraju et al., 2011). The mechanisms responsible for NET-induced tissue damage involve NET components such as NE and other proteases that induce cell death in multiple cell types (Yang et al., 1996; Hou et al., 2014; Grechowa et al., 2017; Daniel et al., 2019; Hiyoshi et al., 2019).

PNEUMOCOCCAL VIRULENCE FACTORS CONTRIBUTE TO EVASION FROM PHAGOCYTOSIS AND INDUCE NEUTROPHIL DEATH

A variety of pneumococcal virulence factors have been identified (Brooks and Mias, 2018; Feldman and Anderson, 2020). In the present review, we discuss the virulence factors associated with immune evasion.

The autolytic enzyme, autolysin, is known to be responsible for the characteristic autolytic behavior associated with pneumococci. The major autolysin of *S. pneumoniae* is *N*-acetylmuramyl-$_L$-alanine amidase (LytA), which breaks down peptidoglycan (Höltje and Tomasz, 1976). Although the exact *in vivo* function of autolysis in pneumococcal pathogenesis is unclear, animal studies have demonstrated that pneumococcal strains deficient in LytA are less virulent than wild-type pneumococci (Berry et al., 1989a; Hirst et al., 2008). Recently, Kietzman et al. identified a novel physiological function of LytA. This enzyme was shown to drive rapid capsule shedding in response to antimicrobial peptides in the initial phases of infection (Kietzman et al., 2016). This response increases bacterial resistance to peptides, as well as invasion of the alveolar epithelium. LytA may also contribute to pneumococcal pathogenesis by catalyzing the release of the intracellular toxin Ply (Martner et al., 2008; Domon et al., 2016; Domon et al., 2018a), cell wall degradation products (Tuomanen et al., 1985), and cytosolic proteins (Nagai et al., 2018), which induce immune responses. Additionally, fragments from autolyzed bacteria inhibit phagocytosis of intact bacteria by peripheral blood mononuclear cells (Martner et al., 2009).

Ply is a potent intracellular toxin possessing multiple functions that augment pneumococcal virulence. Ply-deficient mutant strains of *S. pneumoniae* showed a significant reduction in virulence related to both intranasal and systemic infection (Berry et al., 1989b). Ply toxicity is mainly associated with its ability to induce ring-shaped pores in cholesterol-containing membranes (Tilley et al., 2005). In this regard, Ply has cytotoxic effects on various cell types, including alveolar epithelial cells (Rubins et al., 1993), microvascular endothelial cells (Zysk et al., 2001), and monocytes (Hirst et al., 2002). Thus, the direct cytotoxicity of Ply towards lung tissue is considered to play a primary role in lung injury in pneumococcal pneumonia. However, several studies have demonstrated that *in vivo* lung injury could be due to inflammation and microvascular leakage caused by Ply, rather than its cytotoxic activity (Witzenrath et al., 2006; García-Suárez et al., 2007; Witzenrath et al., 2007). Although neutrophils are required for the clearance of *S. pneumoniae*, intranasal or intratracheal infection of mice with wild-type *S. pneumoniae* demonstrated an increased neutrophil recruitment, increased bacterial burden in the lungs, and a higher prevalence of bacteremia compared to infection with Ply-negative mutant strains (Rubins et al., 1995; Kadioglu et al., 2000). Therefore, the proinflammatory interactions between Ply and neutrophils are considered to play a role in the aggravation of pneumococcal pneumonia. Indeed, Ply is cytotoxic to neutrophils (Cockeran et al., 2001). We demonstrated that Ply induces neutrophil cell death through specific interactions with the $P2X_7$ receptor; whereas Ply is less cytotoxic against $P2X_7$ receptor-negative alveolar epithelial cells and macrophages. This suggests that neutrophils are the primary target cells of Ply (Domon et al., 2016). The subsequent leakage of NE from dead neutrophils disrupts the pulmonary epithelial barrier. Another study demonstrated that Ply induces NET formation, which contains high levels of NE (Nel et al., 2016). Several others have reported pneumococcal evasion of NETs. Pneumococcal surface protein A plays a role in the resistance to NET-mediated killing (Martinez et al., 2019). Meanwhile pneumococcal endonucleases, EndA and TatD, allow the bacterium to degrade the DNA scaffolds of NETs and escape, followed by the release of NE from the NETs (Beiter et al., 2006; Jhelum et al., 2018).

A comprehensive review of the capsule, including its regulation in pathogenesis, capsule synthesis, and the genetic basis for serotype differences, has been published elsewhere (Paton and Trappetti, 2019). Accordingly, current mini reviews

mainly focus on immune evasion related to virulence factors. The capsule, which confers protection against phagocytosis, has been extensively studied in the context of pneumococcal virulence (Jonsson et al., 1985; Andre et al., 2017). The capsule impairs bacterial opsonization with C3b/iC3b by the classical and alternative complement pathways and also inhibits the conversion of C3b, which bound to the bacterial surface, to iC3b, thus resulting in a profound inhibition of opsonophagocytosis by neutrophils (Hyams et al., 2010). Additionally, the capsule plays a role in bacterial adherence, colonization of the nasopharynx, and entry into alveolar epithelial cells (Hammerschmidt et al., 2005). Although capsules protect *S. pneumoniae* against trapping by NETs (Wartha et al., 2007), it has recently been observed that capsules of virulent pneumococcal serotypes enhance the formation of NETs during pneumonia (Moorthy et al., 2016). Moreover, NETs and neutrophil activity in the lungs generally correspond to disease severity after pneumococcal infection (Moorthy et al., 2016).

LEAKAGE OF NE CAUSES ACUTE LUNG INJURY DURING PNEUMONIA

Neutrophil serine proteases, including NE, CG, and proteinase 3, are critical for the effective functioning of neutrophils, and contribute to immune protection (Pham, 2006). Among these proteases, NE has been well studied in both basic and clinical research. Although NE is a protease that degrades elastin (Janoff and Scherer, 1968), the degradation of foreign organic molecules phagocytosed by neutrophils is considered its main function (Kawabata et al., 2002). NE degrades outer membrane protein localized on the surface of Gram-negative bacteria to exert antimicrobial effects (Belaaouaj et al., 2000). NE-deficient mice are more susceptible to sepsis and death following infection with Gram-negative *Klebsiella pneumoniae* and *Escherichia coli* (Belaaouaj et al., 1998). However, the role of NE in Gram-positive bacterial infections remains controversial. It has been reported that NE does not contribute to neutrophil-mediated killing of Gram-positive *Staphylococcus aureus* (Belaaouaj et al., 1998). Specifically, in *S. pneumoniae*, NE plays an important role in degrading pneumococcal cell wall-localized aminopeptidase N, and mediates opsonophagocytic killing by neutrophils (Standish and Weiser, 2009; Nganje et al., 2019). However, some pneumococcal strains exhibit resistance to extracellular NE-mediated killing (Van der Windt et al., 2012; Domon et al., 2016).

Despite its fundamental importance in innate immunity, excessive neutrophil activation causes the release of NE, which contributes to tissue damage (Fox et al., 2013; Kovtun et al., 2018). In general, NE exerts potent catalytic effects against a broad array of host extracellular matrix components, including elastin, proteoglycan, fibronectin, and several collagen types (Janusz and Doherty, 1991; Taylor et al., 2018). The cross-linking of collagen and elastin imparts stability and functionality to the lung extracellular matrix, which plays an important role in the formation of alveolar gas exchange units (Mižíková et al., 2015). Many studies have indicated that increased NE activity in the lung is involved in the pathogenesis of various lung diseases such as pneumonia, acute lung injury, exacerbated chronic obstructive pulmonary disease, and cystic fibrosis (Polverino et al., 2017). Indeed, it has been reported that NE-deficient mice are protected to a significantly greater extent from the development of emphysema than wild-type mice (Shapiro et al., 2003). Additionally, N-formyl-methionyl-leucyl-phenylalanine-induced neutrophil influx in alveolar spaces results in decreased lung elastin content and the development of emphysema in mice (Cavarra et al., 1996). Moreover, instillation of NE into the lungs results in the destruction of alveolar walls in animals (Campbell, 2000). One possible mechanism could be that NE cleaves E-cadherin in alveolar epithelial cells which interferes with cell-cell adhesion (Boxio et al., 2016). As for bacterial infection, patients with bacterial pneumonia exhibit increased levels of NE in bronchoalveolar lavage fluid (BALF) (Boutten et al., 1996; Wilkinson et al., 2012), which may result in excessive proteolytic damage and worse clinical outcomes. Generally, NE is inhibited by serum α_1-antitrypsin. However, neutrophils also release matrix metalloproteinases (MMPs) that inactivate α_1-antitrypsin (Michaelis et al., 1990). Thus, the proteinase inhibitory capacity is decreased in the BALF in patients with bacterial pneumonia compared to that of healthy controls (Abrams et al., 1984).

In animal models, intratracheal pneumococcal infection causes acute lung injury, characterized by an increase in neutrophil accumulation and NE activity in the BALF (Yanagihara et al., 2007; Hagio et al., 2008). Subsequently, extracellular NE impairs the phagocytic activity of macrophages (Domon et al., 2016). Furthermore, NE cleaves extracellular matrix proteins, and proteins associated with the host immune response. In a murine model of bacterial pneumonia, NE, CG, and proteinase 3 cleave surfactant protein D, reducing the ability of the protein to promote bacterial aggregation (Hirche et al., 2004). NE also cleaves multiple cell surface receptors such as TLR2, TLR4, CD14, tumor necrosis factor receptor, and the C5a receptor, leading to an inhibition of downstream signaling (Wiedow and Meyer-Hoffert, 2005; Van den Berg et al., 2014; Domon et al., 2018b). Additionally, multiple cytokines and chemokines, such as interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-12p40, IL-12p70, and tumor necrosis factor, are degraded and inactivated by NE (Wiedow and Meyer-Hoffert, 2005; Clancy et al., 2018; Domon et al., 2018b). Furthermore, we recently reported that NE cleaves human leukocyte antigen class II molecules in both cultured macrophages and *in vivo* mouse models, indicating that NE may disrupt antigen presentation and T-cell activation (Domon et al., 2021). In contrast, NE cleaves and activates MMP-9, which may also have a destructive role in lung diseases (Jackson et al., 2010). Collectively, these data imply that NE cleaves a variety of host immune proteins, induces lung injury, and may assist pneumococci in evading the immune system during pneumonia (**Figure 2**).

EFFECT OF NE INHIBITORS ON BACTERIAL PNEUMONIA

Multiple studies have investigated the potential role of various NE inhibitors, such as sivelestat (ONO-5046), AZD9668, EPI-hNE-4, KRP-109, and pre-elafin in different lung diseases (Polverino et al., 2017). In animal models of LPS- or chemical-induced non-infectious acute lung injury, symptoms have been observed to be ameliorated upon treatment with EPI-hNE-4 (Honoré et al., 2004), sivelestat (Sakamaki et al., 1996; Inoue et al., 2005; Iba et al., 2006), or pre-elafin (Vachon et al., 2002). Preclinical and clinical studies have also demonstrated the efficacy of sivelestat and AZD9668 in treating acute lung injury and bronchiectasis, respectively (Tamakuma et al., 2004; Inoue et al., 2006; Fujii et al., 2010; Aikawa et al., 2011; Stockley et al., 2013). In animal models of pneumococcal pneumonia, the administration of sivelestat resulted in higher survival rates and decreased bacterial counts in the blood (Yanagihara et al., 2007; Domon et al., 2018b), suggesting that NE-induced lung injury and immune subversion cause bacterial invasion of the bloodstream followed by death. Mice treated with KRP-109 showed lower neutrophil infiltration and inflammation than control mice, with no effects on viable pneumococcal numbers in the lungs (Yamada et al., 2011). These findings suggest that NE contributes, at least in part, to the pathogenesis of pneumococcal pneumonia. Although only a few studies have investigated the effects of NE inhibitors in patients with bacterial pneumonia, a retrospective study suggested that the early administration of sivelestat improves patient survival rate (Nakamura et al., 2008). Although this finding provides convincing evidence of NE-induced tissue destruction in pneumonia in humans, since neutrophils are the first phagocytic cells recruited to the lung infection site, further randomized controlled trials are required to examine the efficacy of NE inhibitors against bacterial pneumonia.

CONCLUSION

Accumulating evidence indicates that both pneumococcal virulence factors and host proteases, including NE, are major mediators of lung injury during severe pneumococcal infections. Although the activation of PRRs in response to pneumococcal stimuli, followed by neutrophil infiltration, is key to the initiation of the innate immune response, this host defense strategy can be exploited by pneumococcus in lung tissues. *S. pneumoniae* targets infiltrated neutrophils and promotes the formation of NETs and cell lysis by

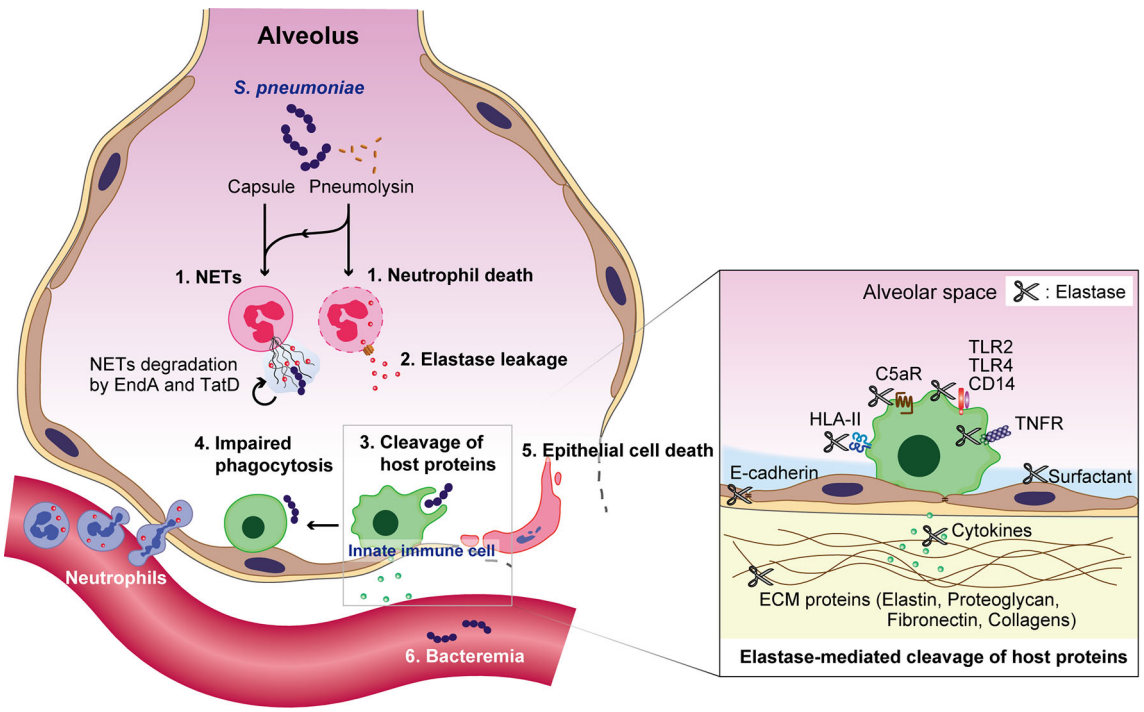

FIGURE 2 | Overview of *S. pneumoniae*-induced immune subversion by the exploitation of neutrophils during pneumonia. Pneumococcal capsules and pneumolysin enhance the formation of neutrophil extracellular traps (NETs), which are subsequently degraded by the pneumococcal endonucleases EndA and TatD. Pneumolysin also exerts cytotoxicity against neutrophils. The subsequent leakage of neutrophil elastase induces the degradation of surfactant protein D, cell-cell adhesion molecule E-cadherin, and extracellular matrix components, such as elastin, proteoglycan, fibronectin, and several collagen types. Additionally, neutrophil elastase impairs the phagocytic activity of macrophages, induces the death of alveolar epithelial cells, and diminishes the pulmonary epithelial barrier. Furthermore, neutrophil elastase cleaves multiple cell surface proteins, such as toll-like receptor (TLR)2, TLR4, CD14, tumor necrosis factor receptor (TNFR), the C5a receptor (C5aR), and human leukocyte antigen class II (HLA-II); followed by the degradation of multiple cytokines and chemokines, such as interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-12p40, IL-12p70, and tumor necrosis factor, which eventually disrupts the pulmonary immune defense.

utilizing Ply and other virulence factors, which in turn could increase the local NE concentration (Mori et al., 2012; Domon et al., 2016; Nel et al., 2016). Subsequently, elastase-induced proteolysis of extracellular matrix components (Taylor et al., 2018), cell-cell adhesion molecules (Boxio et al., 2016), and host immune molecules (Hirche et al., 2004; Wiedow and Meyer-Hoffert, 2005; Van den Berg et al., 2014; Clancy et al., 2018; Domon et al., 2018b) results in disruption of the alveolar epithelial barrier, which may allow pneumococci to invade the bloodstream. Additionally, several host proteases, including MMPs (Davey et al., 2011), CG, and proteinase 3 (Guyot et al., 2014), may contribute to lung injury. Thus, further basic research is still needed to understand the mechanisms of disease initiation, and to develop novel therapies for lung injury during bacterial pneumonia.

AUTHOR CONTRIBUTIONS

HD wrote the paper. HD and YT further developed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant (JP20K09903, JP20K21671, and JP20H03858), the Takeda Science Foundation, and the Kobayashi International Scholarship Foundation.

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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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frontiers in Cellular and Infection Microbiology

BRIEF RESEARCH REPORT
published: 19 March 2021
doi: 10.3389/fcimb.2021.617573

Distribution and Variation of Serotypes and Pneumococcal Surface Protein A Clades of *Streptococcus pneumoniae* Strains Isolated From Adult Patients With Invasive Pneumococcal Disease in Japan

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Pneumococcal surface protein A (PspA) is a surface protein of *Streptococcus pneumoniae* that may be a candidate antigen for new pneumococcal vaccines. This study investigates the distribution of PspA clades of the causative strains of adult invasive pneumococcal disease (IPD) in Japan. Of the 1,939 strains isolated from cases of adult IPD during 2014–2019, the PspA clades of 1,932 (99.6%) strains were determined, and no *pspA* was detected in the remaining 7 strains (0.4%). PspA clades 1–6 were detected in 786 (40.5%), 291 (15.0%), 443 (22.8%), 369 (19.0%), 33 (1.7%), and 6 (0.3%) strains, respectively. New PspA clades (0.2%) were identified in two non-typeable and two serotype 35B pneumococci. The proportions of clade 1 and clade 2 showed significantly decreased and increased trends, respectively. Furthermore, the PspA clade of pneumococcal strains was partially serotype- and sequence type-dependent. The majority of strains belonging to serotypes contained in both the 13-valent pneumococcal conjugate vaccine (PCV13) and the 23-valent pneumococcal polysaccharide vaccine (PPSV23) belonged to PspA clades 1 or 3. In contrast, the distribution of clades in non-

OPEN ACCESS

Edited by:
Ghassan M. Matar, American University of Beirut, Lebanon

Reviewed by:
Samantha J. King, The Ohio State University, United States
Luchang Zhu, Houston Methodist Research Institute, United States

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Specialty section:
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *21 October 2020*
Accepted: *26 February 2021*
Published: *19 March 2021*

Citation:
Chang B, Kinjo Y, Morita M, Tamura K, Watanabe H, Tanabe Y, Kuronuma K, Fujita J, Oshima K, Maruyama T, Abe S, Kasahara K, Nishi J, Kubota T, Ohnishi M, Suga S and Oishi K (2021) Distribution and Variation of Serotypes and Pneumococcal Surface Protein A Clades of Streptococcus pneumoniae Strains Isolated From Adult Patients With Invasive Pneumococcal Disease in Japan. Front. Cell. Infect. Microbiol. 11:617573. doi: 10.3389/fcimb.2021.617573

vaccine serotypes was wider than that of vaccine serotype pneumococci. Our findings demonstrate that almost all pneumococcal strains from adult IPD express PspA clades 1–4, especially for non-vaccine serotypes. These results may be useful for the development of a new pneumococcal vaccine with PspA.

Keywords: *Streptococcus pneumoniae*, invasive pneumococcal disease, serotype, pneumococcal surface protein A clade, vaccine, Japan, adults

INTRODUCTION

Streptococcus pneumoniae is the most common cause of pneumonia, bloodstream infections, and meningitis in young children and adults 65 years or older (Centers for Disease Control and Prevention, 2020). To date, 100 pneumococcal capsular serotypes have been identified (Ganaie et al., 2020). Currently, vaccines for the prevention of *S. pneumoniae* infections include the 23-valent pneumococcal polysaccharide vaccine (PPSV23) and the 13-valent pneumococcal conjugate vaccine (PCV13). These vaccines only cover some pneumococcal serotypes and cannot protect against infections due to non-vaccine serotypes and unencapsulated *S. pneumoniae* (Briles et al., 2019). The 7-valent pneumococcal conjugate vaccine (PCV7) was available for children in Japan in 2010 and was replaced by PCV13 in November 2013. PCVs have been included in the national immunization program (NIP) since April 2013. After the introduction of PCVs, the incidence of IPD in children aged <5 years decreased by >50%; however, this has not decreased further (Suga et al., 2015). PCV13 and PPSV23 have already been licensed for adults, and PPSV23 for those aged ≥65 years has been included in the Japanese NIP from October 2014. We have been studying epidemiology and clinical features of IPD in adults residing in 10 prefectures of Japan (the same area as this study) since 2013 (Fukusumi et al., 2017; Shimbashi et al., 2019; Shimbashi et al., 2020).

The direct and indirect protective effects of PCV have been reported worldwide (Berical et al., 2016). However, an increase in invasive pneumococcal disease (IPD) caused by non-vaccine serotypes (serotype replacement) occurred after the introduction of PCV, especially in children (Hausdorff and Hanage, 2016). The serotype replacement was also observed in adult IPD (Kendall et al., 2016). Because of the limitations of capsular polysaccharide vaccines, there is an urgent need to develop new, effective, and affordable pneumococcal vaccines covering a wide range of serotypes. These candidates include protein-based pneumococcal vaccines using conserved pneumococcal antigens, such as surface-exposed protein, and detoxified pneumolysin.

Pneumococcal surface protein A (PspA) is a choline-binding protein on the cell surface of almost all pneumococcal strains that inhibits the complement-mediated clearance of pneumococci (McDaniel et al., 1984). PspA comprises five domains, namely, a signal peptide, an α-helical highly charged domain, a proline-rich region domain, a choline-binding domain, and a short hydrophobic tail (Hollingshead et al., 2000). The α-helical highly charged domain has an α-helical coiled-coil structure that is further divided into regions A, B, and C (McDaniel et al., 1994). The B region is the clade-defining region, and amino acid residues 192 and 260 of PspA are protection-eliciting epitopes. The sequence variations of this region are used to classify pneumococcal strains into three families and six clades (Pimenta et al., 2006). It was reported that almost 100% of clinical isolates from cases of IPD in adults and non-IPD in children in Japan belonged to either PspA family 1 (clades 1 and 2) or 2 (clades 3–5) (Piao et al., 2014; Kawaguchiya et al., 2018). However, the *S. pneumoniae* strains analyzed in these studies were from adult cases of IPD before the introduction of PCVs or from noninvasive diseases in children. Therefore, the PspA clade distribution of a relatively large number of *S. pneumoniae* strains from adult IPD cases after PCV introduction has not yet been reported.

This study determined the PspA clade of 1,932 *S. pneumoniae* strains isolated from adult IPD cases in Japan between 2014 and 2019 to elucidate whether PCV introduction influenced the PspA clade distribution. We also analyzed the relationships between PspA clade, serotype, and clonal complex (CC).

MATERIALS AND METHODS

IPD Case Definition and Bacterial Strains

The adult IPD Study Group implemented population-based surveillance in Japan in 2013 of IPD cases occurring in people over the age of 15 years old who resided in 10 prefectures of Japan (Hokkaido, Miyagi, Yamagata, Niigata, Mie, Nara, Kochi, Fukuoka, Kagoshima, and Okinawa). When IPD occurred, the clinical information and the causative pneumococcal strains were simultaneously collected and sent to the National Institute of Infectious Diseases (NIID). The clinical information included the patient's sex, age, and history of PCV13 and PPSV23 vaccinations.

A case of IPD was defined as the detection of pneumococci by bacterial culture from normally sterile sites. Our study analyzed *S. pneumoniae* strains isolated from adult IPD patients from January 2014 to December 2019. One isolate per case was included.

Serotyping, Multilocus Sequencing Typing (MLST), and PspA Clades

All *S. pneumoniae* strains were plated on Columbia agar with 5% sheep blood (Nippon Becton Dickinson Co. Ltd., Tokyo, Japan). The quellung reaction was used for serotype determination with pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark). Serotype 11A/E and non-typeable (NT) were determined as described previously (Shimbashi et al., 2020).

The genomic DNA of the pneumococcal isolates was purified using a High Pure PCR Template Purification Kit (Roche Diagnostics, Tokyo, Japan). MLST was performed as described by Enright and Spratt (1998). Allelic numbers and sequence types (STs) were assigned using the pneumococcal MLST website (https://pubmlst.org/spneumoniae/). Strains where ≥5/7 alleles were identical were classified as a CC (Gertz et al., 2003). PspA clade determination was performed as described by Pimenta et al. (2006). For strains where the PCR fragment was not amplified, an additional PCR reaction was performed using primers upstream (*pspA*-up: 5′-CACACGAGATTATGCTA GTC-3′) and downstream (*pspA*-dn: 5′-CTGCTCCTTGAGC AAAAGAG-3′) of *pspA*, and if a PCR fragment and *pspA* sequence was then obtained, the PspA clade was determined. For strains having sequences that were <90% identical to the sequences of known clades 1–6, BLAST search in nucleotide database of NCBI was performed.

Whole Genome Sequencing Analysis

Genomic DNA libraries of the pneumococcal strains in which *pspA* could not be detected by PCR were constructed using the Nextera XT DNA sample prep kit (Illumina, San Diego, CA, USA) and then sequenced using an MiSeq (Illumina). After genome assembly was performed using the SPAdes version 3.13.1 with the careful option and a read coverage cutoff value of 10 (Bankevich et al., 2012), homology search of *pspA* gene on the genomes was performed by GENETYX-MAC (GENETYX, Tokyo, Japan).

The whole genome sequences (accession numbers: DRX251224-DRR251230) as well as new (LC597020-LC597021) and deletion mutant (LC597022-LC597023) *pspA* sequences of pneumococci have been deposited in the DNA Data Bank of Japan.

Statistical Analysis

The proportions of each clade were compared using χ^2-test or Fisher's exact test. The Mantel–Haenszel test was used to reveal the trend of the proportions for each clade from 2014 to 2019. Multiple comparisons were corrected using Bonferroni's method. P values <0.05 were considered to be significant. All statistical analyses were performed using IBM SPSS Statistics version 24 (IBM Corp., Armonk, NY, USA).

Ethics Statement

This study was reviewed and approved by the Ethics Committee of the NIID and was conducted according to the principles expressed in the Declaration of Helsinki. Informed consent was waived because the data did not contain any patient identifiers, and the samples were taken in the course of standard patient care.

RESULTS

Characteristics of IPD Cases

A total of 1,963 IPD cases occurred from January 2014 to December 2019 that were reported to the Adult IPD Study Group. Of them, 24 cases were excluded from our study because the *S. pneumoniae* strains isolated from 20 cases had died, and live bacteria could not be isolated from the remaining 4 cases. The *S. pneumoniae* strains from the remaining 1,939 IPD cases underwent serotyping, MLST, and PspA clade determination. Another 5 cases were excluded because of incomplete clinical data. The remaining 1,934 patients were 15–103 years old, with a median age of 71. Sixty percent of the patients were men.

Serotype Distribution of the Pneumococcal Strains

The most prevalent serotypes were 3 (230 strains; 11.9%), 12F (195 strains; 10.1%), 19A (166 strains; 8.6%), 10A (148 strains; 7.7%), and 23A (135 strains; 7.0%). In total, 32.5% of them belonged to PCV13 serotypes and 63.0% belonged to PPSV23 serotypes.

The prevalence of each pneumococcal serotype for each year is shown in the **Supplementary Figure 1**. The number of IPD cases each year was 203, 220, 291, 416, 391, and 418 between 2014 and 2019. The isolation rates of serotypes 3 and 19A peaked in 2015 and decreased from 2016. Conversely, the isolation rate of 12F increased from 2016, peaked in 2017, and decreased in 2018 (Shimbashi et al., 2019) before further decreasing in 2019 (**Supplementary Figure 1**). During 2014–2019, the isolation rates of pneumococcal serotypes 10A and 23A were consistently high, whereas those of the PCV7 serotypes were low. The coverage rate of the pneumococcal strains by PCV13 decreased from 44.8% to 27.0%. On the other hand, the coverage rate of PPSV23 remained >60% from 2014 to 2018, decreasing by approximately 5% in 2019.

PspA Clades of the Pneumococcal Strains

We determined the *pspA* sequences of 1,932/1,939 (99.6%) *S. pneumoniae* strains. The sequences of 1,928 strains (99.4%) shared >90% identity with the sequences of clades 1–6. Two serotype 35B CC558 strains, which were isolated in 2018, had deletions between 601 and 948 bp and between 601 and 957 bp of *pspA*, respectively. Deletion of amino acid residues was noted at positions 201-316 and 201-319, but no stop codons were found. The sequences upstream and downstream of the deleted region of the two strains were identical to each other and also showed high homology with the sequence of clade 4. No similar deletion sequence could be found in the NCBI database. Because PspA between amino acid residues 192 and 260 has been reported to have protection-eliciting epitopes (McDaniel et al., 1994), it is possible that the two strains have different antigenicity with clade 4. Therefore, the *pspA* genes of the two strains were determined to be a new clade (**Table 1**).

Additionally, the sequences of two non-typeable strains, which were isolated in 2019, showed less than 90% identity with all sequences of clades 1–6. The sequence of the CC230 strain between primers SKH2 and LSM12 had 76% identity with that of clade 6, and the CC15490 strain was 72% of that of clade 3. The sequences of the two strains also showed low identity (56%) with each other. BLAST in the NCBI database showed that

TABLE 1 | Number of the pneumococcal strains with different serotypes and different clonal complexes (CCs).

Serotype	CC[#]	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	Clade 6	New clade	Without *pspA* sequence
4*	CC695	0	0	3	0	0	0	0	0
	CC5872	0	0	0	1	0	0	0	0
6B*	CC90	21	1	3	0	0	0	0	0
	CC902	8	0	0	2	0	0	0	1
	CC2224	0	0	3	0	0	0	0	0
	CC338	3	0	0	0	0	0	0	0
9V*	CC166	0	0	2	2	0	0	0	0
	CC280	0	1	4	0	0	0	0	0
14*	CC13	18	0	0	0	0	0	0	0
	CC343	3	0	0	0	0	0	0	0
18C*	CC870	0	0	0	2	0	0	0	0
	CC3594	3	0	0	0	0	0	0	0
19F*	CC236	0	0	34	2	2	0	0	2
	CC242	0	0	0	0	0	0	0	2
23F*	CC242	0	0	0	0	9	0	0	0
	CC1437	0	0	0	0	9	0	0	0
	CC338	0	2	0	0	2	0	0	0
1*	CC306	3	0	0	0	0	0	0	0
3*	CC180	206	3	8	0	1	0	0	0
6A*	CC3113	11	0	0	0	0	0	0	0
	CC81	0	0	7	0	0	0	0	0
	CC90	2	0	0	0	0	0	0	0
7F*	CC191	0	0	36	0	0	0	0	0
19A*	CC320	0	0	6	0	0	0	0	0
	CC2331	0	0	34	0	0	0	0	0
	CC3111	0	0	124	2	0	0	0	0
10A[§]	CC1263	40	0	0	0	0	0	0	0
	CC5236	104	0	0	1	0	0	0	0
15B[§]	CC199	0	0	0	30	0	0	0	0
22F[§]	CC433	105	0	0	0	0	0	0	0
33F[§]	CC717	21	0	0	0	0	0	0	0
9N[§]	CC66	0	0	2	0	0	0	0	0
12F[§]	CC4846	0	0	117	0	0	0	0	0
	CC6945	0	77	0	0	0	0	0	0
8[§]	CC2234	0	0	0	0	1	0	0	0
	CC3500	2	0	0	0	0	0	0	0
11A/E[§]	CC99	0	1	0	71	0	0	0	0
20[§]	CC4745	20	0	0	0	0	0	0	0
15A	CC63	0	0	0	108	0	0	0	1
	CC81	0	0	1	0	0	0	0	1
15C	CC199	0	0	0	21	0	0	0	0
23A	CC338	76	39	0	0	0	0	0	0
24F	CC2572	0	36	0	0	0	0	0	0
24B	CC2572	0	0	3	0	0	0	0	0
	CC2754	0	0	8	0	0	0	0	0
6C	CC2924	1	40	0	0	0	0	0	0
	CC2923	0	0	0	11	0	0	0	0
	CC5241	0	31	0	1	0	0	0	0
7C	CC2758	12	0	0	0	0	0	0	0
38	CC6429	26	0	0	0	0	0	0	0
35B	CC156	0	0	8	0	0	0	0	0
	CC2755	0	0	0	42	0	0	0	0
	CC558	0	1	2	53	0	0	2	0
34	CC3116	29	0	0	0	0	0	0	0
	CC7338	14	0	0	0	0	0	0	0
31	CC11184	0	26	0	0	0	0	0	0
37	CC447	0	0	0	0	0	6	0	0
Non-typeable	CC230	0	0	0	0	0	0	1	0
	CC338	0	1	0	0	0	0	0	0
	CC2331	0	0	1	0	0	0	0	0
	CC2572	0	1	0	0	0	0	0	0
	CC3116	1	0	0	0	0	0	0	0
	CC15490	0	0	0	0	0	0	1	0

**Serotypes belonging to PCV13.*

[§]Serotypes belonging to PPSV23 but not to PCV13.

[#]Strains where ≥5 of the 7 alleles in MLST analysis are classified as a CC.

the sequence of the CC230 strain was 99% identical to that of isolate 34YLE (LT669627) and the sequence of CC15490 strain was 99% identical to that of isolate R34-3088 (LT669632) (Croucher et al., 2017). Therefore, the *pspA* genes of the two strains were determined to be new clades (**Table 1**).

Besides these strains, the *pspA* fragment of seven strains (0.4%) was not amplified by PCR using the two primer sets. To identify the presence or absence of *pspA*, whole genome sequences of these strains were determined and no gene similar to *pspA* was found by homology search. Therefore, *pspA* is absent in the seven strains. All seven strains were isolated in 2019, and their serotypes were 19F, 15A, and 6B in four, two, and one strain, respectively (**Table 1**).

In summary, 786 (40.5%), 291 (15.0%), 443 (22.8%), 369 (19.0%), 33 (1.7%), and 6 (0.3%) strains belonged to clades 1–6, respectively. Four (0.2%) strains possessed new clades. The *pspA* gene was not detected in seven (0.4%) strains.

The annual distribution of PspA clades of the *S. pneumoniae* strains is summarized in **Figure 1**. The statistical analysis of the trends for the proportions of each clade from 2014 to 2019 revealed significantly decreased and increased trends for the proportions of clade 1 and clade 2, respectively (**Figure 1**). Clades 5 and 6 had lower isolation rates than the other clades during the study period (<5%). The total isolation rates of PspA clades 1–4 were 96.1%, 97.3%, 98.3%, 98.8%, 97.2%, and 96.4% from 2014 to 2019, respectively.

Relationship Between PspA Clade and Serotype and Between PspA Clade and Clonal Complexes

We compared differences in the proportions of PspA clades of the *S. pneumoniae* strains belonging to the vaccine and non-vaccine serotypes. The *S. pneumoniae* strains belonging to PCV13 and PPSV23 had higher rates in PspA clades 1 and 3. On the other hand, the non-PCV13 and non-PPSV23 strains had higher rates in PspA clades 2 and 4 (**Figure 2**).

The PspA clades of the *S. pneumoniae* strains that belonged to the vaccine serotypes and other major serotypes are summarized in **Table 1**, and the major CCs of these serotypes are also recorded. All strains of serotype 14, 7F, 15B, 22F, 33F, 9N, 20, 15C, 16F, 21, 24F, 24B, 7C, 38, 34, 29, 31, and 37 comprised a single clade. The PspA clade for serotypes 18C, 6A, and 12F was CC-dependent. Serotypes 6B, 19F, 23F, 3, 8, 11A/E, 15A, 23A, 23B, 6C, 6D, 35B, and non-typeable strains were found in multiple clades.

The CCs of the seven strains that did not possess *pspA* were CC236 (19F, two strains), CC242 (19F, two strains), CC63 (15A, one strain), CC81 (15A, one strain), and CC902 (6B, one strain). Among the four strains that showed new PspA clades, the two 35B strains were CC558 and the two non-typeable strains were CC230 and CC15490 (**Table 1**).

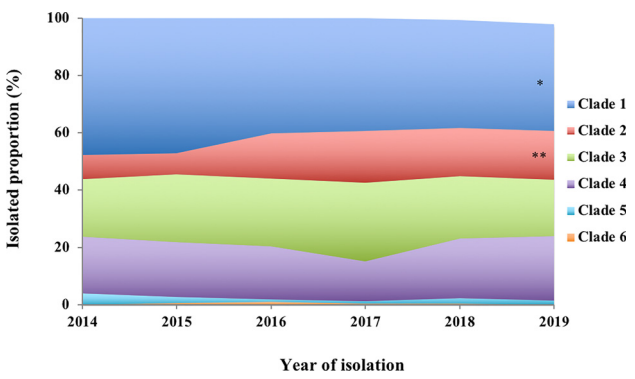

FIGURE 1 | Annual proportion of PspA clades 1–6 among the *S. pneumoniae* strains isolated from invasive pneumococcal diseases in 2014–2019. Mantel–Haenszel test of trends corrected using Bonferroni's method to perform an analysis of the trends for the proportions of each clade from 2014 to 2019. *$p < 0.05$, **$p < 0.01$.

DISCUSSION

Our study summarized the distribution of the PspA clades of 1,928 *S. pneumoniae* strains isolated from cases of adult IPD in Japan during 2014–2019. This is the first report of PspA clade distribution of *S. pneumoniae* strains isolated from adult cases of IPD after PCV introduction for children in Japan. The proportions of PspA clades 1–6 were 40.5%, 15.0%, 22.8%, 19.0%, 1.7%, and 0.3%, respectively. A previous study of 68 strains isolated from adult cases of IPD in Japan during 2010–2011 reported that the major PspA clades were 1 (50%) and 3 (28%) (Piao et al., 2014). The other clades 2, 4, and 5 comprised minor proportions of 4%, 9%, and 9% of the cases, respectively. There was no clade 6. The median age of the 68 patients was 68 years, similar to the median age 71 years in this study. Because pneumococcal strains isolated from adult IPD before PCV introduction were limited in Japan, only 68 strains from adult IPD were analyzed in the previous study (Piao et al., 2014). However, we determined PspA clade distribution of 250 pneumococcal strains isolated from pediatric IPD cases that occurred before the introduction of PCVs (Suga et al., 2015), and these data show a trend similar to that obtained from the 68 adult IPD strains. The major PspA clades of the pneumococci from children were 1 (42%) and 3 (32%), whereas the other clades 2, 4, and 5 comprised minor proportions of 4%, 9%, and 13% of the cases, respectively. There was no clade 6 (**Supplementary Figure 2**). These strains were isolated from pediatric IPD patients who resided in 10 prefectures of Japan (Suga et al., 2015); among the 10 prefectures, seven (Hokkaido, Niigata, Mie, Kochi, Fukuoka, Kagoshima, and Okinawa) were the same as those surveyed in this study. Therefore, the PspA clade distribution of 68 strains from adult patients with IPD and 250 strains from pediatric patients with IPD could be used as controls for this study. Because PCVs have been part of the NIP for children in Japan since 2013, our findings suggested that the PspA clade distribution of strains that caused adult IPD changed considerably after PCV introduction in children.

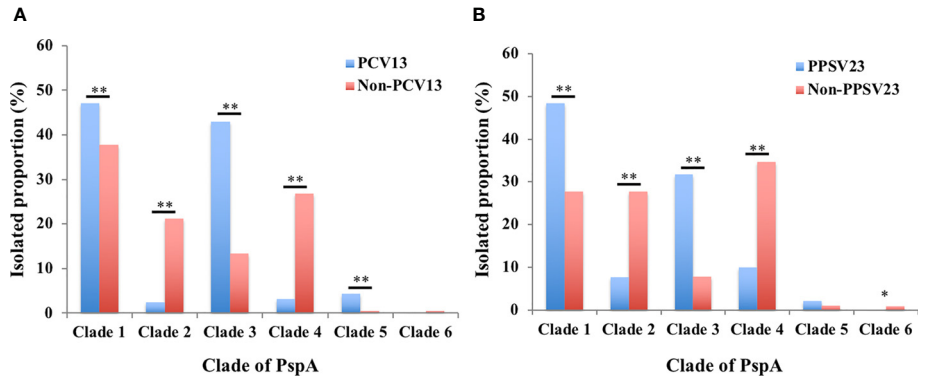

FIGURE 2 | Isolation proportions of PspA clades 1–6 among the *S. pneumoniae* strains of vaccine and non-vaccine serotypes. **(A)** PCV13 and non-PCV13 serotypes, **(B)** PPSV23 and non-PPSV23 serotypes. The proportions of vaccine and non-vaccine serotypes were compared using χ^2-test (clades 1–5) or Fisher's exact test (clade 6) corrected using Bonferroni's method. * $p < 0.05$, ** $p < 0.01$. PCV13: 13-valent pneumococcal conjugate vaccine; PPSV23: 23-valent pneumococcal polysaccharide vaccine.

During 2014–2019, our data demonstrated that the annual distributions of PspA clade 1 and clade 2 exhibited significantly decreased and increased trends, respectively (**Figure 1**). We compared the PspA clade distribution before and after PCV using pediatric IPD and adult IPD data in 2019, respectively. Compared to the data before PCV, clades 2 and 4 were significantly increased and clades 3 and 5 were significantly decreased after PCV. Although there was a tendency for clade 1 to decrease after PCV, the difference was not statistically significant (**Supplementary Figure 3**). Furthermore, *S. pneumoniae* strains of PCV13 and PPSV23 serotypes had higher rates in PspA clades 1 and 3 (**Figure 2**). These results suggest that the serotype replacement might have influenced (**Supplementary Figure 1**), but only partially, the change in PspA clade distributions.

In this study, 4 strains (0.2%) were classified as new clades because they had the *pspA* sequences that differed from the well-known clades 1–6 (**Table 1**). Moreover, there were 7 *pspA*-negative strains (0.4%). Although the total rate of strains without *pspA* and having new PspA clade is low (0.6%), continuous surveillance would be important to determine if there will be any changes in the distribution of PspA clades.

The protective effects of PCV have been well-recognized worldwide (Berical et al., 2016; Kim et al., 2016). The introduction of PCV7 and PCV13 in children dramatically decreased the incidence of IPD caused by vaccine serotypes, and annual incidence of IPD in children aged <5 years decreased significantly by 57% in 2013 compared with that in 2008 before PCV introduction in Japan (Suga et al., 2015; Nakano et al., 2020). In addition to direct effect, PCV in national immunization programs for children has had a significant indirect effect on pneumococcal diseases in adults (Berical et al., 2016; Kim et al., 2016). Compared with PCV, the protective effects of PPSV23 are controversial. However, a systematic review and meta-analysis reported the effectiveness of PPSV23 against IPD in adults aged >50 years to be 54% (Kraicer-Melamed et al., 2016). PPSV23 was included for those aged ≥65 years in Japanese national vaccine program in 2014. The adjusted vaccine effectiveness of PPSV23 against adult IPD caused by vaccine serotypes was 42.2% (Shimbashi et al., 2020). Furthermore, it was shown that prior vaccination with either PCV13 or PPSV23 decreased the risk of pneumococcal carriage in adults aged ≥65 years (Branche et al., 2018). These studies indicate that not only PCV13 but also PPSV23 have protective effects against pneumococcal diseases. However, because of the serotype replacement, limitations of the PCV13 and PPSV23 exist. Therefore, there is an urgent need for a new, effective, and affordable pneumococcal vaccine that covers a wide range of serotypes for both children and adults in Japan.

The PspA clades of *S. pneumoniae* strains from adult IPD cases were partially serotype- and CC-dependent (**Table 1**). More importantly, our data demonstrated that the isolation rates of the PspA clades 1–4 were maintained at high levels (96.1%–98.8%), and clades 5 and 6 had low rates. The studies of IPD strains from Spain, China, and Korea showed that the rate of PspA clade 5 was 9.1%, 8.2%, and 12.4%, respectively (Rolo et al., 2009; Qian et al., 2012; Yun et al., 2017). The clade 6 was found in only 1 strain from Korea and in no other strains from geographies. However, the number of pneumococci analyzed in these studies was limited as 66, 171, and 190 strains, respectively. Therefore, further studies would be needed to determine the actual rates of clades 5 and 6 among pneumococcal strains from IPD patients in these studies because the rate of clade 5 was 9% in the previous report with 68 adult IPD strains in Japan (Piao et al., 2014). Collectively, our results indicate that new vaccines under development targeting PspA should include at least clades 1–4.

In conclusion, the distribution of the PspA clades of *S. pneumoniae* strains isolated from adult IPD cases during 2014–2019 in Japan were determined. These data may be useful for designing new PspA-based pneumococcal vaccines. We aim to continue examining the serotypes, STs, and PspA

clades for an in-depth understanding of *S. pneumoniae* strains that cause IPD in adults.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the National Institute of infectious Diseases. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BC, YK, and KaO designed the study. HW, YT, KoK, JF, KeO, TM, SA, KeK, JN, and TK collected clinical data and bacterial strains. KT performed statistical analyses. BC carried out the microbiological analysis and analyzed the data. MM and MO carried out the whole genome sequencing. BC and SS provided PspA distribution results of pneumococci from children with IPD. BC and YK drafted the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Ministry of Health and Labour Sciences HA Program Grant Number JPMH20HA1005, the Japan Society for the Promotion of Science KAKENHI Grant number 19H03705 and the Japan Agency for Medical Research and Development (AMED; JP20fk0108139j1901).

ACKNOWLEDGMENTS

We sincerely thank the staff of the local public health centers who collected bacterial isolates and case report forms from the hospitals and the staff of the public health institutes and laboratories who worked for the Adult IPD Study Group. In addition to the authors, the following investigators and the institutions participated in the Adult IPD Study Group: Hokkaido Institute of Public Health; Michiharu Nakano, Sendai City Public Health Center; anonymous, Yamagata Prefectural Bureau of Public Health and Welfare; Tatsuya Ikeda, Yamagata Prefectural Institute of Public Health; Yu Suzuki, Junji Seto, Kazue Yahagi, Welfare and Public Health Bureau of Niigata Prefecture; Kurumi Kazama, Health and Sanitation Department of Niigata City; Satoru Yamazaki and Emiko Kumakura, Department of Health and Welfare, Mie Prefecture; Minoru Hiraoka, Mie Prefecture Health and Environment Research Institute; Yuhki Nagai, Sagako Naraya, Department of Medical Policy, Nara Prefecture; Eri Tsujimoto, The Public Health Institute of Kochi Prefecture; Michiaki Matsumoto, Fukuoka Institute of Health and Environmental Science; Hiroaki Shigemura, Fuyuki Okamoto, Nobuyuki Sera, Center for Environment and Health, Kagoshima Prefecture; Mutsuyo Gokuden, Welfare and Public Health Bureau of Okinawa Prefecture; Toru Itokazu, Okinawa Prefectural Institute of Health and Environment; Jun Kudaka, Kochi Medical School, Kochi University; Akihito Yokoyama, Sapporo Medical University School of Medicine; Hiroki Takahashi, Tohoku University Graduate School of Medicine; Tetsuji Aoyagi, Yamagata Saiseikai Hospital; Hiroaki Takeda, Niigata University Graduate School of Medical and Dental Sciences; Chikako Tsubata, Chikamori Hospital; Masayuki Ishida and University of the Ryukyus; Masashi Nakamatsu.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.617573/full#supplementary-material

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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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frontiers in Cellular and Infection Microbiology

REVIEW
published: 22 March 2021
doi: 10.3389/fcimb.2021.643326

Virus-Induced Changes of the Respiratory Tract Environment Promote Secondary Infections With *Streptococcus pneumoniae*

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 17 December 2020
Accepted: 01 March 2021
Published: 22 March 2021

Citation:
Sender V, Hentrich K and Henriques-Normark B (2021) Virus-Induced Changes of the Respiratory Tract Environment Promote Secondary Infections With Streptococcus pneumoniae. Front. Cell. Infect. Microbiol. 11:643326. doi: 10.3389/fcimb.2021.643326

Secondary bacterial infections enhance the disease burden of influenza infections substantially. *Streptococcus pneumoniae* (the pneumococcus) plays a major role in the synergism between bacterial and viral pathogens, which is based on complex interactions between the pathogen and the host immune response. Here, we discuss mechanisms that drive the pathogenesis of a secondary pneumococcal infection after an influenza infection with a focus on how pneumococci senses and adapts to the influenza-modified environment. We briefly summarize what is known regarding secondary bacterial infection in relation to COVID-19 and highlight the need to improve our current strategies to prevent and treat viral bacterial coinfections.

Keywords: *Streptococcus pneumoniae*, pneumococci, influenza virus, COVID-19, respiratory tract infections, coinfection, influenza-pneumococcal coinfection

INTRODUCTION

The primary function of the respiratory system is to exchange oxygen and carbon dioxide by inhaling air. The average person inhales about 10,000 liters of air per day, which is laden with pollutants, allergens, and pathogens. The intake of contaminated air inevitably allows inhaled microorganisms to colonize the respiratory tract. One of the most commonly found bacterial pathogens in the respiratory tract is the gram-positive bacterium *Streptococcus pneumoniae* (the pneumococcus). The pneumococcus dynamically colonizes up to 30-75% of healthy children, especially those attending day care centers, as well as up to 20-30% of healthy adults (Ghaffar et al., 1999; Bogaert et al., 2004; Hjalmarsdottir et al., 2016; Lindstrand et al., 2016). Colonization is usually asymptomatic, but pneumococci can also spread into the lower respiratory tract to cause pneumonia, and to other sites of the body where it causes invasive diseases such as bacteremia and meningitis. Risk groups for developing a severe pneumococcal disease include young children and the elderly (<2 yrs and >65 yrs), immunodeficiencies, and comorbidities like diabetes. Also preceding virus infections constitute a major risk for developing severe pneumococcal diseases (Madhi et al., 2004). Pneumococci are most successful in causing disease, especially when risk factors are present, making them one of the leading causes of lower respiratory tract infections

(LRTI) worldwide (GBD 2016 Lower Respiratory Infections Collaborators, 2018). Increased morbidity and mortality due to pneumococcal infections is closely linked to underlying virus infections, mainly caused by influenza virus (Morris et al., 2017).

The influenza virus causes a highly contagious respiratory illness, also known as the flu that is responsible for significant morbidity and mortality. Influenza-induced epidemics result in 3 - 5 million cases of severe illness, and up to 650 thousand deaths worldwide each year (World Health Organization, 2018). Besides the seasonal epidemics we witness every year, four influenza pandemics have occurred, since the beginning of the 20th century: the Spanish influenza (H1N1) in 1918/1919, Asian influenza (H2N2) in 1957, Hong Kong influenza (H3N2) in 1968, and H1N1 swine influenza in 2009. Of these pandemic viruses, the 1918 virus was the most devastating resulting in 50 - 100 million deaths worldwide (Taubenberger and Morens, 2020). Many of the victims were rather young and secondary bacterial pneumonia, mainly caused by pneumococci, was a major cause of death among those infected with the virus (Morens et al., 2008). Also during the global outbreak of the H1N1 swine influenza in 2009, up to 34% of the fatal cases were associated with secondary bacterial infection, predominantly caused by *S. pneumoniae* (Centers for Disease Control and Prevention, 2009). Secondary pneumococcal infections occurring during or after a viral infection are often associated with negative outcomes. A combined infection of influenza and pneumococci can be either a coinfection or a secondary bacterial infection following influenza. Clinically, it is difficult to distinguish between a coinfection and a secondary pneumococcal infection and the term superinfection is commonly used for the incidence of a second infection superimposed on an earlier infection, often caused by a pathogen of different origin. In this review we mainly focus on secondary pneumococcal infection which is clinically more important and the unidirectional effects that the influenza virus has on pneumococcal disease are well-studied. However, research shows that the interaction is bidirectional and bacterial infection also affects the virus, which has been reviewed previously (Short et al., 2012).

In this review we summarize how a preceding influenza infection predisposes the host to secondary bacterial infection with pneumococci. We outline how virus-induced alterations of the pulmonary immune system promote a secondary bacterial infection with a focus on the two most common pathogens, influenza virus and pneumococci. We provide an overview of the recently emerging role of specific pneumococcal factors favoring secondary bacterial infection, and explain how bacterial sensing and adaptation in the virally modified environment contributes to disease severity. Finally, we summarize what is known about secondary bacterial infection in the current coronavirus disease 2019 (COVID-19) pandemic and highlight the need for development of new alternative therapies to prevent and treat viral-bacterial coinfections. The available data support the theory that influenza-induced modulation of host immune responses and the ability of pneumococci to sense and adapt to virus-modified environments drive the overwhelming severe lung infection.

THE CLINICAL SITUATION—COINFECTIONS IN CAP AND HAP

Worldwide, lower respiratory tract infections are major causes of morbidity and mortality and are frequently caused by coinfecting pathogens. Coinfections are increasingly recognized as an underlying etiology to community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP). Both the influenza virus and *Streptococcus pneumoniae* are among the most common causative agents of lower respiratory tract infections. The Global Burden of Disease study (GBD) 2017, estimated that lower respiratory tract infection caused by influenza accounted for 9,459 000 hospitalization and 145,000 deaths among all age groups with the highest mortality rate among adults older than 70 years (GBD 2017 Influenza Collaborators, 2019). In 2016, *Streptococcus pneumoniae* was identified as the leading cause of morbidity and mortality from lower respiratory infections globally, contributing to 1,189 937 deaths (GBD 2016 Lower Respiratory Infections Collaborators, 2018). The main age groups at risk are children younger than 5 years and adults older than 65 years. Improved molecular testing allows increased detection and thereby extends our epidemiologic understanding of coinfections. Treatment, however, is often limited or done as prevention without specific etiology. Identification of the etiologic agent promotes implications for infection prevention and control, and has important impacts for public health initiatives, such as encouragement for vaccination (Cawcutt and Kalil, 2017). Treatment of bacterial pneumonia relies on antibiotics and treatment of influenza infection on antivirals, and supportive care is often needed for hospitalized patients. Patients with CAP, showing symptoms of flu or are diagnosed with flu in the days or weeks before the onset of CAP, are often empirically treated with antibiotics and possibly antivirals. Such antibiotics target the most common pathogens causing the most severe secondary infections, like *S. pneumoniae* and *Staphylococcus aureus*, often as broad-spectrum antibiotics (Leekha et al., 2011). Antibiotic coverage for methicillin resistant *Staphylococcus aureus* can be initiated when patients have signs of necrotizing pneumonia, including rapid onset of acute respiratory distress or hemoptysis. However, the desired treatment needs to be tailored antibiotic treatment for specific bacterial pathogens isolated from blood or a high-quality sputum specimen (Chertow and Memoli, 2013). In 2019, WHO classified antibiotic resistance as one of the top ten threats to global health (World Health Organization, 2019). A recent study investigating the use of antibiotics in 76 countries over 15 years revealed that antimicrobial resistance is increasing worldwide (Klein et al., 2021), and a major driver of antibiotic resistance is overuse and misuse of antibiotics. The currently ongoing COVID-19 pandemic is linked to higher use of antibiotics which may lead to an increase of antibiotic resistance (Bengoechea and Bamford, 2020; Canton et al., 2020; Dona et al., 2020; Getahun et al., 2020; Murray, 2020). There is currently only few treatment options available for patients with viral infections who also get infected with multidrug resistant bacteria. This indicates the urgent need for developing new antimicrobial therapies to treat coinfections.

Influenza-Induced Alterations of the Pulmonary Host Response

Increased morbidity and mortality from infections with influenza virus are often linked to bacterial superinfection. The complications associated with viral-bacterial coinfections are a result of altered host responses due to the virus infection (**Figure 1**). The innate immune response has a key role in protecting us against viral infections. Unfortunately, aspects of this immune reaction are also responsible for increased morbidity and mortality. We currently experience this from SARS-CoV-2 where interactions between the virus and immune cells lead to dysregulated immune responses, ultimately accelerating disease progression and severity, especially in older individuals (Guan et al., 2020; Huang et al., 2020; Qin et al., 2020; Zhang et al., 2020)

The lung epithelium covered with mucus provides the first line of defense against microbes entering the respiratory tract by brushing pathogens upwards through the mucociliary escalator. Once a virus successfully breaks the secretory mucus barrier, it invades epithelial cells to replicate. Influenza virus replication in the respiratory epithelium alters mucus production and reduces ciliary beating, which results in lower mucociliary clearance of pneumococci *in vivo* (Pittet et al., 2010). In bronchial epithelial cells, the influenza virus reduces the secretion of Chitinase-3-like 1, a protein involved in anti-pneumococcal host response, and thereby promotes secondary pneumococcal infection (Dela Cruz et al., 2012; Karwelat et al., 2020). The influenza-induced epithelial damage exposes more attachment sites for bacteria, thus promoting invasion and severe disease (Plotkowski et al., 1986). Also, direct binding of influenza to pneumococci promotes adhesion to respiratory epithelial cells (Rowe et al., 2020). The influenza-induced tissue damage is greatest at around day 7 after infection which is the time where both humans and mice are most susceptible to secondary bacterial infection (Nugent and Pesanti, 1983). The 2009 H1N1 pandemic virus destroyed basal airway epithelial cells which affected lung repair mechanisms, thus explaining the high fatality rate of coinfections with this pandemic virus compared to the seasonal H1N1 virus (Kash et al., 2011). In addition to the direct effect on the airway

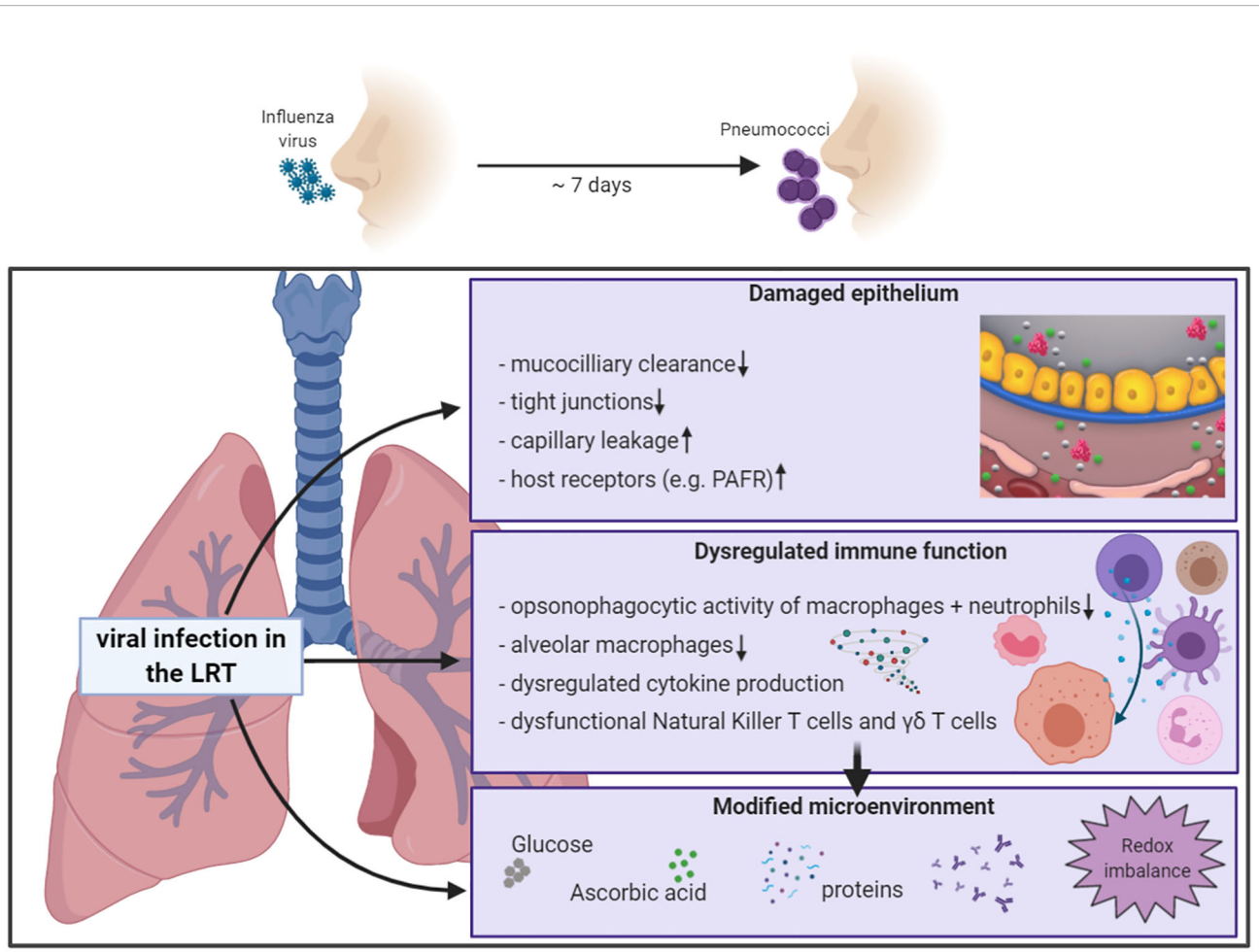

FIGURE 1 | Schematic overview of influenza-induced alterations of the pulmonary host response. Increased sensitivity to secondary bacterial infection is partly mediated by influenza-induced effects on the pulmonary host response, including compromised epithelial barrier functions, innate and adaptive immune responses, and changes of the microenvironment in the respiratory tract. Partly adopted from Sender et al., 2020. *Created with BioRender.com*.

epithelium, recruited inflammatory monocytes induce TRAIL-mediated lung damage, which facilitates pneumococcal invasion (Ellis et al., 2015). The activity of viral neuraminidases further promotes invasion by stripping sialic acids off the lung epithelium, which exposes adhesion receptors for pneumococci to bind (McCullers and Bartmess, 2003). Indeed, influenza A virus (IAV) infection increases the amount of the adhesion receptor platelet-activating factor (PAFR) (Van Der Sluijs et al., 2006b). However, neither mice deficient in PAFR nor PAFR antagonist treatment *in vivo* improved the outcome of secondary bacterial infections (McCullers and Rehg, 2002; Van der Sluijs et al., 2006b). Viral neuraminidase inhibitors only partially protect from bacterial complications following influenza virus infection (McCullers, 2004), and also, the use of neuraminidase treatment to inactive viruses does not affect the outcome of secondary bacterial infection in mice (Chockalingam et al., 2012). Thus, additional factors must play a role for the increased susceptibility to secondary pneumococcal infection, besides the influenza-mediated impact on the lung epithelium.

Phagocytic cells, including macrophages and neutrophils eliminate invading pathogens through opsonophagocytosis. We know that influenza virus infection suppresses the function of such phagocytic cells (Abramson et al., 1982; Debets-Ossenkopp et al., 1982; Astry and Jakab, 1984). Recent studies investigated how influenza virus infections affect the antibacterial activity of phagocytic cells in more detail. Sun & Metzger found that influenza-induced IFN-gamma impairs bacterial clearance by alveolar macrophages through downregulation of the class A scavenger receptor MARCO (Sun and Metzger, 2008). The scavenger receptor MARCO plays an important role in host defense against pneumococcal pneumonia (Arredouani et al., 2004). The antioxidant sulforaphane enhances MARCO expression and thereby improves pneumococcal clearance and host survival during secondary pneumococcal pneumonia (Wu et al., 2017). Similarly, IL-6 protects mice from secondary pneumococcal infection. Administration of recombinant IL-6 rescues macrophages from influenza-induced apoptosis and increases MARCO expression which promotes phagocytosis of bacteria (Gou et al., 2019). The functional impairment of alveolar macrophages allows noninvasive pneumococcal strains to cause deadly disease (Verma et al., 2020). In addition to functional departures, defects in antibacterial activity are also related to lower numbers of alveolar macrophages. The number of alveolar macrophages decreases to 85-90% compared with baseline levels, within 7 days after virus infection (Ghoneim et al., 2013; Smith et al., 2013). Also, dysfunctional neutrophils contribute to defects in antibacterial immunity during coinfection (Levine et al., 2001; McNamee and Harmsen, 2006). In coinfected lungs bacterial numbers remain high, despite the pro-inflammatory state with increased cytokines and more neutrophils (Levine et al., 2001). The reduced phagocytic activity of neutrophils is associated with higher expression of the inhibitory cytokine IL-10 (Van der Sluijs et al., 2004; Van der Sluijs et al., 2006a). Type I IFNs, which are essential for antiviral immunity during influenza infection (Muller et al., 1994), disrupt the migration of neutrophils, thus sensitizing the host for secondary bacterial infection (Shahangian et al., 2009; Nakamura et al., 2011). Considering the detrimental effects dysregulated cytokine production has on phagocytic cell function during influenza-pneumococcal coinfection, it is not surprising that also other key players of the cellular immune defense in the lungs are affected.

Dendritic cells bridge the innate to the adaptive immune response by producing cytokines and presenting antigens. Influenza-pneumococcal coinfection in dendritic cells synergistically upregulates pro-inflammatory cytokines, whereas anti-inflammatory cytokines, like IL-10, are downregulated by influenza, which might contribute to the immunopathology during coinfection (Wu et al., 2011). A study from our group showed that influenza-induced type I IFNs trigger the secretion of the pro-inflammatory cytokines IL-6 and IL-12 in dendritic cells (Kuri et al., 2013). However, co-infecting pathogens do not only affect the production and release of cytokine, but also modulate the expression and activation of pattern recognition receptors. W linked more IL-12p70 production during influenza infection to higher levels of Toll-like receptor (TLR) 3, which recognizes pneumococcal RNA, thus activating TRIF-dependent pro-inflammatory signaling in dendritic cells (Spelmink et al., 2016). Influenza and pneumococci also synergistically activate other Toll-like receptors (TLRs) and TLR-dependent signaling pathways, thus generating inflammation and promoting disease progression during coinfection (Karlstrom et al., 2011; Stegemann-Koniszewski et al., 2013; Rodriguez et al., 2019).

The preceding production of type I IFNs by the influenza virus also affects antibacterial T cell responses. An *in vivo* study in mice revealed that influenza-induced type I IFNs repress γδ T cell function and their production of IL-17 which is responsible for recruitment and activation of neutrophils. This is abrogated in mice lacking the IFN receptor and the adoptive transfer of γδ T cells from IFN receptor KO mice improves the pulmonary clearance of pneumococci in wild type mice (Li et al., 2012). This inhibitory effect of type I IFNs on IL-17 production by γδ T cells promotes secondary pneumococcal pneumonia by inhibiting neutrophil recruitment and thus bacterial clearance is mediated through type I IFN-dependent production of pulmonary IL-27 (Cao et al., 2014). T cell-derived IFN-gamma inhibits pneumococcal clearance by alveolar macrophages in influenza infected lungs (Sun and Metzger, 2008). Besides activation of antibacterial immunity, T cells also play a role in maintenance of tissue homeostasis and tissue repair and the tissue protective cytokine IL-22 limits secondary pneumococcal infection (Ivanov et al., 2013). CD8+ effector T cells produce the anti-inflammatory cytokine IL-10, thereby contributing to resolve lung inflammation during acute influenza infection (Sun et al., 2009). However, this regenerating response can lead to enhanced susceptibility to superinfecting bacterial pathogens. Indeed, the regeneration process creates a favorable environment for opportunistic pathogens like pneumococci, eventually resulting in pneumococcal superinfection.

The tight regulation of the pulmonary immune system that constantly balances pro- and anti-inflammatory signals to maintain immune homeostasis, can be disturbed by infections

and especially polymicrobial infections. While synergistic immune activation by influenza and pneumococci generally leads to hyper-inflammation and tissue damage as described above, subsequent infection with these two pathogens can also result in desensitization. Alveolar macrophages isolated after a resolved influenza infection respond poorly to TLR stimuli, which prevents the initiation of antibacterial responses and allows outgrowth of bacteria such as pneumococci *in vivo* (Didierlaurent et al., 2008). The increased susceptibility to a pneumococcal infection after a primary influenza infection can last up to six weeks. Similarly, peripheral blood mononuclear cells isolated from influenza-infected patients show selective defects in the production of TNFα and IFNγ after stimulation with heat-killed pneumococci (Giamarellos-Bourboulis et al., 2009). Although only partly understood, TLR desensitization and inability to recruit effector cells might be caused by higher numbers of alternatively activated macrophages that support tissue repair and immune homeostasis, but that also suppress immune responses (Chen et al., 2012). The anti-inflammatory state during tissue repair and restoration of lung immune homeostasis involves multiple immune-suppressive mechanisms (Snelgrove et al., 2008; Hussell and Cavanagh, 2009). One example is the increased expression of the CD200 receptor for the negative regulatory ligand CD200 on myeloid cells during viral infections which raises the activation threshold for these cells to superinfecting bacteria, allowing pneumococcal outgrowth (Goulding et al., 2011). The imbalanced pulmonary immune homeostasis greatly contributes to the pathology during influenza-pneumococcal coinfection.

In view of the two major effects influenza infections have on the pulmonary immune response, hyper-inflammation and desensitization, it is important to keep a balance between immune and inflammatory mechanisms to minimize the damage of the lung tissue, while also ensuring adequate defense to infections by other pathogens. However, in addition to the effects on clearance and immune homeostasis, influenza infection also changes the environment in the lower respiratory tract (LRT). Pathogen adaptation to these changed conditions determines if the bacteria can survive, grow and successfully establish disease.

Pneumococcal Growth and Adaptation in the Influenza-Infected Environment

To survive during influenza-infected conditions in the respiratory tract, bacteria must adapt to the environment by increased expression and/or function of virulence determinants (**Figure 2** and **Table 1**). The role of bacterial factors and their implications in driving secondary bacterial pneumonia is only emerging recently. Some strains of pneumococci are more successful in causing disease after pre-infection with influenza

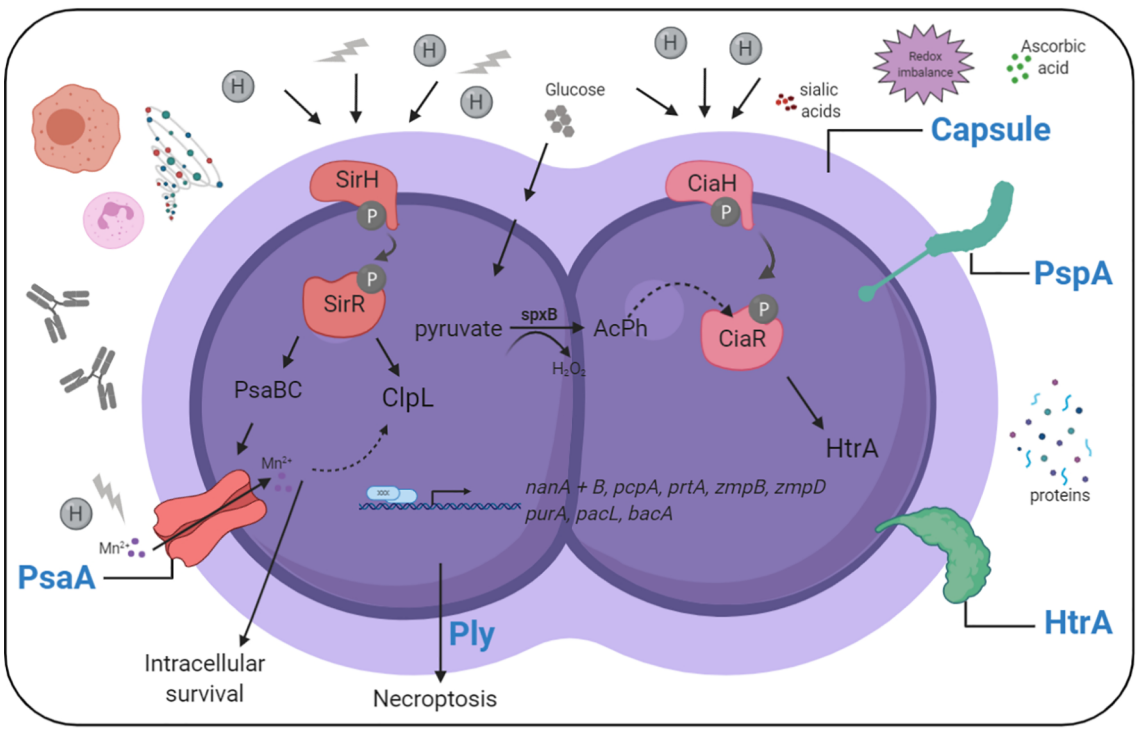

FIGURE 2 | Simplified overview of pneumococcal sensing and adaptation in the influenza-infected respiratory tract. Pneumococci need to adapt to nutritional and environmental changes in the influenza-infected respiratory tract to cause disease. Sensing and adaptation of pneumococci in the influenza-infected respiratory tract includes activation of two component systems, and the expression of effector proteins helping the bacteria to grow and resist stress in this environment. *Created with BioRender.com*.

TABLE 1 | Pneumococcal virulence determinants and their effects on influenza-pneumococcal coinfection.

Pneumococcal virulence determinants	Effect on influenza-pneumococcal coinfection
Sequence type / serotype	Affects *in vivo* transmission in ferrets (Mccullers et al., 2010) Impacts pneumococcal virulence in mice (Mccullers et al., 2010; Sharma-Chawla et al., 2016)
-Carbohydrate transport and metabolism (Glucose, mannose, galactolitol) -Bacteriocins -Virulence factors (e.g. choline-binding protein A (PcpA), IgA proteases *zmpB* and *zmpD*)	Increased transcription in pneumococci from influenza-dispersed biofilms *in vitro* (Pettigrew et al., 2014)
Sialic acid metabolism and transport (Sialidase NanA, Sialic acid transporter SatABC)	Higher *NanA* transcription in bacteria from influenza-dispersed biofilms (Pettigrew et al., 2014), higher bacterial loads in *in vivo* mouse models of colonization and otitis media, better adherence to epithelial cells *in vitro* (Wren et al., 2017) No effect of *nanA* deletion in a mouse pneumonia model (King et al., 2009) Enhanced bacterial load in presence of the main sialic acid transporter SatABC (+/- sialidases NanA and NanB) in a mouse pneumonia model (Siegel et al., 2014)
Pneumococcal surface protein A PspA	Increased virulence in a mouse pneumonia model (King and Harmsen, 2009), higher transcription in pneumococci isolated from influenza-dispersed biofilms (Pettigrew et al., 2014)
High temperature requirement A HtrA	Increased bacterial load in a mouse pneumonia model (Sender et al., 2020)
Pneumolysin Ply	Contributes to necroptosis and virulence in epithelial cells *in vitro* and in a mouse pneumonia model (Gonzalez-Juarbe et al., 2020) No effect on virulence *in vitro* and *in vivo* using CRISPRi-Seq (Liu et al., 2020)
-Adenylsuccinate synthetase PurA capsular operon -Calcium-transporting ATPase pacL bacA	Increased pneumococcal virulence in a mouse pneumonia model (Liu et al., 2020)
Two-component system SirRH and ClpL and PsaB	Higher pneumococcal survival in influenza-infected epithelial cells *in vitro* (Reinoso-Vizcaino et al., 2020)
Two-component system CiaRH	Increased bacterial load in a mouse model (Sender et al., 2020)

than others. We know from *in vivo* studies in mice and ferrets, that the potential of pneumococci to cause severe disease during influenza coinfection, and to spread within a population, depends to a major part on the capsular serotype (McCullers et al., 2010). The pulmonary immune response during coinfections is pneumococcal strain-specific, where more virulent pneumococcal strains are associated with more severe secondary pneumonia (Sharma-Chawla et al., 2016). However, noninvasive strains can also become more invasive and cause lethal disease in influenza-infected mice (Verma et al., 2020). This suggests that additional, serotype-independent factors, contribute to the potential of the bacteria to cause disease.

We are only beginning to understand the role of specific pneumococcal factors in secondary bacterial infection. Transcriptional changes of pneumococci dispersed from influenza-induced biofilms suggest that many factors and adaptive changes help pneumococci to survive and thrive in influenza-infected conditions (Pettigrew et al., 2014). The upregulated genes indicate that bacteria adapt to changes of nutrients and stress. High presences of the Neuraminidase A (NanA), NanB and PTS transporters for rapid uptake of carbohydrates, such as mannose/fructose, glucose, and galactitol, demonstrate a clear link to carbohydrate metabolism. Virulence factors that are increased in pneumococci recovered from influenza-dispersed biofilms include *nanB*, *pcpA*, *pspA*, *prtA*, and the IgA proteases *zmpB* and *zmpD* (Pettigrew et al., 2014). Pneumococci depend on carbohydrates as a carbon source (Buckwalter and King, 2012). Sialic acids are one such carbon source utilized by pneumococci (Marion et al., 2011). Intrinsic neuraminidase activity releases sialic acids which promote pneumococcal growth (Burnaugh et al., 2008) and serve as a signal that increases virulence (Trappetti et al., 2009). During coinfections, pneumococci feed on influenza-provided sialic acids which promotes colonization and development of pneumonia through aspiration (Siegel et al., 2014). However, the activity of viral neuraminidases is insufficient to fully compensate for the absence of NanA in pneumococci, suggesting an important role for NanA properties other than its enzymatic activity in pneumococcal pathogenesis (Wren et al., 2017). Interestingly, another study observed that NanA was dispensable for pneumococcal outgrowth during coinfection (King et al., 2009), which can be explained by the niche-specific expression patterns of NanA, with more NanA in the nasopharynx than in the lungs (Lemessurier et al., 2006). The overall data indicate that the specific conditions in the local environment determine which genes/proteins are induced to achieve an advantage for pneumococci in just that specific conditions at that time.

The LRT provides, especially during influenza infection, additional nutrients such as glucose, which leaks into the lungs from the blood (Sender et al., 2020). Glucose is the preferred carbon source for pneumococci and glucose-mediated catabolite repression further explains why sialic acid-dependent growth is more important in the nasopharynx. This study also shows that not only glucose, but also antioxidants derived from influenza-induced inflammation and capillary leakage allow pneumococcal

outgrowth in the LRT (Sender et al., 2020). We describe influenza-induced redox imbalances in the LRT to which pneumococci adapt by inducing the pneumococcal surface protease/chaperone high temperature requirement A (HtrA), that helps the bacteria to grow under oxidative stress condition *in vitro* and *in vivo*, and protects them from host-mediated opsonophagocytosis by maintaining capsular production (Sender et al., 2020). Hemoglobin, the iron-containing metalloprotein in erythrocytes also supports pneumococcal growth *in vitro* and enhances the ability of pneumococci to feed on host glycoproteins, providing an advantage during colonization and infection (Akhter et al., 2020), especially under influenza-infected conditions where host hemoproteins may be available in the lungs. These studies highlight the ability of pneumococci to adapt to nutritional changes and stressors during influenza infection and imply that complex bacterial adaptation to multiple site- and time-specific changes plays a key role for the development of severe pneumococcal infection.

In the LRT of influenza-infected mice, we found increased cytokines, more immune cells, more antimicrobial peptides, and high levels of plasma proteins (Sender et al., 2020), suggesting that pneumococcal clearance would be promoted in this location. However, the high numbers of proteins present in the lower airways in the highly oxidizing environment during influenza infection likely also induces membrane stress for pneumococci, which the protease HtrA can help to reduce by digesting denatured proteins (Cassone et al., 2012). Another study showed that membrane stress induced by the antimicrobial peptide LL-37 leads to cell surface accumulation of HtrA (Mucke et al., 2020). The major environmental changes pneumococci need to adapt to in influenza-infected conditions include nutritional changes and oxidative stress, which affect surface protein expression.

Influenza-induced oxidative stress also promotes necroptosis caused by the pneumococcal cytotoxin, pneumolysin (ply) (Gonzalez-Juarbe et al., 2020). Necroptosis is a form of regulated inflammatory cell death which can be induced by both influenza infection (Nogusa et al., 2016) and the pore-forming toxin, ply, of pneumococci (Gonzalez-Juarbe et al., 2015), resulting in release of molecules that enhance pro-inflammatory processes and viral clearance in the lungs, but can also disrupt immune homeostasis. The study of Gonzalez-Juarbe et al., 2020 investigated the role of necroptosis during influenza-pneumococcal coinfection and in a series of experiments they show that influenza-induced necroptosis can be inhibited by antioxidant treatment, resulting in reduced disease severity and less tissue damage during secondary pneumococcal infection (Gonzalez-Juarbe et al., 2020). A potential advantageous effect of antioxidant treatment for pneumococci themselves and pneumococcal growth, as observed in our study (Sender et al., 2020), was not investigated in this study. However, antioxidant treatment was performed at 12 and 24 hrs after bacterial infection, whereas our study focused on early bacterial growth between 4-6 hrs after pneumococcal infection. The question remains if antioxidant treatment during coinfection is beneficial or detrimental and it might depend on timing, delivery route and dose. The role of ply in coinfection is controversial. Whereas the previous study suggests that ply contributes to mortality during coinfection, another study did not find any difference in a coinfection of wt and ply-lacking pneumococci (Gonzalez-Juarbe et al., 2020; Liu et al., 2020). However, these discrepancies can be due to variations in the coinfection model (such as C57BL/6 vs BALB/c mice and day 5 vs day 7 after virus infection) and the pneumococcal strains used (TIGR4 vs D39). The latter study also identified *purA*, the capsule operon, pacL and *bacA*, as essential genes for pneumococcal growth during influenza infection when compared to *in vitro* growth in C+Y medium (Liu et al., 2020). The authors confirmed the importance of the capsule gene locus in an *in vivo* coinfection model. This supports the data from our study where pneumococci lacking HtrA were phagocytosed more due to lower capsule production, indicating that the capsule is indeed an important virulence factor during coinfection (Sender et al., 2020). This underlines the concept that rapid bacterial clearance is a major factor influencing the severity of coinfections, which can be disturbed by the influenza-mediated dysfunction of major phagocytic cells and by bacterial adaptation to inflammatory environments.

Another virulence factor that interferes with host-mediated bacterial killing and also contributes to bacterial outgrowth during secondary pneumococcal infection in mice, is the pneumococcal surface protein A (PspA). Immunization with PspA reduces the bacterial load in the lungs early during coinfection (King et al., 2009). This demonstrates that our constantly increasing knowledge regarding the role of specific pneumococcal proteins and a better understanding of how they contribute to severe secondary pneumonia, will help us to develop alternative treatment options.

Pneumococcal Sensing in the Influenza-Infected Environment

It is evident that pneumococci need to adapt to nutritional and environmental changes in the influenza-infected respiratory tract to cause disease. Transcriptomic analyses, combined with *in vivo* experiments using pneumococci with specific gene deletion, convincingly demonstrate the importance of certain genes/proteins for pneumococci during coinfection (**Figure 2** and **Table 1**). However, how exactly influenza-modified environments enable different bacterial factors to promote disease is a recently emerging field.

Pneumococci sense and respond to environmental changes with the help of two component systems (TCS), which consist of a membrane-bound histidine kinase (HK) that is autophosphorylated when sensing a signal, and transfers phosphate to a cytoplasmatic response regulator (RR), then acting as a transcriptional regulator (Stock et al., 1989). Pneumococci possess 13 TCSs and a single RR of which several are associated with virulence regulation (Throup et al., 2000). TCS1, also known as SirRH, senses influenza-induced acidic and oxidative stress, and controls pneumococcal adaptation *via* induction of *clpL* and *psaB*, which are required for intracellular survival of pneumococci (Reinoso-Vizcaino et al., 2020). In our study, TCS05, also known as CiaRH, induces *htrA* under influenza-

infected conditions which helps the bacteria to cope with oxidative stress on their cell surface and protects them from host-mediated killing (Sender et al., 2020). CiaR phosphorylation, and hence *htrA* induction, can also be accomplished by internal acetyl phosphate generated by SpxB oxidation of pyruvate (Pericone et al., 2003; Hentrich et al., 2016). Free sialic acids, which we found to be increased in influenza-infected conditions, are taken up by pneumococci and converted by the SpxB pyruvate oxidase to acetyl-phosphate and hydrogen peroxide, which allows transcriptional activation of the *htrA* promoter *via* phosphorylated CiaR (Hentrich et al., 2016).

In mice and most other mammals, the dominating sialic acid is N-glycolylneuraminic acid (Neu5Gc) whereas in humans, due to a mutation in the *CMAH* gene, N-acetylneuraminic acid (Neu5Ac) decorates the glycan chains (Chou et al., 1998; Muchmore et al., 1998). The pneumococcus has higher transcription of *htrA* and *nanA* and increased sialidase activity in response to human-like Neu5Ac as compared with Neu5Gc (Parker et al., 2012; Hentrich et al., 2016; McCombs and Kohler, 2016), suggesting a specific pneumococcal adaptation to the virally inflamed human LRT where the synergistic activity of viral and bacterial neuraminidases contributes to the pathology of viral-bacterial coinfection.

Similarities and Differences Between Influenza A Virus (IAV) and Other Respiratory Viruses With a Focus on SARS-CoV-2

Neuraminidase (NA) and hemagglutinin (HA) are the two major glycoproteins present on the surface of IAV, and they interact with the host sialic acids to invade cells and replicate (Gottschalk, 1958). The different forms of these surface glycoproteins determine the influenza virus subtype. To date, 16 HA (H1-16) and 9 NA (N1-9) subtypes have been identified in birds (McAuley et al., 2019). The subtype H1N1 and H3N2 are endemic in humans, circulating constantly within the population and cause seasonal outbreaks. Subtypes H5N1, H7N9 and H9N2 occasionally occur *via* zoonotic transmission from birds and swine, but additional mutations are required to allow for those viruses to transmit between humans (Cox and Subbarao, 2000; Harris et al., 2017).

The host tropism of the influenza virus is determined by the sialic acid species and its linkage to the underlying glycan. Most genomes of members of the deuterostomes contain a gene encoding for CMP-Neu5Ac hydroxylase (CMAH), the enzyme responsible for converting Neu5Ac to Neu5Gc (Peri et al., 2018). Deletions in CMAH have been described in humans (Chou et al., 1998), platypus (Schauer et al., 2009), ferrets (Ng et al., 2014), and New world monkeys (Springer et al., 2014), preventing the endogenous production of Neu5Gc, instead allowing decoration of glycans with Neu5Ac. All neuraminidases isolated from influenza viruses since year 1967 cleave both, α2,3- and α2,6-sialic acids (Baum and Paulson, 1990; Kobasa et al., 1999; Franca de Barros et al., 2003), with the neuraminidases of H1N1 and H3N2 cleaving α2,3-sialic acid more efficiently (Ng et al., 2014). While epithelial cells in the respiratory tract and intestine of birds, and tracheal cells of horses, mainly carry glycoconjugates having α2,3-linked sialic acids, the human trachea mainly contains cells carrying glycans with α2,6-linked sialic acids. Tracheal cells in the pigs express α2,6-linked and α2,3-linked sialic acids (Baum and Paulson, 1990; Ito et al., 1998). Accordingly, HA of avian and equine influenza viruses preferentially bind to α2,3-linked sialic acids, while HA of human influenza viruses has a higher affinity towards α2,6-linked sialic acids and swine influenza viruses bind both, α2,6-linked and α2,3-linked sialic acids (Krizanova and Rathova, 1969; Rogers and Paulson, 1983; Couceiro et al., 1993; Suzuki, 2005). Despite having different affinities towards the Sia-linkage to galactose, IAV is also influenced by the Sia species, although the specificity varies greatly among isolates.

Most IAV neuraminidases scavenge Neu5Ac and Neu5Gc from glycoconjugates, but have a lower efficiency for Neu5Gc (Xu et al., 1995; Broszeit et al., 2019; Barnard et al., 2020). Exceptions are NAs of H1N1 or viruses isolated between 1967 and 1969, which prefer Neu5Gc- over Neu5Ac-containing substrates (Kobasa et al., 1999; Ng et al., 2014). The interplay and balance in the specificities of the IAV hemagglutinin and neuraminidase is needed for successful viral infection (Guo et al., 2018; Broszeit et al., 2019). However, not only the sialic acid species and linkage determine IAV binding, but also modifications of other underlying carbohydrates of the glycan strand, like fucosylation, sulfation or phosphorylation of non-sialylated glycans affect IAV binding (Stevens et al., 2006; Byrd-Leotis et al., 2019). O-acetyl modification can inhibit HA binding and neuraminidase activity (Zimmer et al., 1994; Schauer, 2004; Barnard et al., 2020), but it is required for infection by other viruses like human coronaviruses OC43 and HKU1, as well as influenza C and D virus (Hulswit et al., 2019).

Interestingly, α2-6 sialylated glycans, expressed on the epithelial cells of the upper respiratory tract in humans attract seasonal influenza viruses, with inflammation limited to this location and usually milder disease. The highly pathogenic avian H5N1 influenza virus mainly binds to α2-3 sialylated glycans and primarily infects type 2 pneumocytes in the human lung, often leading to severe pneumonia (Shinya et al., 2006). Due to mutations in the HA, H5N1 viruses can bind both α2-3 and α2-6 sialylated glycans (Yamada et al., 2006), making it easier for the virus to spread from human to human. The H1N1 2009 virus is special as it acquired a D222G substitution in HA, detected in severe and fatal cases, which changes the receptor binding specificity from α2-6 to α2-3 sialylated glycans and allows the virus to infect ciliated bronchial cells, possibly increasing the severity of pneumonia (Liu et al., 2010; Mak et al., 2010).

Other viruses that predispose the host for secondary bacterial infections include respiratory syncytial virus (RSV), rhinovirus (RV), human coronavirus, parainfluenza virus and adenovirus (AV) (Falsey et al., 2013). Whereas parainfluenza virus also uses host sialic acids to attach to cells, syncytial virus, rhinovirus, parainfluenza virus and adenovirus utilize diverse attachment receptors (Moscona, 2005; Bochkov and Gern, 2016; Battles and McLellan, 2019; Stasiak and Stehle, 2020). The novel coronavirus SARS-CoV-2 binds the cellular receptor Angiotensin-converting

enzyme 2 (ACE2) to cause coronavirus disease 2019 (COVID-19) (Yan et al., 2020). SARS-CoV-2 may be better in causing lung infection due to its greater binding affinity for the ACE2 receptors, which are present on epithelial cells in the lower airways. ACE2 receptors are also expressed on endothelial cells, allowing the virus to cause thrombosis and other vascular effects that greatly contribute to morbidity in COVID-19 patients (Ackermann et al., 2020; Cure and Cure, 2020; Sardu et al., 2020).

The mechanisms driving viral bacterial co-pathogenesis are diverse and complex, but often similar for the different viruses, including damage of the airways and dysregulated immune responses which, in turn, supports bacterial growth, adherence and invasion into normally sterile body sites. Similarities between influenza- and SARS-CoV-2-mediated host immune responses in severely sick patients that might favor bacterial coinfection include the damaged lung epithelium and the hyperactive immune response with increased levels of cytokines and pulmonary infiltration of immune cells (de Jong et al., 2006; Kash et al., 2006; Perrone et al., 2008; Huang et al., 2020; Lucas et al., 2020; Karki et al., 2021). Until now, we know little regarding potential bacterial, especially, pneumococcal coinfections, in COVID-19 patients. Frequencies of coinfections in COVID-19 patients range from 3.5% for confirmed community-onset bacterial infection (Vaughn et al., 2020) to 28% in severely ill patients from intensive care units (ICUs) (Contou et al., 2020; Feng et al., 2020; Zhou et al., 2020). In one study secondary bacterial infection, defined as a positive blood or LRT culture, occurred in 15% of all patients with 50% frequency in non-survivors compared to only 1% in survivors (Zhou et al., 2020). Another study, using throat swab samples and PCR, identified 24 different respiratory pathogens of which *S. pneumoniae* was the most common, followed by *Klebsiella pneumoniae* and *Haemophilus influenzae* (Zhu et al., 2020). A recent review summarized that only 1.3% of 522 patients in ICUs developed nosocomial superinfections with antimicrobial resistant bacteria, suggesting that COVID-19 overall associates less with bacterial infections, and the isolated bacterial pathogens differ from those causing lower respiratory tract infections during influenza pandemics, with *S. pneumoniae* isolated rarely (Fattorini et al., 2020). However, the methods and definitions used to identify bacterial coinfections are diverse, and the role of coinfection on clinical course and outcomes of COVID-19 has not been investigated yet. A recent summary demonstrates that about 16% of hospitalized COVID-19 patients develop secondary bacterial infection (Rawson et al., 2020) which requires antibiotic therapy. Another study analyzing the antibiotic use in patients with COVID-19 revealed that the prevalence of antibiotic prescribing was around 75% and it was higher with increasing patient age and with increasing proportion of patients requiring mechanical ventilation (Langford et al., 2021). In general, antimicrobial resistance is increasing worldwide (Klein et al., 2021) and a major driver is overuse and misuse of antibiotics. Thus, the ongoing pandemic of antimicrobial resistance may further increase, urging us to develop new strategies that help to prevent and treat viral-bacterial coinfection.

CURRENT TREATMENT APPROACHES FOR SECONDARY PNEUMOCOCCAL PNEUMONIA

In general, prevention may be easier than cure, and vaccines against both influenza and pneumococci can reduce the coinfection aspect. Vaccines against influenza have been shown to reduce both the viral infection and associated secondary pneumococcal infections in mice (Huber et al., 2010; Sun et al., 2011; Haynes et al., 2012; Mina et al., 2013). However, the strong adaptive immune response evoked by viral vaccination compromises innate antibacterial defenses similar to what is observed for the viral infection itself. Indeed, vaccination of mice with live attenuated influenza virus primes the upper respiratory tract for increased bacterial colonization and promotes pneumococcal transmigration to other body sites as seen following influenza virus infection (Mina et al., 2014; Mina et al., 2015), but it can prevent invasive bacterial disease (Sun et al., 2011). In humans, presence of virus is associated with increased pneumococcal carriage (Glennie et al., 2016), and the symptoms humans experience during live viral vaccination are linked to nasal colonization with pneumococci (Hales et al., 2020), suggesting that the immunological changes occurring as a result of host-microbial interactions in the upper respiratory tract might allow aspiration of the bacteria and thus promote infection in the lower airways. Studies in both humans and mice agree that initial contact with influenza (or live-attenuated vaccine) increases the susceptibility to *Streptococcus pneumoniae* infection (de Steenhuijsen Piters et al., 2019; Rylance et al., 2019).

Pneumococcal conjugate vaccines are successful in reducing the overall incidence of invasive pneumococcal disease (IPD) in vaccinated children (Klugman, 2001), and reduce severe influenza-pneumococcal coinfections of the LRT in vaccinated individuals (Madhi et al., 2004). However, vaccines against pneumococci have limited efficiency in other older age groups due to the emergence of non-vaccine type pneumococcal strains in IPD and carriage. Thus, their effectiveness in reducing co-infections between influenza and pneumococci might be limited, and in mice and humans pneumococcal conjugate vaccine have been shown to protect only about 50% of the vaccinated individuals against secondary pneumococcal infection (Madhi et al., 2004; Mina et al., 2013; Metzger et al., 2015). Even though vaccines might be useful to reduce secondary pneumococcal infections, we have to bear in mind that unintended consequences can appear. Additionally, vaccines are not available against other bacteria that commonly cause secondary bacterial infection and the influenza virus is not the only virus predisposing the host for secondary bacterial infection. Thus, these strategies may only influence parts of the problem.

Besides vaccination, antiviral agents that repress viral replication like neuraminidase inhibitors such as Zanamivir and Oseltamivir effectively inhibit disease progression and reduce influenza-related symptoms (Von Itzstein et al., 1993; Gubareva et al., 2000). However, this treatment does not reduce the viral load and has limited effects when administered later

during infection (McCullers and Bartmess, 2003; McCullers, 2004). Despite the limited antiviral effect when treatment is given later during the course of the infection, delayed therapy until up to 5 days post infection improves survival, but does not completely prevent mortality in a mouse model of secondary pneumococcal pneumonia (McCullers, 2004). The underlying mechanism is unclear, but can possibly be explained by an antiviral effect on increased viral loads post-bacterial infection as detected during secondary bacterial infection (Smith et al., 2013). Fludase, a recombinant sialidase that prevents viral entry into epithelial cells by cleaving sialic acids (Malakhov et al., 2006), was suggested to reduce the risk of secondary pneumococcal infection in mice (Hedlund et al., 2010). Surprisingly, Fludase treatment 3 days prior to bacterial infection did not alter bacterial numbers, despite the ability of pneumococci to feed on free sialic acids (Hedlund et al., 2010; Siegel et al., 2014). Also treatment with neutralizing influenza antibodies reduces the disease severity with lower viral and bacterial numbers and reduced lung injury in a mouse model of secondary pneumococcal infection (Van Someren Greve et al., 2018). However, in both studies the effects of repetitive treatment, and treatment at later time points and/or during bacterial infection remain to be determined.

Antimicrobial agents also reduce disease severity and occurrence of secondary bacterial pneumonia (McCullers, 2004; Karlstrom et al., 2009; Karlstrom et al., 2011; Ghoneim et al., 2013), but treatment may be insufficient in improving mortality (Karlstrom et al., 2009), and antibiotic resistant bacteria may further complicate the use of antibiotics. Phage therapies may provide a valuable alternative to antibiotics for treating secondary bacterial infections, but its efficacy in virus-infected patients must be evaluated in clinical studies (Manohar et al., 2020).

Immunomodulatory therapies, like treatment with IFNs or IFN antagonist, have been suggested earlier, but seem to induce more complex effects on the immune response than previously expected (Davidson et al., 2015; Metzger et al., 2015). The inflammation-induced leakage that does not only lead to acute respiratory distress syndrome, but also provides nutrient for bacteria to feed on (Sender et al., 2020), can be at least partly prevented by treatment with soluble ligands that reduce vascular permeability in the lungs and other organs and improve survival in animal models (London et al., 2010). Systemic administration of antioxidants as immunomodulatory therapy to neutralize virus-induced oxidative stress and increase macrophage activity improves survival in influenza-pneumococci coinfected mice (Wu et al., 2017; Gonzalez-Juarbe et al., 2020). However, antioxidant treatment may affect pneumococcal growth, as observed in our study (Sender et al., 2020), but whether route, dose and time of antioxidant administration may affect disease outcome differently remains to be determined. A recent study suggests a dual-functioning broad-spectrum virus- and host-targeting peptide against respiratory viruses, including influenza virus and SARS-CoV-2, as a promising candidate to prevent viral infection (Zhao et al., 2020). Even though an encouraging approach, where one compound combines two targets (virus and host), has been suggested, further studies are needed to elucidate its impact on secondary bacterial infection.

In general, more detailed knowledge is needed on how infection processes change over time and the interaction between involved pathogens and host factors in order to improve our ability to develop new therapeutic strategies and/or targets that effectively abrogate and/or cure secondary bacterial infection. A recently evolving research avenue is to target specific bacterial factors. In that regard, targeting pneumococcal surface protein A (PspA), a major surface protein of pneumococci and a promising vaccine target, might be an interesting approach to evoke protective antibody responses and promotion of bacterial clearance during secondary bacterial infection, (King et al., 2009; Kong et al., 2013; Greene et al., 2016), but the effect depends on the infectious pneumococcal dose (Roberts et al., 2019). The limited protection of currently available therapies, including their time-dependent efficacy and possible adverse effects, in addition to the growing problem of antibiotic resistance, underlines the need of new preventative and therapeutic strategies. Kinetic models can help us to determine the efficacy needed for successful treatment, identify potential immune effects, and show how the regulation of underlying mechanisms can be used to design new therapeutic strategies (Smith, 2017). An attractive alternative approach to improve treatment success or even prevent secondary bacterial infection could be to combine targeted antibacterial therapy with antiviral and/or immunomodulatory therapy. Conflicting results and the problem with extrapolating results from animal models to human therapy should be considered in the attempts to identify and implement novel more specific and effective treatments.

CONCLUDING REMARKS

Worldwide, LRTI and pneumonia is the leading cause of morbidity and mortality, accounting for more than 4 million deaths yearly (World Health Organization, 2017). A systematic analysis of the global burden of LRTI estimated that these diseases caused about 2.4 million deaths in 2016, of which almost 1.2 million deaths were attributed to the pneumococcus, the leading cause of both morbidity and mortality among LRTIs (GBD 2016 Lower Respiratory Infections Collaborators, 2018). One of the major risk factors for the development of severe pneumococcal disease is preceding viral infections, especially with influenza A virus. LRTIs linked to influenza caused about 145,000 deaths worldwide in 2017, according to an analysis from the Global Burden of Disease Study (GBD 2017 Influenza Collaborators, 2019). Fatality from influenza is often linked to secondary bacterial infections. The mechanisms driving virulent coinfections are complex and replete, including dysregulated lung physiology, with impaired mucociliary clearance, and modulation of host immune responses caused by the virus, which in turn promotes bacterial growth, adherence and invasion into normally sterile sites of the lungs. Recently evolving research focuses on the role of specific bacterial factors and investigates how pneumococci sense and adapt to virus-induced changes in the environment.

The currently ongoing COVID-19 pandemic has already caused more than 2.3 million deaths worldwide (World Health Organization, 2020) with numbers increasing. Our current knowledge regarding secondary bacterial infections in COVID-19 patients is still limited, but considering that both influenza and SARS-CoV-2 cause similar disease symptoms with a massive inflammatory immune response in the lower respiratory tract, ultimately leading to acute respiratory distress syndrome, a predisposition for bacterial superinfections is likely. The prophylactic use of antibiotics has increased due to the currently ongoing SARS-Cov-2 pandemic, enhancing the risk for increasing resistance to antibiotics. A better understanding of the mechanisms that promote bacterial superinfection, and more knowledge regarding the processes and factors bacteria use to successfully establish disease in virally infected environments, will help us to develop new therapeutic strategies and identify targets that effectively abrogate and/or cure secondary bacterial infections.

AUTHOR CONTRIBUTIONS

VS, KH, and BH-N all contributed to the design, analysis, and collection of data, as well as to write the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Fundings were provided by Knut and Alice Wallenberg foundation, the Swedish Research Council, and Stockholm County Council.

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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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frontiers in Cellular and Infection Microbiology

REVIEW
published: 23 March 2021
doi: 10.3389/fcimb.2021.613623

Media Matters, Examining Historical and Modern *Streptococcus pneumoniae* Growth Media and the Experiments They Affect

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While some bacteria can thrive for generations in minerals and salts, many require lavish nutrition and specific chemicals to survive to the point where they can be observed and researched. Although researchers once boiled and rendered animal flesh and bones to obtain a media that facilitated bacterial growth, we now have a plethora of formulations and manufacturers to provide dehydrated flavors of historical, modified, and modern media. The purpose of media has evolved from simple isolation to more measured study. However, in some instances, media formulated to aid the metabolic, nutritional, or physical properties of microbes may not be best suited for studying pathogen behavior or resilience as a function of host interactions. While there have been comparative studies on handfuls of these media in *Streptococcus pneumoniae*, this review focuses on describing both the historical and modern composition of common complex (Todd Hewitt and M17), semi-defined (Adams and Roe), and defined pneumococcal media (RPMI and Van de Rijn and Kessler), key components discovered/needed for cultivation/growth enhancement, and effects these different media have on bacterial phenotypes and experimental outcomes. While many researchers find the best conditions to grow and experiment on their bacteria of choice, the reasons for some researchers to use a specific medium is at best, not discussed, and at worst, arbitrary. As such, the goal of this review is to highlight the differences in pneumococcal media to encourage investigators to challenge their decisions on why they use a given medium, discuss the recipe, and explain their reasoning.

Keywords: ***Streptococcus pneumoniae*** **(pneumococcus), media, metabolism, growth, experimental rigor**

OPEN ACCESS

Edited by:
Victor Nizet,
University of California, San Diego,
United States

Reviewed by:
Aruni Wilson,
Loma Linda University, United States
Ramesh Vemulapalli,
Texas A&M University, United States

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *02 October 2020*
Accepted: *02 March 2021*
Published: *23 March 2021*

Citation:
Sanchez-Rosario Y and Johnson MDL (2021) Media Matters, Examining Historical and Modern Streptococcus pneumoniae Growth Media and the Experiments They Affect. Front. Cell. Infect. Microbiol. 11:613623. doi: 10.3389/fcimb.2021.613623

ISOLATION MEDIA FOR PNEUMOCOCCUS IN THE 19TH CENTURY

Streptococcus pneumoniae, or the pneumococcus, was fortuitously discovered on two continents simultaneously. In France, Louis Pasteur was studying the etiology of rabies, and in North America, George M. Sternberg was studying malaria. In North America, the future Surgeon General Sternberg inoculated two rabbits with his saliva to test whether an innocuous substance would produce an

immune response. To his amazement, both rabbits died from a cause of death distinct from malaria (Sternberg, 1881). Sternberg inoculated additional rabbits with his saliva as well as dogs, chickens, guinea pigs, and rats. This led to septicemia in the rabbits and guinea pigs, but not in the dogs or chickens. However, 1 ml of serum from a recently diseased rabbit put into a dog proved fatal (Sternberg, 1881). Three months later, Pasteur investigated a hospitalized child in the later stages of rabies to procure a saliva sample. When the sample was later inoculated into rabbits two days later, the rabbits died, but not from rabies. Upon microscopic examination, Pasteur observed an "8" shaped microorganism with a halo surrounding it and was only successful in obtaining cultures in veal broth (Pasteur, 1881).

Sternberg encouraged other investigators to test their saliva and later learned that saliva from some, but not all, led to death of the rabbits. This result created speculation about the nature of sepsis and whether it was attributed to putrefaction (decaying of organic matter) (Sternberg, 1881; Talamon et al., 1883). Other types of putrid inoculums, such as sewage, could produce sepsis. However, this, at the time, was mostly attributed to bacilli (Sternberg, 1881). Sternberg found that incubating saliva to the point of putrefaction (usually 24 h at 37° C) curtailed its lethality. Further, attempts to isolate the organism (which he would later call *Micrococcus pasteuri*) straight from saliva into artificial media proved impossible. It is unknown whether this was due to the nature of these media, or what we know now to be the bacterium's inherent autolytic ability. He tried cultivating the organism in urine, gelatin, and dog bouillon (a broth or extract made by stewing the subject in water) unsuccessfully; however, similarly to Pasteur, if the inoculum was amplified in rabbit's blood, then it would grow on rabbit flesh bouillon (Sternberg, 1881). A bacteriology manual from 1896 written by Sternberg explains bouillon preparation as ~500 g of chopped meat per liter of water, cooked for 30 minutes in a glass flask or enameled iron kettle, then filtered, neutralized, cooked again to coagulate and remove albuminoids, filtered, and finally sterilized (Sternberg, 1896). Others from the era utilize a different procedure; half a kg of chopped meat is soaked in 1 L of cold water for 24 hours, strained to the same volume, combined with 1% peptone w/w (partial enzymatic digestion or acid hydrolysis of yeast, animal, or plant tissues), and 0.5% NaCl w/w (Stedman, 1900). The mixture was then alkalinized by addition of sodium carbonate, heated to coagulate and precipitate albumins, filtered, and finally sterilized by autoclave.

In November 1883, French physician Charles Talamon was the first to publish the isolation of a pure culture of the pneumococcus (*Coccus lanceolatus*) (Talamon et al., 1883). He described taking his inoculum from the blood of rabbits and culturing it in "bouillon de Liebig," from Liebig's Extract of Meat Company which was famous in the late 1800s for making a variety of products from meat sources (Cansler, 2013). The preparation of bouillon de Liebig included peptone plus sodium chloride, in combination with meat (by record, most likely beef) infusion (the product of allowing the meat to stay in a solvent for a prolonged period of time), and then alkalizing by either adding $NaHCO_3$ directly or passing the medium through a carbonate column. The addition of the infusion or extract supplemented the peptone by adding minerals, phosphates, and vitamins. This medium differed from those prepared in labs by skipping the coagulation and removal of albumin from the meat infusion. However, it is now known that albumin can actually promote pneumococcal growth at low concentrations (Ruiz et al., 2011).

In 1892, William H. Welch published a research article describing the history of the pneumococcus (*Micrococcus lanceolatus*), as well as many characteristics of the organism (Welch, 1892). He described the pneumococcus' preference for alkaline media and its ability to grow on solid media such as nutrient agar, which is 5 g Liebig bouillon, 30 g peptone, 5 g cane sugar (sucrose), and 15 g agar, and "glycerine agar," which is nutrient agar + 5% glycerine/glycerol. In his communication, Welch mentioned his preference for a modification of Guarnieri's formulation which contains per liter 950 g of meat infusion, 5 g of NaCl, 10 g of peptone, 6-8 g agar, and 30-40 g of gelatin (a product of cooking collagen) with slightly alkaline pH (Welch, 1892).

SOURCES OF VARIABILITY IN MEDIA COMPONENTS

Variability in media was a major problem during earlier investigations on *S. pneumoniae*. Media were composed of a few undefined components and prepared by different methods. Different meat sources, qualities, and preparation (soaked vs. cooked) greatly affected the nutritional components of the media. As such, Welch and others had challenges in obtaining quality peptone capable of sustaining pneumococcal growth, even within the same brand (Fennel and Fisher, 1919). Peptones supply a source of amino acids and nitrogen for bacterial growth. However, because the raw input can be sourced from plants (soy), animals (milk, fetal sera) and microbes (yeast), their amino acid profiles are diverse. There are several methods used to process raw protein sources. For example, peptones derived from albumins are subjected to gastric and pancreatic digestion, acid hydrolysis, heating, and precipitation (Zimbro and Power, 2009). Thermal degradation of bulk protein can convert amino acids such as tryptophan to indoles or phenols (Kato et al., 1971). During acid hydrolysis, tryptophan can be completely lost, cystine can be partially broken down, and labile vitamins are mostly destroyed (Zimbro and Power, 2009). Enzymatic digest is gentler, but each proteolytic enzyme has a distinct cleavage pattern, which leads to the diverse array of molecular weight products. Finally, all of these changes take place *before* the media are autoclaved.

Another nuisance involving peptones is their ability to retain carbohydrate contaminants. In 1950, *The American Journal of Public Health* conducted a survey that evaluated 450 samples of peptones and 60 samples of bacteriological culture media for the presence of fermentable carbohydrates (Vera, 1950). The samples were tested against several bacteria including *Streptococcal* spp.

The study concluded that all peptone sample sources, and most meat sources were not suitable for bacterial fermentation tests as they still contained fermentable substances detected *via* acid or acid gas production. Recent documentation provided by different manufacturers also indicates the presence of carbohydrates in peptones from diverse origins (Klotz et al., 2017). This observation was significant, given that large segments of pneumococcal DNA are devoted to carbohydrate metabolism and transport (Bidossi et al., 2012). Additional work by Troxler et al. demonstrated that choice of carbon source had an impact on capsule expression and growth as sugars like fructose are not converted into capsule precursors thus limiting capsule production (Troxler et al., 2019).

Other points of media variability include vitamin and trace metal content. Not surprisingly, the accumulation of vitamins and trace metals vastly differs between animal and plant sources of peptones. For example, while biotin (vitamin B7), nicotinic acid (vitamin B3, nicotinic acid, or niacin is a precursor for nicotinamide adenine dinucleotide), and pantothenic acid (vitamin B5 and necessary for Coenzyme A production) were deemed important vitamins (Rane and Subbarow, 1940; Badger, 1944), Stokes et al. reported that the concentration of these three vitamins varied significantly between different nitrogenous sources (Stokes et al., 1944). This report describes a 60-fold difference in pantothenic acid concentration, 34-fold in nicotinic acid, and 17-fold on biotin among different nitrogen sources.

Trace metals are often important catalytic components for enzymatic reactions, and in doing so, affect gene expression, bacterial growth, and development. They can also be toxic in excess. As such, a bacterium's ability to maintain metal homeostasis is essential for survival. However, historically, there have been discrepancies in trace metal concentrations (Grant and Pramer, 1962; Nolan and Nolan, 1972). A modern analysis reports 2-fold differences in zinc and iron concentrations, and a 40-fold difference for copper between yeast extracts and casein or meat sources (Klotz et al., 2017). In high concentrations, copper has been shown to be toxic to pneumococcal growth by inhibiting nucleotide synthesis (Johnson et al., 2015). High zinc concentrations have also shown to be toxic to the pneumococcus by competing with manganese uptake (Eijkelkamp et al., 2014). Elevated exogenous iron has been implicated in enhancing pneumococcal biofilm formation and competence (Trappetti et al., 2011).

Perhaps not part of a specific medium perse, but the culturing environment is also important for growing bacteria. In the 1930's Gladstone et al. illustrated the importance that CO_2 can have in the cultivation of other bacteria (Gladstone et al., 1935). CO_2 greatly improves *S. pneumoniae* growth as specifically demonstrated in 1942 by Kempner and Schlayer (Kempner and Schlayer, 1942). Under ambient CO_2 concentrations (~300 ppm), *S. pneumoniae* grew in peptone, beef infusion, and glucose broth at higher pH (7.8 vs. 7.4). They postulated that adding CO_2 should lower the pH and thus, reduce pneumococcal growth, but that the opposite occurred. It is known that the pneumococcus possesses a highly conserved, β-class carbonic anhydrase (turning CO_2 and water into carbonic acid) that is necessary for growth in ambient air (Smith and Ferry, 2000; Burghout et al., 2010). As such, modern aerobic cultivation of pneumococcal species often requires, or is improved by providing an enriched 5% CO_2-atmosphere as the benefits of CO_2 outweighed the resultant media acidity.

Growth media development and use have come full circle. From local raw feedstocks, to gradually refined requirements for optimal cultivation, and back to undefined media for convenience's sake. However, while most manufacturing processes have been standardized to minimize variance across batches in modern media, they still rely on disparate ingredients for the cultivation of microorganisms. The road to discovering essential nutrients required for pneumococcal growth has been challenging given the fact that so many serotypes exist (100 different capsules define these serotypes) with some being more fastidious than others. While in some cases, the determination of specific ingredients tested a hypothesis, in other cases, it was trial and error to achieve a predetermined goal such as obtaining a pure culture. In the following paragraphs, we introduce some of these media (**Table 1**) and define them to the extent that historical records allow.

PNEUMOCOCCAL MEDIA

Todd Hewitt

Todd Hewitt broth with yeast extract is a popular liquid media used for the cultivation of *S. pneumoniae*. Its inception stems from studies by Todd and Hewitt on the metabolic functions of the pneumococcus (Todd and Hewitt, 1932). The earlier version of this broth constituted of a meat broth with added 2% proteose peptone (enzymatic digest of protein), diluted tenfold in a buffered salt medium, bubbled with CO_2 to reach a pH of 6.8, and lastly supplemented with glucose (**Table 2**). They later added to this medium by supplementing with metals and phosphate (Hewitt, 1932). Hewitt's experiments (discussed below) not only demonstrated the importance of phosphate addition to media as a buffering agent, but it was also significant for the determining metabolism of *Streptococci*. These studies culminated with the current version of Todd Hewitt broth, later streamlined by Updyke with Difco in the commercially available dehydrated version (Updyke et al., 1953) (**Table 3**).

The original Todd Hewitt formulation was used to describe lactic acid formation from glucose breakdown in cultures of hemolytic *Streptococci* sp. (Todd and Hewitt, 1932). Moreover, Todd and Hewitt found that when inorganic phosphate was added to their formulation, there was a significant increase in the percentage of glucose breakdown and this effect was concentration dependent. Experiments by Mason et al., 1981 expanded on the glucose-phosphate idea. They discovered a correlation between high phosphate concentrations inhibiting pyruvate kinase in starved *Streptococcus lactis* cells (Mason et al., 1981). Starved cells returned to homeostasis after glucose addition (Mason et al., 1981).

Currently, different manufacturers of Todd Hewitt broth further adjust their formulations, for example: BD Bacto and Hardy Diagnostics add 3.1 g/L of beef heart infusion, while

TABLE 1 | Media Quick Guide.

Medium	Defined or Complex	Short Summary of Findings	Citations
Todd Hewitt	Complex	Used to describe lactic acid formation from glucose breakdown; importance of inorganic phosphate	Todd and Hewitt, 1932 and Hewitt, 1932
Rane and Subarrow	Defined	Defined vitamins, choline, and amino acids necessary for pneumococcal growth of different serotypes	Rane and Subbarow, 1940
Adams and Roe	Semi-defined	Subculture of fastidious serotypes; asparagine crucial for sulfonamide-resistant *Streptococci*	Adams and Roe, 1945
Lacks and Hotchkiss	Semi-defined	Common base for semi-defined media such as C+Y, added catalase as a component	Lacks and Hotchkiss, 1960 and Lacks, 1966
Sicard	Defined	Growth of fastidious serotype (type-2 Avery's strain), vitamin solution used for RPMI to facilitate better growth	Sicard, 1964
M17	Complex	Cultivating lactic acid *Streptococci*, uses metal compatible disodium glycerol-2-phosphat e as buffer	Terzaghi and Sandine, 1975
Van de Rijn and Kessler	Defined	Designed for high yield and short lag to prevent adaptation, mostly for virulence factors, made originally for GAS	Van De Rijn and Kessler, 1980
RPMI 1640	Defined	Common medium for culturing host cells assuring common nutrient aviablity during examine host-pathogen interactions	Sicard, 1964; Brown et al., 2001 and Schulz et al., 2014

TABLE 2 | Original Todd Hewitt.

Todd Hewitt early 1932	Todd Hewitt late 1932
Meat broth 100 ml/L	Meat broth 100 ml/L
2% proteose peptone 0.2%	2% proteose peptone 20 g
$(NH_4)_2SO_4$ 5 g/L	$(NH_4)_2SO_4$ 5 g/L
$NaHCO_3$ 4 or 10 g/L	$NaHCO_3$ 8 or 10 g/L
$MgSO_4$ 0.01 g/L	$MgSO_4$ 0.01 g/L
Glucose 6 or 10 g/L	Glucose 5 or 10 g/L
KCl 1g/L	KCl 1g/L
	Sodium phosphate 0, 4, or 8 g/L

HiMedia adds 10.1 g/L, and Millipore includes 10 g/L (**Table 3**). Further, Hardy Diagnostics adds yeast in their powder while other formulations, it can be added separately. Additionally, the other peptone sources also differ by including enzymatic digest of milk protein or enzymatic digestion of animal tissues. Due to the known carbohydrates in peptone, and their effect on many cellular processes not limited to metabolism and capsule production, it is conceivable that these differences could affect the outcome of an experiment.

Rane and Subarrow

Rane and Subarrow (**Table 4**) devised a defined medium to determine the nutritional requirements of at least five different serotypes (Rane and Subbarow, 1940). Rane and Subbarow substituted the complex component gelatin with a defined mixture of amino acids and other additives in their basal medium I to make basal medium II. From this new formulation, they identified choline, pantothenic acid, nicotinic acid, and amino acids arginine, histidine, and glutamic acid as essential for growth (Rane and Subbarow, 1940).

Choline is an important structural component of *Streptococcal* teichoic acid in the cell wall based on cell fractionation experiments (Mosser and Tomasz, 1970). It was also determined that choline incorporation in the cell wall was necessary for the autolytic process, as cells treated with ethanolamine (EA), which can incorporate itself into the teichoic acid, were resistant to autolysis. Briles et al. in 1970 furthered these studies and demonstrated that cells initially grown with ^{3}H-labeled choline, which were later moved to EA media, resulted in cells that couldn't separate post division, and thus formed long chains instead of the usual diplococci phenotype (Tomasz, 1968; Briles and Tomasz, 1970). Additionally, it was demonstrated that cells grown without choline were less likely to uptake DNA during transformation citing inability to respond to competence factor (Tomasz, 1968; Tomasz et al., 1971).

TABLE 3 | Todd Hewitt.

BD Bacto	Millipore	Hardy Diagnostics	Himedia
Heart infusion 3.1 g/L	Beef heart infusion 10 g/L	Beef heart infusion 3.1 g/L	Beef heart infusion 10.1 g/L
Neopeptone 20 g/L	Peptic digest of animal tissue 20 g/L	Pancreatic digest of casein 10 g/L	Peptic digest of animal tissue 20 g/L
Dextrose 2 g/L	Dextrose 2 g/L	Dextrose 2 g/L	Dextrose 2 g/L
Disodium Phosphate 0.4 g/L	Disodium phosphate 0.4 g/L	Disodium phosphate 0.4 g/L	Disodium phosphate 0.4 g/L
Sodium Carbonate 2.5 g/L	Sodium Carbonate 2.5 g/L	Sodium Carbonate 2.5 g/L	Sodium Carbonate 2.5 g/L
		Yeast extract 10 g/L	
NaCl 2 g/L	NaCl 2 g/L	NaCl 2 g/L	NaCl 2 g/L

Hardy Diagnostics makes what is typically referred to as THY broth or Todd Hewitt plus yeast.

TABLE 4 | Rane and Subbarow, 1940.

Component	Concentration g/L
Basal Medium I	
Acid hydrolyzed Eastman de-ashed gelatin	6.0 g
D-Glutamic acid	0.1 g
L-Cystein	0.025 g
KH_2PO_4	5.0 g
Basal Medium II	
D-Glutamic acid	1.0 g
Glycine	0.25 g
L-Asparagine	0.2 g
D-L Leucine	0.15 g
D-Arginine carbonate	0.075 g
D-L Alanine	0.05 g
D-L Lysine dihydrochloride	0.05 g
D-L Methionine	0.05 g
L-Cysteine	0.05 g
D-L Histidine monohydrochloride	0.025 g
L-Tryptophan	0.025 g
β-Alanine	0.025 g
Nor-Leucine	0.015 g
L-Phenylalanine	0.01 g
L-Oxyproline	0.01 g
KH_2PO_4	5.0 g
NaCl	2.5 g
Distilled water	800 mL
Vitamins tested	Concentrations range tested
Pantothenic acid	0-5 μg/ml
Nicotinic acid	2-50 μg/ml
Choline	0-50 μg/ml
Thioglycollic acid	0-250 μg/ml
Flavin	0-1 μg/ml

Adams and Roe

Adams and Roe (**Table 5**) devised a medium to grow more fastidious pneumococcal serotypes as they were unable to subculture their isolates with the Rane and Subarrow medium and examine sulfonamide resistant bacteria (Adams and Roe, 1945). This medium is based on a 1942 recipe for growing hemolytic *Streptococci* (Bernheimer et al., 1942). It is a semi-defined medium that contains hydrolyzed casein as an undefined ingredient as meat infusion and peptones had sulfonamide inhibitors. While arginine and histidine were determined to be essential for pneumococcal growth, they were not added explicitly here. However, undefined components (e.g., casein digest) can contain as much as 4.8% arginine (Klotz et al., 2017) and 4.8% histidine (Becton-Dickinson, 2006). After this enzymatic hydrolysis, significant amounts of these amino acids are available in small and more bio-available oligopeptides. With their new medium, Adams and Roe were able to both subculture their strains and identify asparagine as crucial to sulfonamide-resistant *Streptococcus* (Adams and Roe, 1945).

Hoeprich would extensively modify this medium five years later: adding L-tyrosine increasing concentrations of iron, potassium, phosphate, glucose, glutamine, asparagine, uracil, adenine, choline, Vitamins B1-3, 5-7 (thiamine, riboflavin, nicotinic acid, pantothenate, pyridoxine, and biotin respectively) omitting copper and calcium; and decreasing the concentrations of zinc and manganese (Hoeprich, 1955). The addition of uracil to growth media was not pioneered by Hoeprich, as this ingredient appeared

TABLE 5 | Adams and Roe, 1945.

Component	Concentration g/L
Basal Medium I	
Acid hydrolysate of casein	200 ml of 10% solution
L-Cysteine	150 mg
L-Tryptophan	20 mg
KCl	3.0 g
$NaHPO_4.12H_2O$	7.5 g
$MgSO_4.7H_2O$	0.5 g
Distilled Water	900 ml
Vitamin Solution	
Component	Concentration mg/100 ml
Biotin	0.015 mg
Nicotinic acid	15 mg
Pyridoxine	15 mg
Calcium pantothenate	60 mg
Thiamine	15 mg
Riboflavin	7 mg
Adenine sulfate	150 mg
Uracil	150 mg
Solution II Salt Mixture	
Component	Concentration in 100 ml
$FeSO_4.7\ H_2O$	50 mg
$CuSO_4.5\ H_2O$	50 mg
$ZnSO_4.7\ H_2O$	50 mg
$MnCl_2.4\ H_2O$	20 mg
HCl concentrated	1 ml
Add Per Liter of Solution	
Vitamin mixture solution I	8 ml
Salt mixture solution II	2 ml
20% glucose solution	10 ml
Glutamine	200 mg
Asparagine	100 mg
Choline	10 mg
$CaCl_2.2\ H_2O$	10 mg
Distilled water	50 ml
Bicarbonate—thioglycollate mixture*	5%

**Prior to inoculation, add 1 mL of thioglycolic acid to 9 mL of sterile distilled water. Mix and incubate in boiling water bath for 10 minutes. Add 10 mL of sterile distilled water to 200 mg of autoclaved sodium bicarbonate. Add 200 μL of the thioglycolic acid mixture to the bicarbonate mixture.*

in earlier versions of Rane and Subarrow's medium and in other groups studying *Streptococci*. Nevertheless, many of the preceding media include uracil or uridine (glycosylated uracil) as an important component.

The physiological relevance of uracil was explored by Carvalho et al. in 2013 where they demonstrated that the lack of uracil increased the lag phase of D39 but had no effect on the R6 strain (and unencapsulated derivative of D39) (Carvalho et al., 2013). Ultimately, uracil was found to be involved in pneumococcal capsule production by 1) measuring less capsule production in uracil deficient media; 2) a spontaneous D39 capsule production mutant was largely nonresponsive in growth in the presence of uracil; and 3) the native D39 *cps* promoter was 25% lower without uracil present demonstrating effects at a transcriptional level (Carvalho et al., 2018).

Lacks and Hotchkiss

Another widely used, semi-defined media is Lacks and Hotchkiss (**Table 6**), also referred to as a modified Adams and Roe (1945).

TABLE 6 | Lacks and Hotchkiss, 1960.

Component	Amount Per Liter
Casein hydrolysate	5 g
Tryptophan	6 mg
Cysteine	35 mg
Sodium acetate	2.0 g
Salts	
K_2HPO_4	8.5 g
$MgCl_2.6\ H_2O$	0.5 g
$CaCl_2$	2.5 mg
$MnSO_4.4\ H_2O$	25 µg
$FeSO_4.7\ H_2O$	0.5 µg
$CuSO_4.5\ H_2O$	0.5 µg
$ZnSO_4.7\ H_2O$	0.5 µg
Vitamins	
Biotin	0.2 µg
Nicotinic acid	0.2 mg
Pyridoxine –HCl	0.2 mg
Thiamine-HCL	0.2 mg
Riboflavin	0.1 mg
Calcium pantothenate	0.6 mg
Other	
Glucose	2 g
4% Bovine Serum Albumin	12 ml
Fresh yeast extract	30 ml

Maltose medium substituted 2 g of maltose for glucose and yeast extract replaced by 10 mg/L glutamine and 5 mg/L adenine.

It has been the base for many modern media. This medium incorporated complex components such as yeast extract, casein hydrolysate, and BSA (Lacks and Hotchkiss, 1960). It also eliminated pure components that were deemed important to growth in previous work such as choline, arginine, histidine, and glutamic acid/glutamine. In 1966, Lacks media was revised to include: asparagine, glutamine, adenine, choline chloride, sodium bicarbonate and catalase (Lacks, 1966). *S. pneumoniae* is known to produce high levels of H_2O_2 but lacks catalase (Tettelin et al., 2001). In fact, the level of H_2O_2 that *S. pneumoniae* can make approaches low mM levels and can be detected in the breath of pneumococcal infected individuals (Majewska et al., 2004; Erttmann and Gekara, 2019).

Sicard

In 1964, Sicard developed a fully synthetic (defined) medium as the others "have proven inadequate for the culture of strains used in this laboratory" (**Table 7**) (Sicard, 1964). He was working with a type 2 rough strain or Avery's (Avery et al., 1944). While known at the time, Sicard's work solidified that different strains of the pneumococcus varied in the media that they could grow. Sicard used either linoleic acid, spermidine, or bovine serum albumin in this new medium (see **Table 7**). Unsaturated fatty acids (such as oleic, linoleic, and linolenic acids) have been reported to improve the growth of *S. anginosus* as well as increase the production of capsule polysaccharide under low CO_2 conditions (Matsumoto et al., 2019). Furthermore, Gullett et al. (2019) demonstrated that host polyunsaturated fatty acids are bound by a protein called FakB3 to enable the utilization of polyunsaturated fatty acids for incorporation into its membrane

TABLE 7 | Sicard, 1964.

Component	Amount Per Liter
Glucose	4.0 g
Pyruvate, Sodium	0.8 g
Uracil	1.0 mg
*Linoleic Acid	10^{-7} g
*Spermidine Phosphate	10^{-5} g
Minerals and buffer (pH = 7.55)	
NaCl	5.0 g
NH_4Cl	2.0 g
KCl	0.4 g
$MgSO_4$	0.024 g
$CaCl_2$	0.010 g
$FeSO_4\ 7\ H_2O$	0.0055 g
Tris- (hydroxymethyl) aminomethane	4.84 g
Na_2HPO_4	0.12 g
$FeSO_4\ 7H_2O$	0.55 mg
Riboflavin	0.3 mg
Vitamins	
Biotin	0.015 mg
Choline	5.0 mg
Nicotinamide	0.6 mg
Pantothenate, Calcium	2.4 mg
Pyridoxal HCl	0.6 mg
Riboflavin	0.1 mg
Thiamine	0.6 mg
Amino Acids	
L-Arginine	200 mg
L-Asparagine	10 mg
L-Cysteine-HCl	100 mg
L-Glutamine	20 mg
Glycine	120 mg
L-Histidine	150 mg
L-Isoleucine	6.55 mg
L-Leucine	6.55 mg
L-Lysine	420 mg
L-Methionine	180 mg
L-Threonine	175 mg
L-Valine	5.85 mg

**Can be replaced by 0.8 g of BSA fraction V.*

and therefore, minimize the metabolic expenditure of *de novo* membrane precursor synthesis (Gullett et al., 2019).

M17

M17 was developed in 1975 as a complex media for the improved cultivation of lactic acid bacteria particularly, lactic acid producing *Streptococci* sp. (Terzaghi and Sandine, 1975) (**Table 8**). M17 improved on its predecessor M16 with the addition of disodium glycerophosphate (GP). Phosphate was omitted in M16, relying on peptone and acetate for buffering capacity, as it would precipitate with calcium during phage assays. While having buffering capacity, GP can form a soluble complex with calcium. With this increased soluble interaction with metal, GP helped maintain the pH of the growth medium leading to improvement in cultivation as previously with some *Streptococci* sp., they had observed a pH below 6 after 24-hour growth at 30°C. Presently, different manufacturers offer a near-identical product through their websites. However, the Millipore Sigma formulation contains a combination of digested protein source (**Table 8**). Usually, but not in all cases, the product arrives

TABLE 8 | M17.

Original 1975	Difco™ M17	Himedia 1029	Millipore Sigma
Polypeptone 5.0 g	Pancreatic Digest of Casein 5.0 g	Peptic digest of animal tissue 2.5 g Casein enzymic hydrolysate 2.5 g	Tryptone, 2.5 g Meat peptone (peptic), 2.5 g
Phytone peptone 5.0 g	Soy Peptone 5.0 g	Papaic digest of soyabean meal 5.0 g	Soy peptone (papainic), 5.0 g
Yeast Extract 2.5 g	Yeast Extract 2.5 g	Yeast extract 2.5 g	Yeast Extract 2.5 g
Beef Extract 5.0 g	Beef Extract 5.0 g	Beef Extract 5.0 g	Meat Extract 5.0 g
Lactose 5.0 g	Lactose 5.0 g	Lactose 5.0 g	Lactose 5.0 g
Ascorbic Acid 0.5 g	Ascorbic Acid 0.5 g	Ascorbic Acid 0.5 g	Ascorbic Acid 0.5 g
Disodium glycerophosphate 19.0 g	Disodium-β-glycerophosphate 19.0 g	Disodium glycerophosphate 19.0 g	Sodium glycerophosphate, 19.0 g
1.0 M $MgSO_4.7H_2O$ 1 ml.	$MgSO_4$ 0.25 g	$MgSO_4$ 0.25 g	$MgSO_4$ 0.25 g

Recipe is per 950 mL before adding sterile lactose solution with the exception of Himedia in which the lactose is included in the dry powder.
Tryptone is tryptic digest of casein [Trypsin cleaves peptides on the C-terminal side of lysine and arginine amino acid residues].
Peptone is enzymatic digest of animal tissues [pepsin preferentially cleaves at Phe and Leu in position P1].

without a carbon source for the investigator to provide post sterilization. The suggestion is 14.6 mM (5 g/L) of lactose; however, other sugars have also been used in the literature most commonly, glucose.

Van de Rijn and Kessler

Van de Rijn and Kessler formulated a chemically defined medium (CDM) that would yield high growth, short lag phase, and most notably, accurate virulence factor production for Group A streptococci (GAS), after previous CDMs did not support their needs in these areas (**Table 9**) (Van De Rijn and Kessler, 1980). The main purpose of obtaining this faster lag phase was to eliminate the selection and adaptation of bacterial during transfer from complex media, mostly, in reference to making M protein, for which, they were successful. This medium was also successful in that it allowed for growth small inoculum to be used with a short lag time and the doubling time was comparable to other complex media (Todd Hewitt) with a higher buffering capacity. They empirically determined this medium's components which contained vitamins, minerals, trace metals (except zinc and copper), purines, sodium bicarbonate, and phosphates (**Table 9**).

RPMI 1640

RPMI 1640 is a completely defined, well-known cell culture formulated at Roswell Park Memorial Institute (**Table 10**). Growth in this medium was likely adopted due to it being defined and its popularity in culturing mammalian cells, in which both (presumably) host cells and bacteria would have the same nutritional availability. RPMI was used for the cultivation of the pneumococcus, with a few modifications such as the addition of 0.4% BSA, 1% vitamin solution from Sicard, and 2 mM glutamine (Sicard, 1964; Brown et al., 2001). In 2014, Schulz devised a number of supplements suitable for the cultivation of the pneumococcus in RPMI 1640 which included 30.5 mM glucose, 0.65 mM uracil, 0.27 mM adenine, 1.1 mM glycine, 0.24 mM choline chloride, 1.7 mM $NaH_2PO_4{\cdot}H_2O$, 3.8 mM Na_2HPO_4, and 27 mM $NaHCO_3$ (Schulz et al., 2014).

Host-Like or Niche-Specific Media

For early clinical microbiology, a rate-limiting step was consistent isolation and propagation. Understandably, early culture media were nutrient-rich to ensure replication of the inoculum for differential diagnosis or experimentation. We will later highlight that pneumococcal growth rates, gene transcription, protein expression, and virulence (including the capsule) are known to vary by media type and O_2 concentration. However, these rich media were not designed to reflect the abundance (or chemical form) of nutrients *in vivo*. A prime example is the choice of carbon source, or fermentable sugar, where glucose is often used. *S. pneumoniae* grows fast and vigorous in glucose, but rarely encounters this sugar except in the blood and cerebrospinal fluid (CSF) niches. The host-pathogen interface is a critical area of pneumococcal research and several studies have developed or used media intended to replicate conditions within the hosts, especially given the range of niches (e.g., nasopharynx and pulmonary epithelia, blood, CSF) in which *S. pneumoniae* persists.

It has been shown that *S. pneumoniae* grows equally fast in minimal media supplemented with porcine gastric mucin versus culture in BHI (Yesilkaya et al., 2008). Mucins are large proteins, extremely abundant in the inner walls of respiratory epithelia, and heavily glycosylated leading to molecular weights in mega-Dalton range. Despite a relatively small genome, *S. pneumoniae* encodes dozens of deglycosylases, proteases, and importers which enable the pathogen to turn an innate immune barrier (mucous) into a buffet (Bidossi et al., 2012; Buckwalter and King, 2012).

Most recently, van Beek et al. explored metal availability in the host nasopharynx by measuring the levels of free and protein bound metals in human nasal fluid of healthy individuals (van Beek et al., 2020). When comparing their CDM as defined by the Kloosterman et al. paper for "extending the molecular toolbox" of the pneumococcus against nasal fluid, it was found that nasal fluid contained higher levels of calcium (~10x), magnesium (~1.5x), copper (~10x), and iron (~12x) while having lower concentrations of manganese (>250x), cobalt (>15x), and zinc (~1.5x) (Kloosterman et al., 2006; van Beek et al., 2020). Of note, most of the iron, copper, and zinc in the nasal fluid were protein-bound and not free. To better mimic the host environment, van Beek et al. made an *in vivo* mimicking CDM, termed IVM-CDM, that more closely resembled the nasal fluid with no metal concentration differing by more than 20% (van Beek et al., 2020). This host-pathogen interface is of particular interest in pneumococcal colonization and by creating a chemical defined

TABLE 9 | Van De Rijn and Kessler, 1980.

Component	Amount Per Liter
Metals	
$FeSO_4.7H_2O$	5.0 mg
$Fe(NO_3)_2.9\ H_2O$	1.0 mg
K_2HPO_4	200 mg
KH_2PO_4	1000 mg
$MgSO_4.7H_2O$	700 mg
$MnSO_4$	5.0 mg
Amino Acids	
L-Alanine	100 mg
L-Arginine	100 mg
L-Aspartic acid	100 mg
L-Cysteine	50 mg
L-Glutamic acid	100 mg
L-Glutamine	200 mg
Glycine	100 mg
L-Histidine	100 mg
L-Isoleucine	100 mg
L-Leucine	100 mg
L-Lysine	100 mg
L-Methionine	100 mg
L-Phenylalanine	100 mg
L-Proline	100 mg
Hydroxy-L-Proline	100 mg
L-Serine	100 mg
L-Threonine	200 mg
L-Tryptophan	100 mg
L-Tyrosine	100 mg
L-Valine	100 mg
Vitamins	
p-Aminobenzoic acid	0.2 mg
Biotin	0.2 mg
Folic acid	0.8 mg
Niacinamide	1.0 mg
β-Nicotinamide adenine dinucleotide	2.5 mg
Pantothenate calcium salt	2.0 mg
Pyridoxal	1.0 mg
Pyridoxamine dihydrochloride	1.0 mg
Riboflavin	2.0 mg
Thiamine hydrochloride	1.0 mg
Vitamin B12	0.1 mg
Other	
Glucose	10,000 mg
Adenine	20 mg
Guanine hydrochloride	20 mg
Uracil	20 mg
$CaCl_2.6H_2O$	10 mg
$NaC_2H_3O_2.3H_2O$	4,500 mg
$NaHCO_3$	2,500 mg
L- Cysteine	500 mg
$NaH_2PO_4.H_2O$	3,195 mg
$Na_2HPO_4.2H_2O$	7350 mg

media containing similar concentrations of metals available in the nasopharynx, van Beek et al. was able to bring the field closer to a recipe that truly resembles the host niche.

Using the IVM-CDM, van Beek et al. also highlighted nine proteins that were surface exposed, conserved, most part of the core genome (8/9), and expressed in increased abundance during colonization as potential vaccine antigens. Five of these proteins induced antibody responses in mice—PsaA (Pneumococcal surface adhesin A), MetQ (D-methionine-binding lipoprotein), AdcAII (zinc-binding lipoprotein), PrtA (serine protease, and AliA (Oligopeptide-binding protein)—with AliA having the most protective effect as measured by reduced bacterial load (van Beek et al., 2020). As such, growing bacteria in media mimicking their native environment here and detailed in other protocols (Suárez and Texeira, 2019) is an effective method in the quest to make new treatments and vaccines.

Blood Agar Plates

One of the benefits of growing *S. pneumoniae* in blood agar plates is their distinctive colony morphology and alpha-hemolytic characteristic which quickly aids in its identification. However, even this simple method isn't standardized among investigators. Two commonly used bases for blood agar plates are tryptic soy agar or Columbia agar (**Tables 11** and **12**). These base media also differ in their composition, with Columbia agar having a wider source of amino acids as well as the addition of corn starch (Ellner and Stoessel, 1966), which enhances hemolytic reactions as well as marking differences in colony morphology between *Streptococcal* spp. The most used blood sources are defibrinated 3-10% (5% being most common) sheep or horse blood, but rabbit blood has also been used as an agar base. Mammalian blood is a poorly defined media supplement as it contains tens of thousands of protein isotypes in various states (e.g., phosphorylated, glycosylated, lipidated). This is especially true with lysed blood.

Early comparisons between the species' blood (with or without cell lysis) in culture media demonstrated that the sensitivity of bacteria to sulfonamide and diaminopyridine. Ferone et al. reasoned that this effect of lower effectiveness was due to an excess of a supplemental nutrient (Ferone et al., 1975). It was important to remove the source of inhibition—i.e., the active factor(s) in the blood—using solid Mueller-Hinton Broth (MHB) plates. To test their hypothesis, they supplemented this media with a downstream product, thymidine, which requires folate metabolism. By supplying this downstream metabolite, any drug targeting folate or nucleotide synthesis would have little effect. Ferone et al. found that horse and human blood contain thymidine phosphorylase (absent in sheep's blood), also known as Harper-Cawston factor (Harper and Cawston, 1945). This protein phosphorylates, then cleaves thymidine into thymine and 2-deoxyribose-1-phosphate. Naturally, some bacteria are less efficient using thymine over thymidine, which led to results that varied by the species of blood used. It is likely that abundant and resilient proteins (e.g. catalase) retain substantial activity—despite the heat shock of mixing with liquid agar—and interact with media nutrients and bacterial metabolites as small molecules diffuse through the agar matrix demonstrated in antimicrobial disk diffusion assays. However, little is known regarding the persistence and dynamics of blood protein biochemistry in agar or co-culture with pathogens.

Anaerobic Growth

Pneumococcus is habitually cultured as an aerobe with 5% CO_2 atmosphere, where it seems to thrive despite lacking catalase or cytochromes. However, it is a facultative anaerobe.

TABLE 10 | RPMI 1640 Thermo.

Components	mg/L
Amino Acids	
Glycine	10
L-Arginine	200
L-Asparagine	50
L-Aspartic acid	20
L-Cystine 2HCl	65
L-Glutamic Acid	20
L-Glutamine	300
L-Histidine	15
L-Hydroxyproline	20
L-Isoleucine	50
L-Leucine	50
L-Lysine hydrochloride	40
L-Methionine	15
L-Phenylalanine	15
L-Proline	20
L-Serine	30
L-Threonine	20
L-Tryptophan	5
L-Tyrosine disodium salt dihydrate	29
L-Valine	20
Vitamins	
Biotin	0.2
Choline chloride	3
D-Calcium pantothenate	0.25
Folic Acid	1
Niacinamide	1
Para-Aminobenzoic Acid	1
Pyridoxine hydrochloride	1
Riboflavin	0.2
Thiamine hydrochloride	1
Vitamin B12	0.005
i-Inositol	35
Inorganic Salts	
Calcium nitrate ($Ca(NO_3)_2.4H_2O$)	100
Magnesium Sulfate ($MgSO_4$) (anhyd.)	48.84
Potassium Chloride (KCl)	400
Sodium Bicarbonate ($NaHCO_3$)	2000
Sodium Chloride (NaCl)	6000
Sodium Phosphate dibasic (Na_2HPO_4)	800
Other Components	
D-Glucose (Dextrose)	2000
Glutathione (reduced)	1
Supplements from Schulz 2014	Final concentration (mM)
Glucose	30.5
Uracil	0.65
Adenine	0.27
Glycine	1.1
Choline chloride	0.24
NaH_2PO_4	1.7
Na_2HPO_4	3.8
$NaHCO_3$	27

TABLE 11 | Tryptic soy agar Criterion™.

Component	g/L
Pancreatic Digest of Casein	15.0 g
Peptic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g

TABLE 12 | Columbia agar Criterion™.

Component	g/L
Pancreatic Digest of Casein	12.0 g
Peptic Digest of Animal Tissue	5.0 g
Sodium Chloride	5.0 g
Yeast Extract	3.0 g
Beef Extract	3.0 g
Corn Starch	1.0 g
Agar	11.0 g

Many different methods have been used to produce hypoxic environments such as Gas-Pak® systems, anaerobic chambers, and oxygen scavengers. Anoxic growth has been previously reported to facilitate the isolation of clinical samples. Howden used an atmosphere of 90% hydrogen and 10% CO_2 to facilitate the isolation of clinical isolates (Howden, 1976). Four years later Wu et al. compared three methods for the recovery of *Streptococcus* from lower respiratory secretions and found that using a Gas-Pak® allowed them to recover 93% of their clinical specimens (Wu et al., 1980). The increased isolation was hypothesized to be due to a shortened lag phase during anaerobic growth and a reduction in autolysin production under anaerobic conditions (Howden, 1976; Nagaoka et al., 2019). However, conversely, Baesman and Strand (1984) compared isolation using aerobic and anaerobic methods (a Bio-Bag Environmental Chamber A anaerobic system), observing no significant difference between either (Baesman and Strand, 1984).

Despite the hundreds of isolates gathered across these three studies, none of them mentioned the serotype for any isolate. While the CDC published a widely used isolate culture method using broth enrichment (Todd Hewitt broth supplemented with 1 mL of rabbit serum), followed by real-time PCR of *lytA* (pneumococcal autolysin) for identification in 2010 (da Gloria Carvalho et al., 2010), Tothpal et al. performed a major survey of 256 pneumococcal strains under different experimental conditions including temperature and oxygen availability while including typing (Tothpal et al., 2019). For an anaerobic environment, they utilized the oxygen scavenger Oxyrase®. This study showed that there was significant variation between the serotype growth in anaerobic and aerobic environments, and variations thereof—such as serotype 4— which grew to higher optical density when cultured aerobically with catalase than anaerobically (Tothpal et al., 2019). In spite of ambient O_2, catalase use in the aerobic growth media supported higher maximum growth. Therefore, culture density for some serotypes is linked to the flux and concentration of H_2O_2 (in the presence of glucose), and was determined by capsule switching experiments, not linked to the capsular type alone.

While this is not an exhaustive list of all pneumococcus solid and liquid media, it covers most reported growth media.

Considering *S. pneumoniae* is genetically diverse with an estimate of 50% core genome at the species level, there is a strong possibility that variations in the nutritional value of these media can have an impact on bacterial physiology (Donati et al., 2010).

MEDIA MATTERS: EFFECTS OF MEDIA ON GENE EXPRESSION AND METABOLISM

There are many nuances to microbial culture media, especially those reported for growing *S. pneumoniae*. It is evident that these media can vary in amino acid composition, abundance of vitamins, metals, carbon source availability, and more. In the previous sections, we described how different media was developed to grow fastidious pneumococcal serotypes (to higher OD and with shorter lag time) and mimic the host environment. Below we present specific examples of how media can change capsule dynamics, gene expression, metabolism, protein profiles, and antibiotic susceptibility. Ultimately, studies rarely focus on comparing microbial behavior at the genetic, proteomic, and metabolomic level in different media, thus this is a limitation in finding examples.

Antibiotic Susceptibility

While the host makes itself non-hospitable to pathogenic bacteria, sometimes it needs assistance in the form of an antibiotic. Bacteria are quite adaptable in forming resistance to these drugs by mutating the target of the antimicrobial compound or by making efflux pumps to export them. It is important to have a standardized measure of bacterial susceptibly to a given antibiotic. Antimicrobial susceptibility testing (AST) is done *in vitro* using media like blood agar plates usually with the base of Mueller-Hinton Broth (MHB), a rich medium, to find minimum inhibitory concentration (MIC) (Clinical and Laboratory Standards Institute, 2012). For the pneumococcus, the standard AST procedure as defined by the Clinical and Laboratory Standards Institute (CLSI) uses cation adjusted MHB (calcium at 20 to 25 mg/L and magnesium at 10 to 12.5 mg/L) with 2.5-5% lysed horse blood (Clinical and Laboratory Standards Institute, 2012). AST is critical in tracking geographic and phenotypic changes over time in global antibiotic resistance. When developing candidate antibiotics, this same assay is used to establish the feasibility and capability of a hit compound. Given what we have briefly reviewed on media design and use, such a rich media may not be suitable and may be a limitation of these assays.

While several laboratories have investigated AST in other organisms (Rajput et al., 2019; Salazar et al., 2020), Ersoy et al. in 2017 looked at the pneumococcus as a bacterium of study (Ersoy et al., 2017). In comparing these effects of host-like media and other complex media on antibiotic resistance, the MICs of some drugs varied by at least a factor of 4 (Ersoy et al., 2017). Overall, Ersoy et al. found that the standard AST of MHB frequently failed to adequately identify antibiotic susceptibility *in vivo*. They also provide evidence that host-like media could give be more predictive of MIC *in vivo* (Ersoy et al., 2017). In one example, the folate-targeting drug trimethoprim was shown to be effective against most tested serotypes of *S. pneumoniae* using standard AST as defined by the CLSI, but ineffective in the host-like conditions Lacks and modified Lacks medium (Clinical and Laboratory Standards Institute, 2012). Following up, mice were infected with *S. pneumoniae* and treated with trimethoprim, which failed to protect any of the ten mice. In this case, and in many others that they tested, the standard MIC test did not accurately predict the drug's efficacy, but the host-like conditions did. These measurements extended themselves to different serotypes demonstrating that two serotypes (6 and 23) were more resistant to ampicillin and ceftriaxone with the D39 strain exhibiting less resistance to azithromycin and erythromycin. Ersoy et al. also examined the influence of $NaHCO_3$ concentration on resistance finding that as a trend adding physiological levels of sodium bicarbonate to MHB improved the *in vivo* prediction (Ersoy et al., 2017).

While conclusions regarding resistance and susceptibility differ from those obtained by CLSI methods, and thus must be looked at with scrutiny, it is nevertheless striking, but not surprising that host-like media give more reliable results than complex media. Especially in the search for new antibacterial mechanisms, standard AST media may obfuscate the effects of an otherwise promising drug. The MIC of any drug against a target organism not just a function of genome but can be strongly impacted by changes in the environment. It is further complicated by the dynamics of the other bacteria in the environment which could convey unexpected resistance (Sorg et al., 2016). The nutritional environment is a dynamic and strong factor in the laboratory, but especially at the host-pathogen interface.

Capsule

S. pneumoniae dedicates 30% of its membrane transport systems to carbohydrates as it is their only source of energy (Buckwalter and King, 2012). Therefore, it is not far-fetched to imagine that quantity and type of carbon source is linked to gene expression. An example of how carbon sources can impact gene expression is illustrated by Troxler et al. (2019). This work investigated capsule thickness using FITC dextran exclusion assay for serotype 6B and 7F under different carbon sources (glucose, fructose, and sucrose) on CDM, which here was modified Lacks medium with single carbon sources added. They found a significant decrease in capsule thickness when cultured on fructose as the only carbon source and an increase in capsule thickness when grown on glucose or sucrose as confirmed by transmission electron microscopy (TEM). The capsule was mostly generated by the glucose carbons while the fructose carbons were metabolized mostly for glycolysis. Additionally, they tested intracellular metabolites from all three carbon sources using ^{31}P labeled NMR and confirmed the accumulation of capsule precursors made from each carbon source in a capsule knockout model. Finally, gene expression was studied using RNA-seq. This

revealed changes in the peptidoglycan synthesis genes *glmS* and *nagA* as well as pyrimidine synthesis genes changing in a carbon source dependent manner, indicating an important role for carbohydrate sources in nutrient restricted media. This point is especially poignant in the host where these sources are limited and therefore affect capsule size. Capsule size has a negative correlation with binding to luminal mucus and positive correlation with spreading to other host surfaces (Nelson et al., 2007).

Hathaway et al. tested pneumococcal mutants that could switch their capsules between two different types to determine if serotype was an important factor for growth and carriage of pneumococcus (Hathaway et al., 2012). This paper found that making different capsules is energetically costly and as such, available nutrients affect colonization in terms of long-term vs invasiveness, and growth (Hathaway et al., 2012). Inadvertently, this study found that capsule thickness increased when the pneumococcus was cultivated in rich media such as brain heart infusion with fetal calf serum as compared to cultivation in minimal media (Hathaway et al., 2012). Taken together, these studies imply that the choice and components of media directly impact capsule production and therefore can influence measures of infection.

Co-infections can also affect how the pneumococcus obtains and processes carbohydrates and morphology. This is most notably manifested in its co-infections with influenza and *Haemophlius influenzae*. Viral influenza infections increase pneumococcal access to sialic acid in the nasopharynx (Siegel et al., 2014). Sialic acid, which in addition to its ability to be a sole carbon source can also be a major component in pneumococcal capsule (Charland et al., 1995; Marion et al., 2011). During long term co-culture (more than 10 days) with *H. influenzae*, as without *H. influenzae* there was no pneumococcal growth, glucose and lactose produced varying outcomes in culturability with the latter causing a morphology change (small colony variant) and reduce capsule size (Tikhomirova et al., 2018). Co-infection between these two pathogens can also lead to increased pneumococcal biofilm production, however, resulting in decreased infection (Weimer et al., 2011).

Transcriptomics and Proteomics

The availability of resources causes many binary changes leading to a cascade of effects at the genetic level. In an ambitious study, Aprianto et al. categorized the transcriptomes of the pneumococcus by mimicking various host environments and made a graphical user interface (https://veeninglab.com/pneumoexpress) that allows the end user to see the different expression profiles (Aprianto et al., 2018). To achieve the 22 conditions that mimicked various stages and types of infection, they varied sugar type and concentration, protein, CO_2, temperature, acidity, and presence of epithelial cells selecting Sicard's defined medium as the backbone of many of the conditions (Sicard, 1964; Aprianto et al., 2018). This study was notable due to the few comprehensive environment transcriptome databases that exist for model bacteria. The authors state that one limitation is that they did not vary metal ions which also play a large role in infection, however, above, we detailed the effects of metals in finding vaccine candidates (van Beek et al., 2020). Aprianto et al. found that there were 498 conditionally expressed genes based on the media conditions with ~10% of these genes involved in regulating carbohydrate import (many of which are under the control of CcpA (catabolite control protein A). Further, they found that growth in C+Y (not to be confused with CY media ATCC MD-2906) had a vastly different transcription profile than those related to infection or competence but was similar to other conditions like transmission indicating that the response was not completely tied to the media (Aprianto et al., 2018). Mining this data set will occur for years to come, likely finding more differences in how the pneumococcus behaves in various environments.

Another example of additives impacting phenotypes is documented by Ferrándiz et al. (2019). In this work, the authors were characterizing stress proteins in nutrient restricted conditions in semisynthetic AGCH (Adenine Glutamine Casein Hydrolysate) medium also known as modified Lacks media (Ottolenghi and Hotchkiss, 1962), in comparison to effects on nutrient rich conditions (Todd-Hewitt broth + yeast extract) (Ferrándiz et al., 2019). They found that when the general stress gene *gls24B* was knocked out, the mutant was able to grow only in the presence of BSA and growth was concentration dependent. While unable to determine what about the BSA was responsible for the phenotype, the effect was also observed using protease-treated BSA, but not with acid-digested BSA (Ferrándiz et al., 2019). Interestingly, BSA binds fatty acids which still need to be removed and fatty acid metabolism was downregulated in the mutant (Davis and Dubos, 1947; Chen, 1967).

In 2009, Pandya et al. used a combination of microarray and RT-PCR to study the transcriptional profile of a type 4 strain serially cultured on two broths (Pandya et al., 2009). As a control, mRNA from *S. pneumoniae* grown in Todd-Hewitt yeast extract broth was compared to the same strain cultivated 50-100 passages on blood agar plates (Pandya et al., 2009). They found that selection on blood agar increased the number of differentially expressed genes from 113 genes after 50 passages to 706 genes (or approximately 1/3 of the pneumococcus genome) after 100 passages. Of the genes most differentially expressed, roughly a third of the genes (160) were annotated as hypothetical, 60 genes of unknown function, and 93 virulence genes—including the capsule locus. Consistent with previous reports, genes associated with adhesion and colonization (e.g., neuraminidase and choline binding proteins) were downregulated during blood agar cultivation (Gosink et al., 2000; Brittan et al., 2012). While neuraminidase and choline binding proteins support bacterial colonization and dissemination respectively, neuraminidase cleaves sialic acids in the blood which recruits host complement (Attali et al., 2008; Syed et al., 2019).

These transcriptional changes highlight what occurs when the pneumococcus transitions to other environments in the host such as the blood. Bae et al. cultured D39 on Todd Hewitt agar ± 5% sheep blood and documented a more general shift in protein

expression by using 2-D gel electrophoresis and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) spectra (Bae et al., 2006). One major change they saw with added blood was with metabolism. Lactate oxidase (*lctO*), which catalyzes the oxidation of lactate and O_2 to pyruvate and H_2O_2, had lower expression (3.5 fold), L-lactate dehydrogenase (*ldh*), which reversibly converts pyruvate and NADH (nicotinamide adenine dinucleotide with hydrogen) to lactate and NAD^+, had higher expression (4.2 fold), and pyruvate oxidase (*spxB*), which converts pyruvate, phosphate and O_2 to acetyl phosphate and H_2O_2 was observed as a spot, but not increased (Bae et al., 2006). These changes mimic bacterial transition to the anaerobic conditions of the blood, an environment where *ldh* has been found to be essential (Gaspar et al., 2014). Both *spxB* (the major contributor of and making H_2O_2) and *lcto* are upregulated limited aeration conditions compared to anaerobic growth in BHI (Echlin et al., 2016; Lisher et al., 2017). Interestingly, it is H_2O_2 made by SpxB and LctO that causes α-hemolysis in blood (McDevitt et al., 2020).

Zinc import mutants grown on zinc restrictive media demonstrate morphological defects as revealed by bright field microscopy and cryo-electron microscopy (Bayle et al., 2011). These mutants were also unable to colonize a mouse model 48 hours after In or IP inoculation, indicating reduced virulence (Bayle et al., 2011). Carbon source can also play a role in how the pneumococcus maintains metal homeostasis. In fact, the addition of hemoglobin has been shown to stimulate pneumococcal usage of host carbohydrates the essentially helping it to adapt to the host environment (Akhter et al., 2020). Lack of iron also affects the pneumococcus, which produces no known siderophores and thus, must scavenge it *via* hemoglobin, heme, hemin, or take in free iron (uncommon in the host), by hindering virulence and causing dysbiosis in metabolism (Tai et al., 1993; Akhter et al., 2020). Lastly, Afzal et al. demonstrated in D39 that ascorbic acid as the carbon source in M17 led to decrease in intracellular zinc, manganese and iron (Afzal et al., 2015).

Finally, one of the most striking examples of the effects of media on bacterial physiology was illustrated by Hoyer et al. (2018). In this work, an unencapsulated variant of pneumococcus was grown on chemically defined media (RPMI 1640 & supplements) and complex media (Todd-Hewitt) under iron limiting conditions and control. Proteomic analysis was performed comparing gene expression between growth mediums. They found that 43.7% of pneumococcal proteins were uniquely expressed in one of the two mediums. This study highlights how varying a single nutrient impact nearly half of the organisms' proteins. They also noted that many proteins involved in pathogenesis were downregulated in nutrient-restricted conditions. Among the genes differentially expressed was the iron acquisition gene *piuA* (SPD_1652) which was highly expressed under CDM vs. rich medium. Morphological differences were also revealed using scanning electron microscopy. Denser DNA was noted by TEM under nutrient restricted conditions, suggesting a decrease in active transcription. Finally, a decrease in ribosomal proteins detected *via* LC–MS/MS under CDM conditions indicating a slower growth rate.

SUGGESTIONS AND CONCLUSIONS

Throughout this process, the authors encountered numerous reports of papers using "CDM" as their bacterial media. However, as we have seen throughout this process, not all CDMs are the same or created equal. Further, in many cases, primary sources of the media were not cited, but instead, had 3-4 degrees of separation. While we understand that stating "as described in x et al." can save on space, due to the variability in media, we strongly suggest listing the recipe for the CDM used in the paper in the supplemental text, especially if modifications were made (along with listing why modifications were made) or at the very least, citing the primary source paper containing the recipe in addition to the original method. Further, choice of media is often taken as given — based on training, tradition, or studies read and, in many cases, the reasons behind said choice of media are not given. As such, we encourage for the reasoning behind using a given medium be explained in the text.

As discussed, the media choice for *S. pneumoniae* dramatically impacts experimental outcomes. The host-pathogen interface is a critical area of pneumococcal research and several studies have developed or used media intended to replicate conditions within the host. Survival in the disparate niches (e.g., nasopharynx and pulmonary epithelia, blood, cerebrospinal fluid) in which *S. pneumoniae* persist—this is no simple task and a testament to this organism's adaptation. Based on varying media components, it seems possible that many experimental outcomes may have been and could still be accidentally influenced by decisions regarding media. As we illustrated here, the choice of media matters for far more than organism growth and propagation. We sincerely hope that in choosing pneumococcal and all other bacterial media, that the examples provided above will be considered to enhance future research studies.

AUTHOR CONTRIBUTIONS

YS-R and MJ conceptualized, wrote, and edited this manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

NIGMS 1R35128653.

ACKNOWLEDGMENTS

We thank Joe W. Alvin, Magdalene Y. So and Jorge E. Vidal for critically reading the manuscript.

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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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frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 31 March 2021
doi: 10.3389/fcimb.2021.651495

Exposure to Cigarette Smoke Enhances Pneumococcal Transmission Among Littermates in an Infant Mouse Model

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: 10 January 2021
Accepted: 10 March 2021
Published: 31 March 2021

Citation:
Murakami D, Kono M, Nanushaj D, Kaneko F, Zangari T, Muragaki Y, Weiser JN and Hotomi M (2021) Exposure to Cigarette Smoke Enhances Pneumococcal Transmission Among Littermates in an Infant Mouse Model. Front. Cell. Infect. Microbiol. 11:651495. doi: 10.3389/fcimb.2021.651495

Streptococcus pneumoniae, one of the most common commensal pathogens among children, is spread by close contact in daycare centers or within a family. Host innate immune responses and bacterial virulence factors promote pneumococcal transmission. However, investigations into the effects of environmental factors on transmission have been limited. Passive smoking, a great concern for children's health, has been reported to exacerbate pneumococcal diseases. Here, we describe the effect of cigarette smoke exposure on an infant mouse model of pneumococcal transmission. Our findings reveal that the effect of cigarette smoke exposure significantly promotes pneumococcal transmission by enhancing bacterial shedding from the colonized host and by increasing susceptibility to pneumococcal colonization in the new host, both of which are critical steps of transmission. Local inflammation, followed by mucosal changes (such as mucus hypersecretion and disruption of the mucosal barrier), are important underlying mechanisms for promotion of transmission by smoke exposure. These effects were attributable to the constituents of cigarette smoke rather than smoke itself. These findings provide the first experimental evidence of the impact of environmental factors on pneumococcal transmission and the mechanism of pathogenesis.

Keywords: ***Streptococcus pneumoniae*, transmission, cigarette smoke, children, mouse model**

INTRODUCTION

Streptococcus pneumoniae (*Sp.*; the pneumococcus) is one of the leading pathogens responsible for upper respiratory infections or invasive infections during childhood. When pneumococcus moves to body sites that are typically sterile it can cause various diseases (otitis media, rhinosinusitis, pneumonia, meningitis, and sepsis). *S. pneumoniae* can transmit from host to host by close contact with respiratory secretions, which often occurs among families or in daycare centers. Despite near-global use of pneumococcal vaccines, pneumococcal diseases remain among those of greatest medical concerns for children and the elderly. There is a need to investigate mechanisms involved in

transmission and to develop preventive strategies against pneumococcal colonization, the first step in disease.

Important steps for pneumococcal transmission are exit from a colonized host (shedding) and acquisition by a new host (colonization) (Weiser et al., 2018). Animal studies have revealed that the induction of inflammation in the nasal cavity of a *Sp.*-colonized host increases bacterial shedding, resulting in an increase in pneumococcal transmission. Co-infection with influenza A virus (IAV) dramatically increases transmission of the pneumococcus by increasing secretions (which carry *Sp.* to a new host) due to increased nasopharyngeal inflammation (Short et al., 2012; Richard et al., 2014). Host innate immunity factors such as TLR2 and TLR3 were shown to be involved in the mechanism of increased pneumococcal transmission in an IAV co-infection model (Richard et al., 2014; Kono et al., 2016). In the infant mouse model, pneumococcal mono-infection caused a subtle but acute inflammatory response which resulted in pneumococcal shedding and transmission among littermates (Zafar et al., 2016). Pneumolysin, a pivotal pneumococcal virulence factor, contributes not only to pneumococcal colonization density in infant mice but also promotes shedding and transmission due to local inflammation caused by tissue damage (Hotomi et al., 2016; Zafar et al., 2017).

Despite the progress of research on host immunity and pathogens, there is little understanding of the environmental factors that promote pneumococcal transmission. Smoking has long been one of the major health problems in the world: according to the World Health Organization, smoking causes more than 7 million deaths every year (World Health Organization, 2017). Passive smoking or second-hand smoking, defined as involuntary exposure to smoke formed from the burning of cigarettes and smoke exhaled by the smoker (Oberg et al., 2010), significantly elevates the risk for bacterial diseases among children, including invasive pneumococcal disease (Nuorti et al., 2000), middle ear disease, and lower respiratory tract infections (Cao et al., 2015). Some *in vivo* studies have shown that cigarette smoke exposure promotes nasopharyngeal colonization or invasive infection of pneumococci in an adult animal model (Voss et al., 2015; Shen et al., 2016), but the underlying mechanism is not fully understood. Additionally, there are no studies modeling the impact of passive smoking on pneumococcal colonization and transmission during early childhood. It is known that cigarette smoke causes local inflammation and injury of respiratory tissue accompanied by an influx of neutrophils and macrophages (Jaspers, 2014; Strzelak et al., 2018). Inflammation could promote pneumococcal growth and injury could inhibit clearance of the bacteria from the mucosal surface. Cigarette smoke extract (CSE) has been recently used as an alternative to actual cigarette smoking in the laboratory setting (Elliott et al., 2006; Mizutani et al., 2009; Wu et al., 2014; Ueha et al., 2016; Jia et al., 2019). CSE is made by bubbling cigarette smoke through saline, and is reported to have approximately equivalent effect to cigarette smoke on the subjects which received the CSE (Mizutani et al., 2009; Ueha et al., 2016). In the current study, we provided CSE by intranasal administration to model a passive smoking state among the infant mice, thereby also avoiding the confounding effects of maternal exposure to cigarette smoke. We hypothesized that passive smoking would increase pneumococcal colonization and transmission by causing nasopharyngeal inflammation and disruption of the mucosal barrier.

MATERIALS AND METHODS

Ethics Statement

This study was conducted according to the guidelines outlined by National Science Foundation Animal Welfare Requirements and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. This project was approved by the Institutional Animal Care and Use Committee at Wakayama Medical University (approved number: 882).

Bacterial Strain and Growth Conditions

P2431, a serotype 6A *S. pneumoniae* (*Sp.*) strain resistant to streptomycin was used in all experiments. It is already known that transmission ratio varies depending on serotype (Zafar et al., 2016). To investigate the promoting effect on transmission by CSE, we selected serotype 6A which is unlikely to transmit comparing to other serotypes. The bacteria were grown in tryptic soy (TS) broth (Becton Dickinson (BD), Franklin Lakes, NJ) to mid-exponential phase at 37°C. When the bacterial culture reached the desired optical density (OD) at 600 nm, bacteria were washed and diluted in sterile phosphate-buffered saline (PBS) for inoculation. Quantitative culture was performed by plating 10-fold serial dilutions in triplicate on TS agar plates containing streptomycin (200 μg/ml) and catalase (6,300 U/plate) (Worthington Biochemical Corporation, Lakewood, NJ). Plates were incubated overnight at 37°C + 5% CO_2. Bacterial stocks were stored in 20% glycerol at -80°C.

Mice

Pregnant female wildtype C57BL/6 mice were obtained from Charles River Laboratories Japan, INC (Yokohama, Japan). Mice were maintained in a conventional animal facility and pups remained with their dam for the course of the experiment. Pups inoculated intranasally with chemical or infectious agents were monitored throughout the duration of the experiments and appeared healthy and gained weight similar to untreated animals.

Cigarette Smoke Extract (CSE)

CSE was obtained from CMIC Pharma Science Co., Ltd (Yamanashi, Japan). Briefly, CSE is prepared by bubbling a stream of smoke from 50 of Hi-Light cigarettes (Japan Tobacco Inc., Tokyo) through 50ml of saline (Mizutani et al., 2009). For standardization, CSE was diluted 100-fold, and an OD at 267 nm was used as the indicator of concentration; the standardized concentration was adjusted to ~1.308. Undiluted CSE was used for all experiments similarly as previous reports (Mizutani et al., 2009; Ueha et al., 2016; Ueha et al., 2017). CSE was stored at -80°C until use.

Infant Mouse Model of Cigarette Smoke Exposure and Infection

From day 4-7 of life, pups were given CSE (3µl/mouse), intranasally, twice a day without anesthesia; pups in the control group received 3µl PBS. On day 8 of life, pups were inoculated intranasally with 8,000 CFU of *S. pneumoniae* suspended in 3µl of PBS, without anesthesia.

Evaluation of Nasal Colonization and Shedding

The pneumococcal nasal colonization levels were measured by enumerating colonies in a nasal wash or a nasal tissue. Pups were euthanized with isoflurane and the upper respiratory tract was lavaged with 200µl of sterile PBS from a 25G needle inserted in the trachea; lavages were collected from the nares. After washing nasal cavity, the nasal tissue was homogenized in 1ml of sterile PBS. The nasal lavages or homogenized tissues were serially diluted and plated on TS agar plates containing streptomycin (200 µg/ml) and catalase (6,300 U/plate) and incubated overnight at 37°C + 5% CO_2. The pneumococcal colonies were counted to determine the colonization in each mouse. The limit of detection was 666 CFU/ml.

To assess pneumococcal shedding, from day 1 to 4 p.i., daily nasal secretions were collected by gently tapping (10 times) the nares onto TS agar plates containing streptomycin (200 µg/ml) and catalase (6,300 U/plate) as reported previously (Richard et al., 2014; Kono et al., 2016; Zafar et al., 2016; Zafar et al., 2017). The sample was then evenly spread across the plate using a sterile swab and incubated overnight at 37°C + 5% CO_2 for quantitative culture.

Mouse Model of Pneumococcal Transmission

The transmission model was modified for the current study (Richard et al., 2014; Kono et al., 2016). First, all pups were treated intranasally with 3µl of CSE ("index-and-contact CSE" group) or 3µl of PBS ("index-and-contact PBS" group) from days 4-7 of life as described above. Then half of the pups in a litter were intranasally inoculated with *Sp.* (8,000 CFU) at day 8 of life ('index mice'). The pups were then returned to the dam and their uninfected littermates ('contact mice'). At day 4 p.i. (day 12 of life), all pups were euthanized by isoflurane and the nasal lavages were collected. A transmission event was defined as the presence of the pneumococcus in the nasal wash of contact mice.

In other transmission experiments, only index or only contact pups received CSE ("index-CSE" and "contact-CSE" groups). In index-CSE groups, index mice received intranasal CSE and the contact mice received PBS; in contact-CSE groups, contact mice received intranasal CSE and the index mice received PBS. The litters were infected with *Sp.* and transmission was assessed as described above.

Acquisition Model

After the pre-treatment with CSE or PBS on days 4-7 of life, pups were inoculated intranasally with a low dose of *S. pneumoniae* (~1,000 CFU) on day 8 of life. All pups were euthanized 7 or 24 hours after infection; nasal lavage was collected and pneumococcal colonization was determined as described as above.

Histological Analyses

Pups were pre-treated with CSE or PBS and infected with *Sp.* as described above. On day 2 p.i. (day 10 of life), all pups were euthanized. The heads were removed and fixed in 4% paraformaldehyde for 2 days, then decalcified by ethylenediaminetetraacetic acid (EDTA) for 2 weeks. After decalcification, the tissue was dehydrated and embedded into paraffin. Histological analysis was performed with coronal sections. Tissues were sectioned with a thickness of 3µm at the nasal cavity and stained with hematoxylin and eosin (HE). Tissue samples for immunohistochemistry (IHC) double staining of type 6A pneumococcal capsule polysaccharide and Alcian blue for mucus were sectioned with a thickness of 4µm. After the activation of antigen for 20 min at 95°C, samples were incubated with rabbit antisera against type 6A pneumococcal polysaccharide (Statens Serum Institut, Denmark, 1:5,000 diluted in Dako REAL Antibody Diluent (Dako, Santa Clara, CA)) for 30 min at room temperature, and then incubated with un-diluted anti-rabbit secondary antibody (Histofine Simple Stain MAX PO (R), Nichirei Bioscience Inc., Tokyo, Japan) for 30 min at room temperature. In order to develop the color, samples were incubated with diaminobenzidine tetrahydrochloride (DAB) for 5 min at room temperature. The sections were then stained with Alcian blue solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 30 min at room temperature and nuclei were stained by kernechtrot (MUTO PURE CHEMICALS CO., LTD., Tokyo, Japan) for 5 min at room temperature. Images were captured using KEYENCE BZ-X810 All-in-One Fluorescene Microscope (KEYENCE Japan, Osaka, Japan).

Flow Cytometry

Neutrophils in nasal lavages were stained as previously described (Kono et al., 2016; Zafar et al., 2017). Pups exposed to CSE or PBS were pre-treated and infected with 8,000 CFU *Sp.* as described above. Pups were euthanized on day 2 or 4 p.i. and nasal lavages (200 µl) were collected. Pellets of nasal lavages were resuspended with PBS + 1% bovine serum albumin. Cells were stained with a 1:150 dilution of the following antibodies: anti-CD11b-V450 (BD), anti-Ly6G-PerCP-Cy (BD), and anti-CD45-APC-Cy7 (BD) for 30 min on ice in the dark after FcR blocking with a 1:200 dilution of anti-CD16/32 (BioLegend) for 15 min on ice. Cells were then fixed with 4% paraformaldehyde until analysis on FACS Verse (BD). Neutrophils were detected as CD11b+, Ly-6G+, CD45+ events.

Immunoblot

After the pre-treatment of CSE or PBS, pups were intranasally inoculated with ~1,000 CFU and euthanized 24 hours post-infection. Nasal lavage samples (200µl) were 3-fold serially diluted and 300µl of each dilution was applied to a nitrocellulose membrane (Amersham Protran NC 0.2, Cytiva, Tokyo, Japan) with a slot-blot vacuum apparatus. The membranes were blocked with PBS containing 1% BSA, then

probed with 1:500 dilution of the biotin-labeled Maackia Amurensis Lectin II (MAL II) (Vector Laboratories, Burlingame, CA) and then probed with 1:100,000 dilution of streptavidin conjugated to horseradish peroxidase (Abcam plc, Cambridge, UK) for 1 hour each, at room temperature. Immunoreactive bands were visualized by chemiluminescence (ECL Prime Western Blotting Detection Reagent, Cytiva) and detected by imaging apparatus (LuminoGraph II, ATTO, Tokyo, Japan). The relative intensities of the bands on the immunoblot were quantified by measuring the integrated pixel density (IPD) using Adobe Photoshop (21.1.0 Release). For the purpose of analysis, the color of the blot was inverted so the background was black and the bands were white. An ellipse was drawn to encompass a band and the measurement was recorded. The same ellipse was used to measure all bands on the blot. A blank ellipse on the blot was measured to provide a background value that was subtracted from all other band IPD values.

Statistical Analyses

Mann-Whitney U test or Fisher's exact test was used for comparisons between two groups using GraphPad Prism 7 (GraphPad Software Inc., SanDiego, CA). Differences with P values of < 0.05 were considered statistically significant.

RESULTS

Cigarette Smoke Exposure Increases Pneumococcal Colonization in the Upper Respiratory Tract of Infant Mice

To determine how cigarette smoke exposure affects pneumococcal colonization of the nasopharynx, neonatal mice were daily inoculated intranasally with 3µl of CSE or 3µl of phosphate buffered saline (PBS) from day 4-7 of life and on day 8 of life challenged with 8,000 CFU of *Sp*. Type 6A; pneumococcal colonization in the nasopharynx was assessed on day 10 or 12 of life. The experimental schematic is shown in **Figure 1A**. The density of nasal colonization was significantly higher in the CSE than the PBS control group 4 days post-infection (p=0.0079), although there was no difference between the groups on day 2 p.i. (**Figure 1B**). We also examined pneumococcal colonization in nasal tissue, but there were not any significant differences between the CSE and PBS groups (data not shown). These results suggest that passive smoking increases colonization density or the number of bacteria attaching to the epithelial surface rather than contributing to an increased number of bacteria penetrating the nasal subepithelial tissue.

Cigarette Smoke Exposure Increases Pneumococcal Shedding and Neutrophil Influx Into the Nasal Cavity in Colonized Pups

As the number of bacteria shed by colonized pups is one of critical factors impacting transmission, the effect of cigarette smoke exposure on pneumococcal shedding was evaluated. After pre-treatment of with CSE or PBS on days 4-7 of life and infection with *Sp*. P2431 on day 8 of life, pneumococcal shedding was monitored daily from day 1-4 p.i. (**Figure 1A**). Shedding values were significantly higher on day 2 p.i. in CSE-treated pups compared to PBS-treated pups (**Figure 2A**, p=0.030). Comparing the total number of shed *Sp*. from days 1-4 p.i. shows a significant increase in the number of *Sp*. shed in the mice treated with CSE over those which received PBS (**Figure 2B**, p=0.0041). Based on the previous report showing that over 300 CFU/20 taps of shedding were required for effective pneumococcal transmission (Zafar et al., 2016), we evaluated the number of events from days 1-4 p.i. that shedding were over 150 CFU/10 taps and the ratio of positive events was significantly higher in CSE-treated group by Fisher's exact test (p=0.031). As previous reports have shown suggestions of a positive correlation

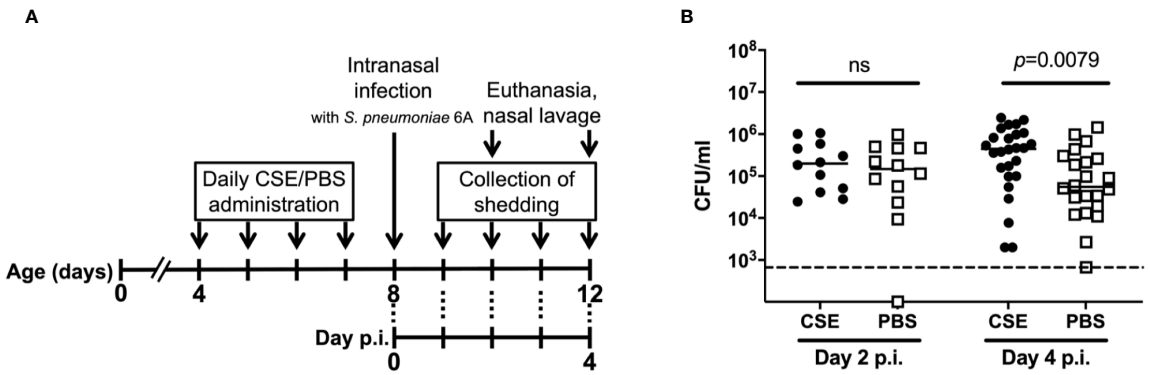

FIGURE 1 | General experimental schematic for pneumococcal shedding and nasal colonization with CSE treatment. **(A)** Schematic of the experimental schedule. From day 4-7 of life, pups were inoculated intranasally with either CSE or PBS twice a day. At day 8 of life, pups were inoculated intranasally with *S. pneumoniae* 6A. Pups were euthanized on day 2 or 4 p.i (day 10 or 12 of life) and the pneumococcal burden obtained from nasal lavages was evaluated. Unless otherwise noted, the experiments were performed in this schedule. **(B)** Nasopharyngeal pneumococcal colonization assessed by nasal lavages. Bar represents the median values and each symbol represents the CFU/ml from a single mouse. CSE group (black circle) (n = 12 in Day 2 p.i. and 26 in Day 4 p.i.) and PBS group (open square) (n = 12 in Day 2 p.i. and 22 in Day 4 p.i.). Dotted line indicates the limit of detection (666 CFU/ml). Mann-Whitney U test was used for the statistical analyses.

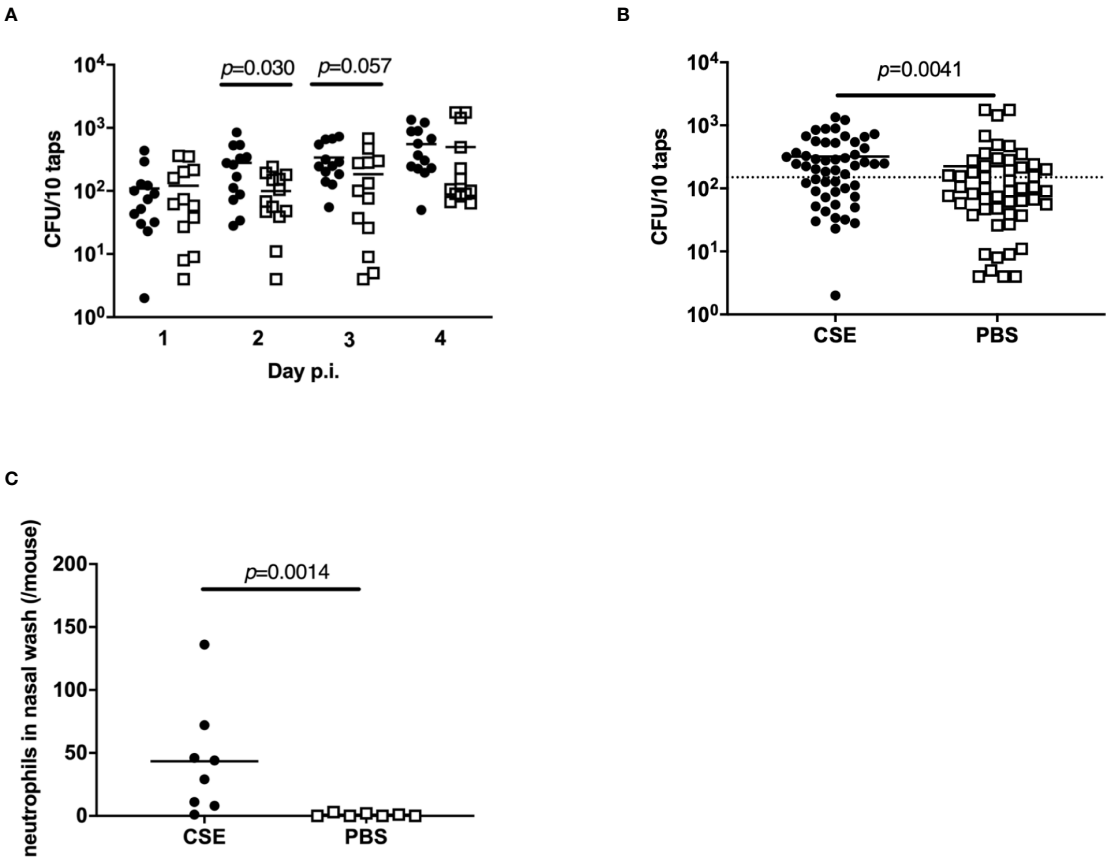

FIGURE 2 | Bacterial shedding in nasal secretions and local neutrophil influx into the nasal cavity. Quantification of daily **(A)** and total (all 4 days) **(B)** bacterial shedding. Shedding values of each day post-infection; median indicated by the bar and each symbol represents the CFU/10 taps from a single mouse on a single day (n = 13 in each group). Dotted line indicates 150 CFU/10 taps. **(C)** Neutrophils in the nasal lavages quantified by flow cytometry. CSE group (black circle) (n = 8) and PBS group (open square) (n = 7). Mann-Whitney U test was used for the statistical analyses.

between pneumococcal shedding and local neutrophil recruitment in the nasal cavity (Richard et al., 2014; Kono et al., 2016; Zafar et al., 2016), we performed flow cytometry with the nasal lavages obtained on day 2 p.i. from CSE or PBS pre-treated pups as this was the day when the bacterial shedding was significantly different between both groups (**Figure 2C**). There were significantly higher numbers of neutrophils in the nasal cavity of the CSE group on day 2 p.i. (p=0.0014). Together, these results suggest that passive smoking increases pneumococcal shedding early in infection by inducing increased inflammation in the URT mucosa and a higher density of colonizing bacteria.

Cigarette Smoke Exposure Promotes Host-to-Host Pneumococcal Transmission

To evaluate the impact of passive smoking on pneumococcal transmission, we compared transmission of *Sp*. among index and contact-CSE group (both index and contact pups were CSE-treated) to transmission within PBS-treated litters (control group). The density of *Sp*. colonization among contact pups in the index and contact-CSE group was significantly higher than the control group (p=0.021; **Figure 3A**) and the transmission rate among the CSE-treated group (55%) was also significantly higher compared to the PBS-treated litters (7%) by Fisher's exact test (p=0.021; **Table 1**). Next, to evaluate whether shedding or acquisition is the factor more influenced by cigarette smoke exposure, we assessed transmission within litters in which only the index pups received CSE (index-CSE group) compared to transmission in litters in which only the contact pups received CSE (contact-CSE group; **Figure 3B** and **Table 1**). There were no transmission events observed in the index-CSE litters, and in the contact-CSE groups the transmission rate was 25%; however, there were not any significant differences compared to the control group (in which both index and contact mice received PBS; **Table 1**). These results suggest that exposure of CSE in both the donor (index) and recipient (contact) pups increases rates of pneumococcal transmission.

Cigarette Smoke Exposure Enhances Susceptibility to Pneumococcal Acquisition

To investigate how passive smoking influences initial pneumococcal colonization, we examined upper respiratory tract

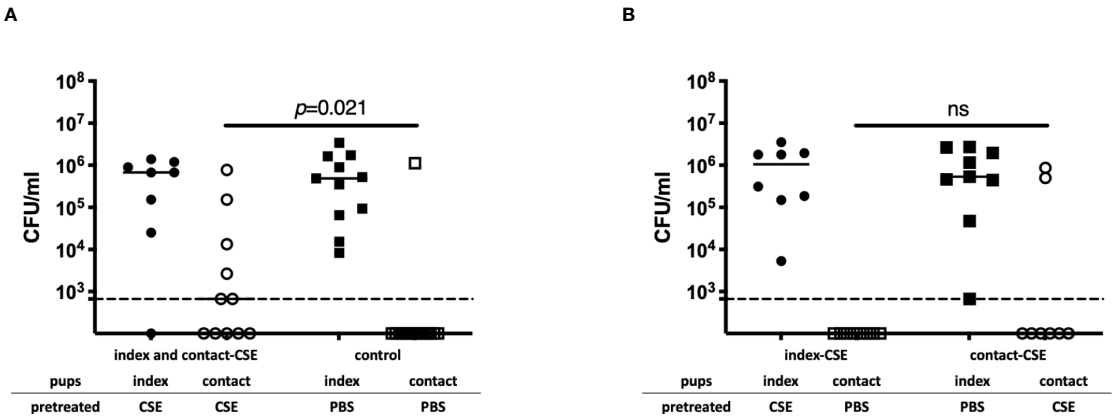

FIGURE 3 | Pneumococcal transmission with or without CSE. **(A)** Pneumococcal colonization of index and contact mice in the index and contact-CSE group (index and contact received CSE) (n = 14) and the control group (index and contact received PBS) (n = 11). **(B)** Pneumococcal colonization of index and contact mice of index-CSE group (n = 8) and the contact-CSE group (n = 8) transmission studies. Each symbol represents the colonization density in nasal lavages for individual pups (CFU/ml), with median values indicated by a bar. CSE group (circles), PBS group (square); index mice (closed symbols), contact mice (open symbols). Dotted line indicates the limit of detection (666 CFU/ml). Mann-Whitney U test was used for the statistical analyses.

TABLE 1 | Summary of pneumococcal transmission experiments. Transmission rates of each group were compared to control group by Fisher's exact test.

Group	Treatment of index	Treatment of contact	Number of contacts colonized/total	Transmission rate	Fisher's exact test compared with control group
control group	PBS	PBS	1/14	7.1%	–
Index and contact- CSE group	CSE	CSE	6/11	54.5%	p=0.021
Index-CSE group	CSE	PBS	0/8	0%	not significant
Contact-CSE group	PBS	CSE	2/8	25%	not significant

colonization density at early points after intranasal infection. For this experiment, we infected pups with a lower dose of *Sp*. to more closely model natural acquisition during transmission experiments. We chose a bacterial dose of >1,000 CFU as the total *Sp*. shed from a single colonized mouse in the CSE-treated group over 4 days was estimated at ~1,000 CFU (**Figure 2B**). In pups given 1000 CFU intranasally after CSE treatment, there were significantly more bacteria at 7 hours (p=0.0065) and 24 hours (p=0.029) post-infection (**Figures 4A, B**) compared to pups that were pre-treated with PBS. On the other hand, there could not find statistical difference in the bacterial density in the nasal cavity at day 4 post-infection (data not shown). This suggests that passive smoking enhances susceptibility to an initial step of colonization by pneumococcus in a new host.

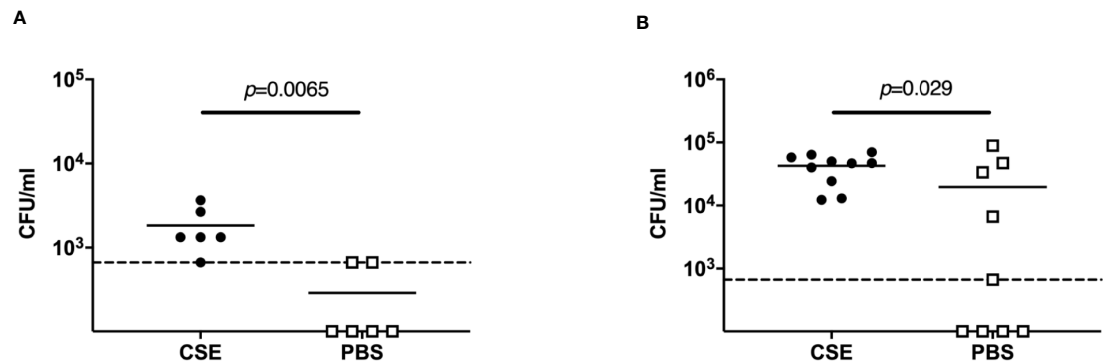

FIGURE 4 | Acquisition model. Nasopharyngeal pneumococcal colonization assessed by nasal lavages 7 hours **(A)** and 24 hours **(B)** post-infection with ~1,000 CFU *Sp*. 6A. Median values are indicated by a bar and each symbol represents the CFU/ml of a single mouse. CSE group (black circle) (n = 6 in 7 hours p.i. and 10 in 24 hours 4 p.i.) and PBS group (open square) (n = 6 in 7 hours p.i. and 9 in 24 hours 4 p.i.). Dotted line indicates the limit of detection (666 CFU/ml). Mann-Whitney U test was used for the statistical analyses.

Nasopharyngeal Inflammation by Cigarette Smoke Exposure Causes Disruption of the Mucosal Barrier and Increased Pneumococcal Attachment

At day 2 p.i., when the shedding of the CSE-treated group was significantly higher than the PBS group (**Figure 2A**), we performed a histological examination of the nasal tissue from CSE and PBS pre-treated pups. Representative images are shown in **Figure 5**. Following staining of nasopharyngeal tissue sections with hematoxylin and eosin (HE), we observed more damaged tissue and vascular leakage (hemorrhage and inflammatory cells) in the nasal cavity of the mice pre-treated with CSE than mice treated with PBS (**Figures 5A, B** compared to **5G, H**). In sections which were stained for the pneumococcal capsule by anti-polysaccharide-specific sera and for mucus by Alcian blue, we identified clusters of *Sp.* surrounded by mucus (**Figures 5E, F**) and disruption of the mucosal membrane, as well as adhesion of the pneumococcus to exposed submucosa in the CSE-treated pups (**Figures 5C, D**). In contrast, the disruption of the mucosal membrane and luminal bacteria were fewer in the PBS group, although the bacteria were still observed along the undisrupted surfaces of the nasal cavity (**Figures 5I–L**). These findings suggest CSE causes tissue damage in upper respiratory tract which appears to facilitate attachment of the pneumococcus to the host mucosal surface.

Cigarette Smoke Exposure Induces Mucus Secretion in the Nasopharynx

To assess the effect of CSE on mucus production in the upper respiratory tract, we quantified sialic acid levels in the nasal lavages by immunoblotting, probing with Maackia Amurensis Lectin II (MAL II), which binds α-2,3-linked sialic acids. We wanted to assess the relationship between CSE treatment and mucus secretion under "acquisition model" conditions (infecting with a low-dose of *Sp.*). After pre-treatment with CSE or PBS (**Figure 1A**), pups were infected with ~1,000 CFU *Sp.*; nasal lavages were obtained 24 hours p.i. The levels of sialic acid were significantly increased in the URT lavages obtained from the CSE-treated group compared to the PBS-treated group (**Figures 6A, B**), suggesting that mucus secretion is elevated by passive smoking in the setting of pneumococcal acquisition in the early phase of infection.

DISCUSSION

In this report, we demonstrate that cigarette smoke exposure promotes host-to-host transmission of *S. pneumoniae* in a neonatal mouse model by enhancing pneumococcal shedding from the colonized host and by increasing susceptibility to infection in a new host. Previous studies revealed that an

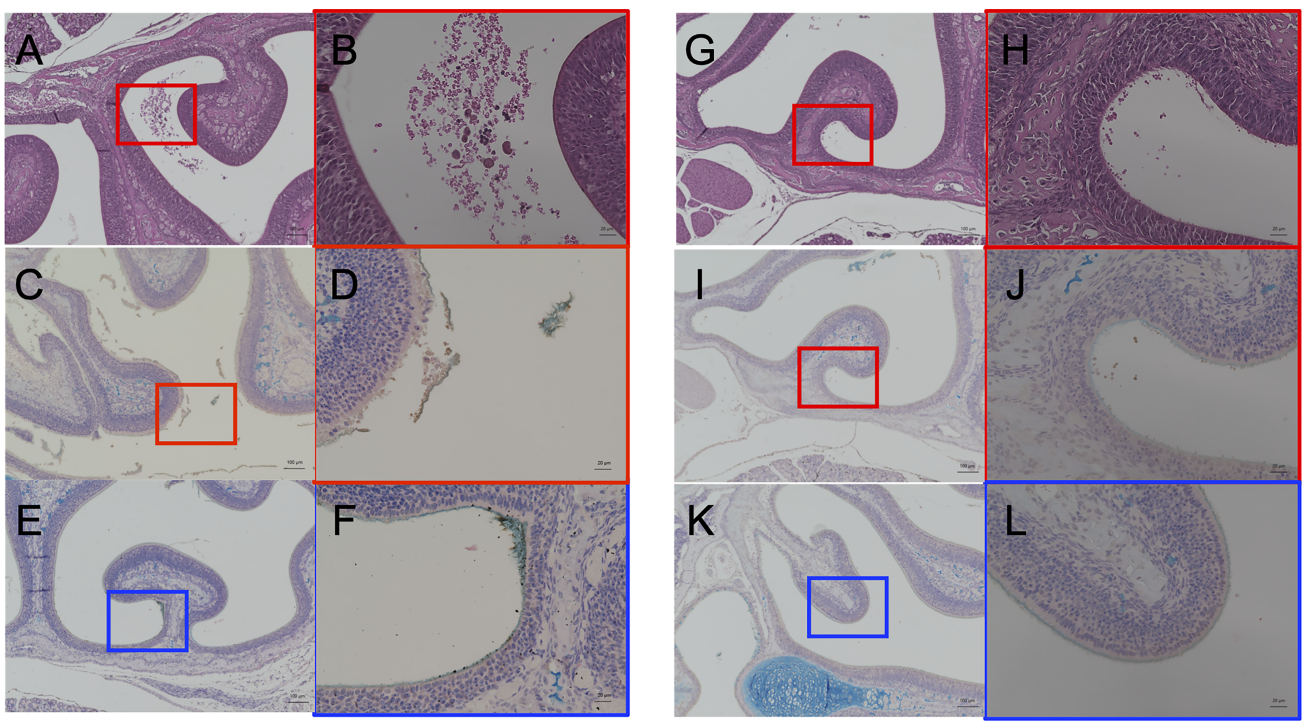

FIGURE 5 | Histological analyses of the nasal cavity of pups by hematoxylin and eosin staining **(A, B, G, H)** and immunohistochemistry double staining of type 6A pneumococcal capsule polysaccharide and Alcian blue for mucus **(C–F, I–L)**. CSE pre-treated group; **(A–F)**, PBS pre-treated group; **(G–L)**. **(A, C, E, G, I, K)** show low-magnification field (100×magnification. Scale bar, 100 μm) and **(B, D, F, H, J, L)** are high-magnification field of boxed sections (400×magnification. Scale bar, 20μm). Three mice for each group were examined for the analyses and the representative images were displayed.

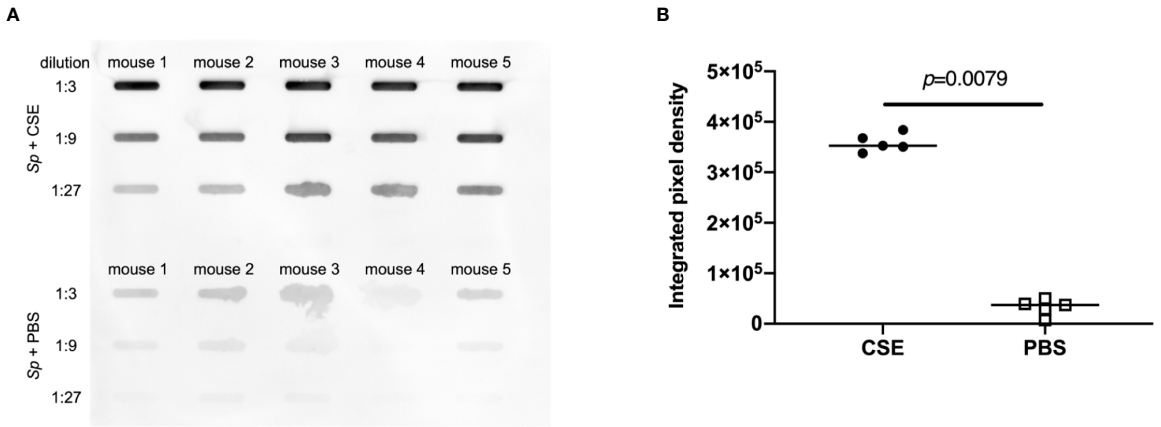

FIGURE 6 | Comparison of sialic acid secretion. Nasal lavages were analyzed by slot-blot immunoblot for α-2,3-linked sialic acids by MAL-II binding. **(A)** Each band represents MAL-II binding to lavages from individual mice. **(B)** The relative intensities of the bands on the immunoblot were quantified by measuring the integrated pixel density (IPD). Bar represents the median and each symbol represents IPD from an individual band. CSE group (black circle) and PBS group (open square) (n = 5 in each group). Mann-Whitney U test was used for the statistical analyses.

inflammatory environment in the upper respiratory tract, caused by co-infection with influenza A virus, was an important factor in promoting colonization and transmission of the pneumococcus (Short et al., 2012; Richard et al., 2014; Kono et al., 2016; Zafar et al., 2016). In particular, co-infection with influenza virus significantly increased bacterial load and neutrophil migration to the nasopharynx, which resulted in a significant increase in bacterial shedding compared to mice that were infected with *Sp*. alone. We have adapted this transmission model to evaluate the effect of cigarette smoke exposure on the donor and recipient by treating pups with CSE in lieu of co-infection with influenza virus.

In the transmission process, both shedding from a colonized host and acquisition in new host are critical factors. In the current study, CSE treatment affected both donor (index) and recipient (contact) pups: the effects of CSE treatment on the index pups include 1) the induction of inflammation in the nasal cavity, 2) increased pneumococcal burden, and 3) increased shedding. Differences were observed in colonization and bacterial shedding between CSE and PBS exposed group. Considering CSE exposure was required to both index and contact group to enhance transmission, this increase of shedding was an important factor for index side in this model. In the CSE-treated group, the increase in pneumococcal shedding positively correlated with the influx of neutrophils to the nasal cavity. Previous reports have shown that pneumococci aggregate with neutrophils that have migrated to the nasal cavity and are then secreted out the nose (Richard et al., 2014) which concurs with the results described in this study. Furthermore, examination of nasopharyngeal tissue sections revealed mucosal epithelial damage of the nasal cavity due to CSE treatment and *Sp*. infection, and, interestingly, we noted many pneumococci adhered to the shed mucosal epithelium. This finding may suggest that the shed mucosal epithelium plays the role of a vehicle that carries pneumococci out of the nose. The expression of mRNA of cytokines (IL-1α, IL-1β, IL-6 and TNF-α) in the nasal lavages were measured by qPCR to evaluate the level of inflammation. But the levels of mRNA expression were low in all pups suggesting limitations of the quantitative evaluation of cytokines in infant upper respiratory tract (data not shown). We showed a significant increase in sialic acid-containing mucus secretions in the nasal washes of the *Sp*.+CSE group compared to *Sp*.+PBS pups by slot blot assay. Together with the pathology findings, this suggests that increased nasal mucus is an important factor responsible for the increased shedding of pneumococci by CSE treatment. Cigarette smoke itself has been reported to enhance mucin (*Muc5AC* and *Muc5b*) expression in the respiratory tract and to promote mucus secretion (Shao et al., 2004; Ueha et al., 2017; Cao et al., 2018).

In previous reports, factors which affect the donor (index pups) and subsequently influence pneumococcal transmission have been well-examined, but the investigation on the recipient (contact pup) side of the process has been lacking. In one of the few studies which examined the role of acquisition, it was shown that when contact pups were immune to *Sp*., transmission was completely prevented, suggesting that immunity before exposure is critical for protection of a new host (Zangari et al., 2017). In this study we also examined the impact of CSE on recipient (contact) pups. CSE-treated pups showed increased *Sp*. colonization in the nasopharynx at early timepoints post-inoculation compared to PBS pups, suggesting that susceptibility to pathogens may be increased. As it is known to provide protection against microbial colonization, we examined the integrity of the mucosal barrier after CSE treatment in *Sp*.-infected mice. In these pups we found that the mucus and epithelial layers had sloughed off, and it appears this creates a favorable environment for the pneumococcus to attach on the mucosal surface and establish colonization. The reports that, in humans, cigarette smoking increases platelet-activating factor receptor (PAFR) expression and stimulates PAFR-dependent

adhesion of the pneumococcus to airway epithelial cells would also be an explanation contributing to the increased susceptibility of these pups (Cundell et al., 1995; Grigg et al., 2012; Shen et al., 2016).

The increased pneumococcal shedding and increased susceptibility to pneumococcal colonization of contact pups due to CSE treatment were both found to be required factors for increased pneumococcal transmission, as no significant increase in transmission was observed when CSE was administered to only the index or contacts pups. Passive smoking is a major public health problem for children, and this study has demonstrated that cigarette smoke exposure promotes pneumococcal colonization and transmission. It is considered that the prevention of passive smoking is effective for prevention of pneumococcal transmission for both *Sp*. carriers and non-carriers, and has high social significance.

The primary limitation of this study is that pups received cigarette smoke by liquid extract (CSE) to avoid the influences of inhalation of cigarette smoke on the dam. CSE is a product in which a mainstream of smoke is dissolved in saline. Although the differences of direct inhalation of cigarette smoke and topical administration of CSE are difficult to evaluate, published reports on the use of CSE inoculation into the respiratory tract of animal models conclude that the results did not conflict when compared to results due to direct inhalation of cigarette smoke (Elliott et al., 2006; Mizutani et al., 2009; Wu et al., 2014; Ueha et al., 2016; Jia et al., 2019). It is difficult to quantify passive smoking and the total exposure may not correlate with the number of cigarettes. When interpreted the current model of mice into human the dose of CSE, while its relatively a lot amount, would be reliable and our model reflects a situation close to the passive smoking in the real life by considering sidestream smoke contains much more chemical component and is thought to be more harmful than mainstream smoke (World Health Organization, 2010).

This is the first study which reports that cigarette smoke exposure promotes pneumococcal host-to-host transmission in a neonatal animal model. Experience from the use of conjugate pneumococcal vaccines has demonstrated that young children are a main source of transmission in the community. Our data suggest the avoidance of passive smoke exposure among children may decrease overall pneumococcal transmission, the first step of invasive pneumococcal disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at Wakayama Medical University.

AUTHOR CONTRIBUTIONS

MK, JNW and MH conceived and designed the experiments. MD, MK, DN and FK performed the experiments. MD, MK, TZ and YM analyzed the data. MD, MK and MH wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This project was supported by grants from Smoking Research Foundation (No. N101) to MK and GlaxoSmithKline Japan Research Grant 2018 (No. C-34) to DM. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

ACKNOWLEDGMENTS

We thank Keisuke Enomoto for the technical support.

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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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REVIEW
published: 28 April 2021
doi: 10.3389/fcimb.2021.639450

Mechanisms Underlying Pneumococcal Transmission and Factors Influencing Host-Pneumococcus Interaction: A Review

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *09 December 2020*
Accepted: *06 April 2021*
Published: *28 April 2021*

Citation:
Morimura A, Hamaguchi S, Akeda Y and Tomono K (2021) Mechanisms Underlying Pneumococcal Transmission and Factors Influencing Host-Pneumococcus Interaction: A Review. Front. Cell. Infect. Microbiol. 11:639450. doi: 10.3389/fcimb.2021.639450

Streptococcus pneumoniae (also called pneumococcus) is not only a commensal that frequently colonizes the human upper respiratory tract but also a pathogen that causes pneumonia, sepsis, and meningitis. The mechanism of pneumococcal infection has been extensively studied, but the process of transmission has not been fully elucidated because of the lack of tractable animal models. Novel animal models of transmission have enabled further progress in investigating pneumococcal transmission mechanisms including the processes such as pneumococcal shedding, survival in the external environment, and adherence to the nasopharynx of a new host. Herein, we present a review on these animal models, recent research findings about pneumococcal transmission, and factors influencing the host-pneumococcus interaction.

Keywords: *Streptococcus pneumoniae*, animal models, bacterial transmission, pneumococcal transmission, bacterial shedding

INTRODUCTION

Streptococcus pneumoniae (also known as pneumococcus) is a commensal that colonizes the upper respiratory tract and a pathogen that causes invasive diseases such as otitis media, pneumonia, sepsis, and meningitis. Pneumococcus is a gram-positive bacterium first isolated in 1881 (Bennet et al., 2015) with 100 distinct serotypes (Ganaie et al., 2020). Despite advances in therapeutics and vaccines, the world continues to experience a high burden of the disease, especially in the vulnerable populations which include young children, older adults, and immunocompromised persons (Centers for Disease Control and Prevention, 2017). In 2017, the World Health Organization published a list of antibiotic-resistant "priority pathogens" which included penicillin-nonsusceptible pneumococcus as one of the 12 families of bacteria that pose the greatest threat to human health (WHO, 2017). Multidrug resistance, defined as resistance to more than any three antimicrobial agents of different classes, was observed in 59.3% of isolates from Asian countries (Kim et al., 2012). As the problem of pneumococcal resistance to antibiotics worsens, the effectiveness of vaccines becomes even more important (Kim et al., 2016). Currently available pneumococcal vaccines include the 23-valent pneumococcal polysaccharide vaccine (PPSV23) and the 10-valent or 13-valent pneumococcal protein-conjugate vaccine (PCV10, PCV13) which replaced the PCV7.

These three vaccines, especially the PCV, have significantly reduced the incidence of invasive pneumococcal disease, and pneumococcal pneumonia (Fine et al., 1994; Simonsen et al., 2011; Suzuki et al., 2017).

To effectively prevent pneumococcal disease, it is important to understand the natural course of infection. Pneumococcal infection can be divided into three stages: transmission, colonization, and invasion (Weiser et al., 2018). To date, various studies have been conducted to understand various aspects of the mechanism of pneumococcal colonization and invasion; however, the process of transmission was not elucidated until the establishment of novel animal models. We present a review on the animal models that have been used to study disease transmission, the underlying mechanism, and factors that influence the host-pneumococcus interaction.

ESTABLISHMENT OF TRACTABLE ANIMAL MODELS TO STUDY PNEUMOCOCCAL TRANSMISSION

For pneumococcal transmission to new hosts, it first has to leave the colonized host (shedding). It should then survive in the environment before reaching the new host, unless it is transmitted through droplets or direct contact. Finally, it needs to be acquired successfully by the new host without being eliminated (**Figure 1**). To examine the mechanisms of each process, establishing tractable animal models is indispensable.

Experimental investigation on pneumococcal transmission has a relatively short history because animal models have only recently become available. There are only a few tractable animal models other than *Streptococcus pneumoniae* for bacterial transmission. These include an infant rat model of intralitter *Haemophilus influenzae* transmission (Halsey et al., 1980) and a possible transmission model of *Salmonella* species: a murine model of chronic *Salmonella* carriage in the gallbladder using *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Gonzalez-Escobedo and Gunn, 2013). In addition, a murine model of *Klebsiella pneumoniae* transmission through the fecal-oral route has recently been described (Young et al., 2020). Epidemiological studies suggest that pneumococcal transmission requires close contact and is more likely to occur in the setting of viral coinfection (Gwaltney et al., 1975). Among respiratory viruses, influenza A virus (IAV) is known to be associated with severe secondary pneumococcal pneumonia (Morens et al., 2008). In contrast, severe secondary pneumococcal pneumonia is less common among people severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (Fattorini et al., 2020; Lansbury et al., 2020; Zhu et al., 2020; Garcia-Vidal et al., 2021), although its severity and poor prognosis are suggested; a severe case has been reported in

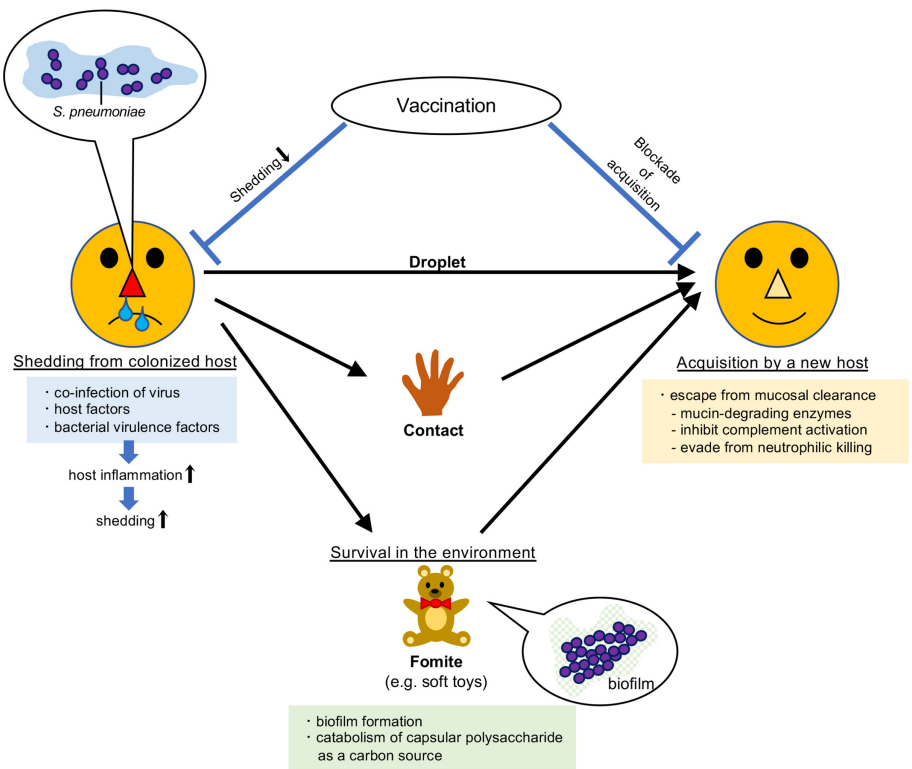

FIGURE 1 | Route of transmission of *Streptococcus pneumoniae*. *Streptococcus pneumoniae* colonizes the mucosal surface of the upper respiratory tract. It can be transmitted to new hosts by droplets, direct contact, or *via* fomites. Vaccination prevents its transmission.

an infant (Nieto-Moro et al., 2020) and high mortality rate in the elderly patients is also reported (Rodriguez-Nava et al., 2020). Surveys of children attending daycare centers have shown that respiratory viral infection and symptoms of rhinitis are associated with bacterial colonization and transmission (Rodrigues et al., 2013; Thors et al., 2019).

As for the pneumococcal transmission animal model, in 1998, it was reported that pretreatment with pneumococcal polysaccharide immunoglobulin led to a significant reduction in nasal colonization in an intralitter transmission model of infant rats (Malley et al., 1998). However, there are no subsequent reports of this model being used.

In 2010, two types of animal transmission models were reported. McCullers et al. demonstrated that aerosol transmission of pneumococcus occurred among adult ferrets that were coinfected with IAV (McCullers et al., 2010). In this model, the ferrets were infected with IAV or *S. pneumoniae* intranasally. They examined four combinations of pairs of ferret infection (the donor ferrets with pneumococcal infection with or without IAV coinfection, cohoused with the contact ferrets infected with IAV or not) at three different distances (the same cage, 3-feet separation, and 10-feet separation). As a result, donors with prior IAV infection were more susceptible to pneumococcal transmission and disease and that of contact ferrets increased their susceptibility to pneumococcal acquisition. In another report, it was shown that increased pneumococcal colonization and disease in the presence of IAV using the model of infant mice colonized with *S. pneumoniae* and subsequently infected with IAV 3 days later (Diavatopoulos et al., 2010). In this model, a litter of 5-day-old C57BL/6 mice was randomly divided into 2 equally sized groups, "index" mice and "contact" mice, and index mice were intranasally colonized with *S. pneumoniae* and infected with IAV 3 days later. Secondary infection with IAV resulted in a strong increase in pneumococcal load and its transmission to contact pups. This model has been of great use for further analyses of both bacterial factors under the condition of transmission. However, the presence of IAV is essential for pneumococcal transmission in this model, which raises the problem that host reactions against pneumococci and IAV are difficult to discern.

In 2016, Zafar et al. developed a murine transmission model without a respiratory virus coinfection, which can be utilized to examine bacterial and host factors that contribute to pneumococcal transmission free from the effects of viral coinfection (Zafar et al., 2016). Four-day-old pups were inoculated intranasally with *S. pneumoniae* without anesthesia, and their pneumococcal shedding from the upper respiratory tract was quantified. This model of pneumococcal transmission was adapted to an infant mouse model of Group A *Streptococcus* (Vega et al., 2020).

In summary, these tractable pneumococcal transmission animal models with or without IAV coinfection have enabled researchers to examine the transmission process in detail since the last decade (**Table 1**). As shown, the models with IAV coinfection are the first animal models of pneumococcal transmission, but the strong inflammation caused by IAV infection makes it difficult to evaluate the host response to pneumococcal infection alone. The pneumococcal monoinfection model is a breakthrough which established pneumococcal transmission independent from IAV, and showed the method of measuring the quantity of shedding.

SHEDDING

The IAV coinfected infant mouse model enabled researchers to assess the effect of inflammation caused by IAV infection on *S. pneumoniae* transmission. IAV infection renders the mucosal surface of the human respiratory tract suitable for pneumococcal proliferation by decreasing mucociliary clearance (Levandowski et al., 1985), enhancing the expression of glycoproteins within mucus (Barbier et al., 2012) and providing sialic acid as a nutrient source (Siegel et al., 2014). Inflammation induced by IAV infection had been demonstrated to promote pneumococcal shedding from index mice at or above a level sufficient to infect uninoculated contact mice by adapting the infant mouse model coinfected with IAV (Richard et al., 2014). In this study, toll-like receptor (TLR) 2 deficiency induced more frequent transmission because of a weakened antiviral response that made the mice more susceptible to IAV infection and caused heightened inflammation. Therefore, increased inflammation is associated with increased transmission rate of *S. pneumoniae* by infected hosts. Furthermore, it was reported that a sufficiently large number of pneumococci on the mucosal surface seemed

TABLE 1 | Remarkable animal transmission models of *Streptococcus pneumoniae*.

Animal type	Co-infection	Transmission	Characteristics	Author	Year
ferret	IAV	co-housed pairs of ferrets	-similar airway symptoms to human -difficult to exclude the influence of IAV -not genetically homogenous	McCullers et al.	2010
infant mouse	IAV	intralitter transmission	-easier to handle than ferrets -difficult to exclude the influence of IAV -genetically homogenous	Diavatopoulos et al.	2010
infant mouse	none	intralitter transmission	-easier to handle than ferrets -can evaluate the effect of pneumococcal infection solely -quantitative evaluation of pneumococcal shedding -no adult models	Zafar et al.	2016

necessary for a single organism to reach the nasal mucosa of a new host because transmission rates correlate with the density of shed organisms using the infant mouse coinfected with IAV model (Kono et al., 2016).

In the infant mouse *S. pneumoniae* monoinfection model of transmission established by Zafar et al., the level of pneumococcal shedding was highest in the pups infected intranasally at age 4 days and this level depended on colonization density and pneumococcal capsule type. To achieve a high (29%) transmission rate, transmission experiments were performed with a 1:1 ratio of index mice to contact mice (Zafar et al., 2016). Both the type and the amount of capsular polysaccharide (CPS) were demonstrated to be determinants of the spread of pneumococci from one host to another with isogenic capsule switch and *cps* promoter switch mutants (Zafar et al., 2017a). In this study, the importance of CPS in shedding was revealed based on the finding that the shedding of a type 23F isolate was significantly less than that of a type 4 isolate, TIGR4 (T4), even though the colonization levels in the nasopharynx were equivalent. The T4 isolate expressing a 23F capsule showed less shedding than that of a 23F isolate expressing a type 4 capsule, and the proportion of high shedding events of the 23F isolate expressed the type 4 CPS increased compared to that of the 23F isolate. In addition, comparing strains of different genetic backgrounds expressing the same capsule type showed that genetic background had little influence on shedding. These findings suggested that CPS type is a more important factor for shedding than genetic background. They also created constructs of the same type that expressed different levels of CPS by switching the *cps* promoter region. They demonstrated that the strain with a weaker *cps* promoter showed significantly reduced shedding and transmission in the model of transmission by infant mice coinfected with IAV. A mucin-binding assay showed that the strains with the CPS type or amount whose shedding was reduced had increased mucin-binding affinity and were more strongly immobilized, suggesting a correlation between the ability to escape from mucus entrapment and pneumococcal shedding. Furthermore, using the infant mouse pneumococcal monoinfection model, two notable effects of a pneumococcal pro-inflammatory pore-forming toxin, pneumolysin (Ply) were demonstrated (Zafar et al., 2017b). First, Ply promotes inflammation, which increases shedding and enables intralitter transmission. Second, Ply increases bacterial survival outside the host. probably because Ply-induced host cell lysis helps pneumococcal survival and growth nutritionally. These findings suggest that sufficient levels of Ply are needed for pneumococcal shedding for transmission to occur. In 2019, it was demonstrated that the *dlt* locus, which is involved in D-alanilation of lipoteichoic acids (LTA) increasing TLR2-mediated inflammation and resistance to antimicrobial peptides, also plays a key role in pneumococcal shedding, using the infant mouse model (Zafar et al., 2019).

Rowe et al. described other pneumococcal genes required for effective transmission using the model of the ferret coinfected with IAV, by screening with a TnSeq library of a pneumococcal strain. They also demonstrated that targeted deletion of the putative C3-degrading protease CppA, iron transporter PiaA, or competence regulatory histidine kinase ComD significantly decreased transmissibility in the infant mouse pneumococcal monoinfection model, confirming the result of the ferret screening (Rowe et al., 2019). In particular, ComD is known to be a receptor for competence stimulating peptide (CSP) and a member of the ComABCDE pathway, which regulates competence and is associated with quorum sensing and biofilm formation (Chandler and Morrison, 1987; Håvarstein et al., 1995; Håvarstein et al., 1996; Pestova et al., 1996; Alloing et al., 1998; Ween et al., 1999; Oggioni et al., 2006). They also showed that maternal vaccination with recombinant surface-exposed PiaA and/or CppA blocked intralitter transmission and was more effective than PCV13 in this study.

Thus, it is underscored that host inflammation plays a key role in pneumococcal shedding and that bacterial factors such as capsule type and capsular amount are important, as shown by quantitative measurement of shedding in the studies using the pneumococcal monoinfection transmission model. The comprehensive genetic screening method, TnSeq, has enabled identification and quantification of pneumococcal factors required for transmission, and can be utilized to search for and prioritize vaccine candidates.

SURVIVAL IN THE EXTERNAL ENVIRONMENT

Transmission can occur through exposure to fomites in addition to direct contact with carriers. For example, the teats of the dam were contaminated with large numbers of pneumococci by infected suckling mice and appeared to be the source of contagion of the pups in the mouse model, even without nasal pneumococcal colonization of the dam (Diavatopoulos et al., 2010). The duration of pneumococcal survival in the external environment ranges widely from a few hours to several months, depending on the situation, and it is longer than that of another important respiratory pathogen, *Haemophilus influenzae* (Walther and Ewald, 2004). In daycare settings, pneumococci can survive for hours and be cultured from environmental surfaces such as soft toys and crib linen (Marks et al., 2014). Furthermore, it has been reported that pneumococci have desiccation tolerance, and viable bacteria are culturable even after four weeks of desiccation at ambient temperature and humidity (Walsh and Camilli, 2011). Various clinically relevant *S. pneumoniae* strains show both survival and growth in saliva under ambient conditions (Verhagen et al., 2014). In contrast, in airway surface fluid where nutrients are limited (Barker et al., 1989; Philips et al., 2003), Ply expression of pneumococcus increases ex vivo survival, and the possible mechanism is that Ply-induced inflammation increases nutrient levels in airway secretions (Zafar et al., 2017b). Under nutrient-poor environmental conditions, pneumococci remain infectious for at least 24 hours and encapsulation prolongs bacterial survival in a serotype-dependent manner, which suggests that pneumococci catabolize their own capsular

polysaccharide as a carbon source when other carbon sources are scarce (Hamaguchi et al., 2018).

Pneumococcus forms biofilms during nasopharyngeal colonization (Gilley and Orihuela, 2014). Biofilms are complex bacterial populations with an extracellular matrix (ECM) adherent to each other and/or to surfaces or interfaces (Costerton et al., 1995; Donlan and Costerton, 2002). The ECM is composed of host factors, polysaccharides, and extracellular DNA and protects bacteria from the host immune system (Moscoso et al., 2006). Pneumococci in biofilms have been shown to be less virulent than their planktonic form (Lizcano et al., 2010; Sanchez et al., 2011; Gilley and Orihuela, 2014). Marks et al. developed a novel model of biofilm on live epithelial cells both *in vitro* and *in vivo* (Marks et al., 2013). In their study, they demonstrated that infection with IAV or the host signals caused by IAV infection (febrile-range temperatures, norepinephrine, extracytoplasmic ATP, and increased nutrient availability) induced the release of bacteria from biofilms and that these dispersed bacteria appeared to be more virulent than both biofilm and broth-grown planktonic bacteria. The bacteria dispersed from biofilms showed enhanced expression of genes associated with virulence, such as the *cps* cassette, pneumolysin, adhesin PavA, and *licD2* locus involved in phosphorylcholine metabolism and promoting the opaque phenotype (Zhang et al., 1999), suggesting that high capsule expression induced high levels of inflammatory cytokines and caused more severe disease in a mouse infection model. These observations are rather phenomenological, and the precise mechanism underlying pneumococcal colony opacity phase variation, dispersal from biofilms, and upregulation of its virulence, remains unclear. Furthermore, the roles and interactions of pneumococcal biofilms, bacteria in biofilms, and hypervirulent dispersed bacteria from biofilms in viral inflammation require further investigation. Recently, it was shown that pneumococcal phase variation of colony opacity occurred by recombination of the DNA methylase genes (*hsdS*, *hsdS'*, and *hsdS''*) in the SpnD39III and Spn556II type I restriction-modification (R-M) systems (Manso et al., 2014; Li et al., 2016). Based on these reports, Oliver et al. created phase-locked mutant strains of TIGR4 background, whose colony phenotypes were stable over multiple serial passages *in vitro* and *in vivo* (Oliver et al., 2017). In this study, it was observed that capsule expression of *hsdS* variants was less than that of the TIGR4 strain, and that the capsule expression of transparent variants was less than that of the opaque strains. In addition, the biofilm formation and viability in the biofilm of the opaque variants were reduced. Furthermore, transcriptome sequencing (RNA-seq) analyses showed that the expression levels of potential virulence factors were altered in a phase-specific manner. The concerning point of this study is its finding that transparent variants were more virulent than opaque ones, which was inconsistent with previous studies (Kim and Weiser, 1998; Kim et al., 1999). The possible reasons of this inconsistency are the difference of the capsular types (TIGR4 vs type 2, 6A, and 18C) or the route of administration (intranasal vs intraperitoneal). However, the epigenetic approach and next generation analysis such as transcriptome sequencing will enable further breakthroughs to be made in researching host-pathogen interactions.

Biofilm formation enhances the survival of pneumococci on fomites (Marks et al., 2014). The relative importance of a contagion through environmental fomites compared to that of direct contact is unclear. However, reports suggest that pneumococci in biofilms survive long enough in the external environment to transmit to new hosts. It has been shown that pneumococci remain fully infectious after an environmental exposure without nutrients for up to 24 h under experimental conditions (Hamaguchi et al., 2018). Therefore, hygiene management, such as washing hands, cleaning the surrounding areas, and disinfecting equipment, is indispensable.

ADHERENCE TO THE NASOPHARYNX OF A NEW HOST

To be acquired by a new host, pneumococci must escape from the host defense mechanism and adhere to the mucous membrane of the nasopharynx. Here, we focus on the pneumococcal mechanism of evading initial mucosal clearance and its impact on transmission.

First, most pneumococcal capsules are negatively charged, which is thought to enable them to evade clearance by mucus (Nelson et al., 2007). Second, pneumococci have several enzymes that degrade mucus, inhibit mucociliary clearance, and enable adhesion to the mucosa. Neuraminidase A (NanA), β-galactosidase (BgaA), and β-N-acetylglucosaminidase (StrH) sequentially deglycosylate N-linked glycans on host defense molecules (King et al., 2006). In addition, NanA and BgaA function as adhesins independently of their enzymatic activities (Uchiyama et al., 2009; Limoli et al., 2011). Furthermore, Ply released on autolysis by autolysin slows ciliary beating and inhibits mucociliary clearance (Steinfort et al., 1989; Feldman et al., 1990; Feldman et al., 2002). Recently, Fliegauf et al. discovered that pneumococci inhibit mechanical cilia-mediated clearance independently of the effect of Ply using high-speed video microscopy and live-cell imaging in a murine *in vitro* airway infection model (Fliegauf et al., 2013). Moreover, two pneumococcal proteins, namely, peptidoglycan-N-acetylglucosamine deacetylase (PgdA) and attenuator of drug resistance (Adr), modify the peptidoglycan of its cell wall and are associated with resistance to lysozyme, which cleaves bacterial peptidoglycan and has antimicrobial effects on the mucosal surface fluid (Davis et al., 2008). Pneumococcal surface protein A (PspA), is another important protein that plays an important role in invasive infections by inhibiting complement activation (Tu et al., 1999) and protecting pneumococcus from killing by apolactoferrin (Shaper et al., 2004). It has been reported that immunization with PspA is protective against pneumococcal infection in mice (Ezoe et al., 2011; Piao et al., 2014). For evasion of neutrophilic killing, pneumococcal choline-binding-protein E (CbpE, also known as Pce) decreases neutrophil recruitment by the inactivation of platelet-activating factor (PAF), a host-derived inflammatory phospholipid that stimulates neutrophil

phagocytic capacity and bactericidal function (Hergott et al., 2015).

The pneumococcal zinc metalloprotease, ZmpA, cleaves immunoglobulin A1 (IgA1), the most abundant immunoglobulin on mucosal surfaces of the human upper respiratory tract, and thereby eliminates the agglutinating activity of IgA1, which prevents mechanical bacterial clearance by the mucociliary flow (Janoff et al., 2014; Roche et al., 2015). Epidemiological studies on PCVs have found that they not only protect children from invasive disease, but also prevent pneumococcal transmission to vulnerable groups, especially older adults, by reducing pneumococcal carriage among vaccinated children (herd immunity) (Whitney et al., 2003; Centers for Disease Control and Prevention, 2005; Miller et al., 2011; Whitney et al., 2014). The infant mouse model has been used to examine the role of anti-pneumococcal immunity in nasal shedding and pneumococcal transmission. It has been shown that shedding is decreased and transmission is blocked by anti-pneumococcal immunity and PCV (Zangari et al., 2017). The agglutinating function of PCV-induced IgG to type-specific pneumococcal polysaccharide appears to reduce pneumococcal shedding. In humans, the Pneumococcal Conjugate Vaccine Experimental Human Pneumococcal Carriage (PCV/EHPC) study was conducted in 2012 (Collins et al., 2015), and the EHPC model was utilized in subsequent studies. The studies revealed that pneumococcal carriage protects healthy adults against re-challenge to the homologous strain (Ferreira et al., 2013), but not against a heterologous strain type (Pennington et al., 2016). Furthermore, high levels of CPS-specific memory B cells are associated with protection against acquisition (Pennington et al., 2016) and pneumococcal agglutination of airway secretions mediated by CPS-specific antibodies after PCV vaccination appears to be a key mechanism of protection against acquisition of carriage (Mitsi et al., 2017).

S. pneumoniae has various strategies to escape from mucosal clearance and successfully colonize new hosts. In humans, PCV prevents not only invasive pneumococcal disease, but also the acquisition of pneumococcus in human pneumococcal challenge experiments. Among the proteins described above, another promising vaccine candidate is PspA. Previous studies have shown that it could be utilized as vaccine with both intranasal and subcutaneous administration, opening a broad range of developmental possibilities.

DISCUSSION

S. pneumoniae is an opportunistic commensal and is at the same time, one of the deadliest bacteria that has a long history of being in an inseparable relationship with humans. The development of antibiotics and vaccines is not yet sufficient to eliminate pneumococcal disease. To protect humans from pneumococcal disease, it is necessary to block pneumococcal transmission in addition to improving bactericidal measures or enhancing the immune mechanisms which confront pneumococcus after its entry into the body. Reducing its shedding, refining environmental hygiene, and blocking its acquisition, are all key.

In summary, this review focused on pneumococcal transmission because there has been great progress in research and understanding over the past decade due to the establishment of tractable animal models. The animal species used in these models are ferrets and mice. There are two major advantages of using ferrets in the models. First, they can transmit pathogens through droplets by sneezing like humans. Second, the glycomic profiles of ferrets are more similar to those of human than mice (Walther et al., 2013; Jia et al., 2014). Meanwhile, the accessibility to genetic engineering techniques such as transgenesis (e.g. humanized mice) and gene knock-out is one of the major advantages in murine models, and these techniques can be more utilized to clarify the specific genetic factors in pneumococcal transmission. There are several limitations of the current animal models of pneumococcal transmission. First, the influence of pneumococcal infection alone is difficult to evaluate in IAV coinfection models. Second, in the infant mouse model of pneumococcal monoinfection, shedding collection is a manual procedure and the result can be dependent on the technique of its measurer. Furthermore, the infant mouse model cannot mimic human adult pneumococcal transmission because the immune system of infant mice is immature. Moreover, the major route of transmission in murine models is thought to be contact transmission, while in humans is thought to be droplet transmission. Therefore, establishing a novel pneumococcal monoinfection model of adult mice with an objectively quantitative method of measuring the amount of shedding, is necessary for further research. Using these tractable animal models, it may be possible to evaluate the detailed effects of available vaccines on each transmission steps. Epigenetic analysis and the next generation sequencing such as RNA-seq and Tn-seq may reveal more dynamic mechanisms of pneumococcal infection and facilitate the search for vaccine candidates and therapeutic targets more readily. Further research on the comprehensive pneumococcal mode of life will enable us to improve our strategies for prevention and treatment of pneumococcal disease.

AUTHOR CONTRIBUTIONS

AM wrote the manuscript. SH and YA reviewed the manuscript. KT supervised this work. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by JSPS KAKENHI Grant Number 19K17924.

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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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frontiers in Cellular and Infection Microbiology

MINI REVIEW
published: 25 May 2021
doi: 10.3389/fcimb.2021.651474

Insights Into the Effects of Mucosal Epithelial and Innate Immune Dysfunction in Older People on Host Interactions With *Streptococcus pneumoniae*

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OPEN ACCESS

Edited by:
Masaya Yamaguchi, Osaka University, Japan

Reviewed by:
Daniel Neill, University of Liverpool, United Kingdom
Stephen I. Pelton, Boston University, United States
Soichiro Kimura, Toho University, Japan

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Specialty section:
This article was submitted to Bacteria and Host, a section of the journal Frontiers in Cellular and Infection Microbiology

Received: *09 January 2021*
Accepted: *10 May 2021*
Published: *25 May 2021*

Citation:
Weight CM, Jochems SP, Adler H, Ferreira DM, Brown JS and Heyderman RS (2021) Insights Into the Effects of Mucosal Epithelial and Innate Immune Dysfunction in Older People on Host Interactions With Streptococcus pneumoniae. Front. Cell. Infect. Microbiol. 11:651474. doi: 10.3389/fcimb.2021.651474

In humans, nasopharyngeal carriage of *Streptococcus pneumoniae* is common and although primarily asymptomatic, is a pre-requisite for pneumonia and invasive pneumococcal disease (IPD). Together, these kill over 500,000 people over the age of 70 years worldwide every year. Pneumococcal conjugate vaccines have been largely successful in reducing IPD in young children and have had considerable indirect impact in protection of older people in industrialized country settings (herd immunity). However, serotype replacement continues to threaten vulnerable populations, particularly older people in whom direct vaccine efficacy is reduced. The early control of pneumococcal colonization at the mucosal surface is mediated through a complex array of epithelial and innate immune cell interactions. Older people often display a state of chronic inflammation, which is associated with an increased mortality risk and has been termed 'Inflammageing'. In this review, we discuss the contribution of an altered microbiome, the impact of inflammageing on human epithelial and innate immunity to *S. pneumoniae*, and how the resulting dysregulation may affect the outcome of pneumococcal infection in older individuals. We describe the impact of the pneumococcal vaccine and highlight potential research approaches which may improve our understanding of respiratory mucosal immunity during pneumococcal colonization in older individuals.

Keywords: epithelium, pneumococcus (*Streptococcus pneumoniae*), innate immunity, inflammageing, older individuals

INTRODUCTION

William Osler, a Canadian physician, who himself died of pneumonia, wrote in his book *The Principles and Practice of Medicine*: "In the aged, the chances are against recovery. So fatal that it has been termed the natural end of the old man" (Osler, 1892).

Much has changed since, with a huge global public health effort to reduce the burden of pneumonia and invasive pneumococcal disease (IPD), particularly in young children. However, the

1.5 billion people worldwide who are >65yrs (older individuals) now outnumber those <5yrs and, by 2050, will outnumber those aged 15–24yrs when there is predicted to be 426 million people >80yrs (U.N, 2019). Community Acquired Pneumonia (CAP) is common in older individuals, particularly men, with infection by *Streptococcus pneumoniae* as the leading cause (Kaplan et al., 2002; Janssens and Krause, 2004; Stupka et al., 2009). In 2016, pneumococcal pneumonia was responsible for ~494,340 deaths globally in individuals >70yrs (Collaborators, 2018).

Why older people are so vulnerable to disease caused by *S. pneumoniae* is likely to be multifactorial including co-morbidities, relative immunodeficiency, malnutrition and defective swallowing (Janssens and Krause, 2004; Zalacain, 2004; Arndt, 2015). Disease follows pneumococcal carriage and reported nasopharyngeal and oropharyngeal carriage rates in older people vary between 0–39% (Krone et al., 2015; Adler et al., 2020; Almeida et al., 2020; Smith et al., 2020; Yasuda et al., 2020). Unlike adults aged 18–64yrs, older adults do not appear to benefit from the natural immune effects of pneumococcal colonization events that are thought to protect against re-colonization and disease (Ferreira et al., 2013; Adler et al., 2020). Older people often display a disorganised inflammatory state, which is associated with an increased mortality risk and has been termed 'Inflammageing' (Franceschi and Campisi, 2014; Ferrucci and Fabbri, 2018). This may compromise upper respiratory mucosal immunity, mediated by the nasopharyngeal epithelium and other cellular and soluble innate immune components (Simell et al., 2012; Wilson et al., 2015; Jochems et al., 2018; Jochems et al., 2019a; Weight et al., 2019).

In this review we discuss the impact of inflammageing on innate immunity to *S. pneumoniae* in older people, summarised in **Figure 1**. We outline the impacts of the pneumococcal vaccine in older individuals and experimental approaches which may lead to deeper understanding of how the pneumococcus affects this vulnerable population.

PHYSIOLOGY AND AGEING

Pulmonary Physiology

In humans, there is a decrease in pulmonary elasticity, loss of respiratory muscular strength, decreased ciliary beating and mucus velocity with age (Ho et al., 2001; Janssens and Krause, 2004). These changes occur from a combination of genetic predisposition, inflammageing and environmental exposure, which involve a wide range of molecular and cellular changes and impairment of cell-cell communications (Brandenberger and Muhlfeld, 2017).

Microbiome

It is becoming increasingly apparent that the microbiome is an important determinant of lung and gut homeostasis and the development of disease, particularly in older individuals (de Steenhuijsen Piters et al., 2016; Schenck et al., 2016; Man et al., 2017; Ragonnaud and Biragyn, 2021). It remains

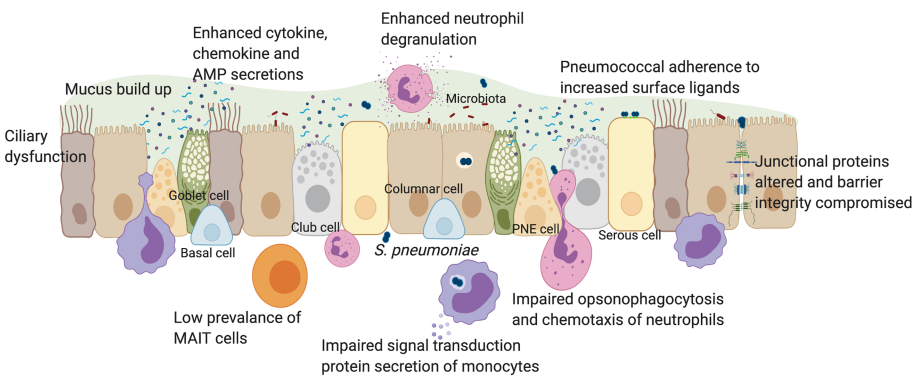

FIGURE 1 | The impact of pneumococcal infection on mucosal immunity in older individuals. The human respiratory epithelium includes many cell types all of which contribute to the development of innate immunity through a physical barrier held together by junctional proteins, and a chemical barrier *via* secretions of mucus, cytokines, chemokines, antimicrobial peptides, vitamin D and retinoic acid. Innate immune cells such as monocytes, neutrophils and MAIT cells are also present in the mucosa. In older individuals, there is a loss of physical movement both at a mechanical elasticity level and a lack of cilia beating which impacts on mucus clearance. This contributes towards increased prevalence of luminal factors such as cell debris, secreted factors, microbiota and pathogens such as *S. pneumoniae* which trigger already elevated baseline levels of cytokines such as IL-6, IL-8 and TNFα. In younger adults, epithelial-derived secretion of anti-microbial peptides such as cathelicidin and NFκB activation, leads to autophagy. Impaired autophagy and type 1 interferon responses in older individuals may lead to suppressed IFNβ levels, increasing pneumococcal load. Increased expression of epithelial senescence markers and pneumococcal ligands such as PAFr in older people enhances pneumococcal colonization, influencing adhesion, micro-invasion and transmigration potential. Vitamin D deficiency in older people may affect epithelial barrier integrity. In younger adults, disruption to barrier function after pneumococcal infection affected the expression of junctional proteins such as Claudins. In older adults, dysregulation of barrier may enhance rates of pneumococcal transmigration, infiltration of innate immune cells and inflammation. Although MAIT cells are rare in the airway of older individuals, neutrophil prevalence is enhanced, which elevates degranulation and reactive oxygen species levels following pneumococcal infection. However, neutrophil ability for opsonophagocytosis and chemotaxis is impaired in older individuals. Monocyte function may also be impaired in signal transduction and secrete less IL-6, IL-8 and TNFα during infection, in comparison to younger adults. PNE cell, pulmonary neuroendocrine cell; AMP, anti-microbial peptides. Created with Biorender.com.

uncertain whether changes in the composition and diversity of microbiota represent a cause or consequence of pneumonia (de Steenhuijsen Piters et al., 2016). Older individuals with pneumonia exhibit increased abundance of species such as *S. pneumoniae*, Rothia and Lactobacilli, but decreased overall anaerobic bacterial diversity in the upper respiratory tract (URT) (de Steenhuijsen Piters et al., 2016). In a human experimental pneumococcal challenge model (EHPC), low density pneumococcal carriage was associated with a stable mucosal microbiome (baseline presence of Corynebacterium/Dolosigranulum species) and a less pro-inflammatory phenotype (de Steenhuijsen Piters et al., 2019). Whether different pneumococcal strains interact differently with the respiratory microbiome during colonization (Cremers et al., 2014) and how this affects older individuals, remains to be determined. The role of intestinal microbiota on lung susceptibility to pneumococcal infection also warrants further investigation in humans as murine studies suggest that Nod-stimulating microbiota in the gut induce GMCSF-dependent immunity, which influences alveolar macrophage function during pneumococcal infection (Schuijt et al., 2016; Brown et al., 2017).

Inflammageing

An imbalance of cytokine expression in older individuals is referred to as "inflammageing", where damage to the tissue, changes in composition of the microbiome and cellular and immune senescence, all contribute to this state of chronic inflammation (Franceschi and Campisi, 2014). The contributors to inflammageing may include microbial translocation, chronic infections, mitochondrial dysfunction and accumulation of DNA damage (Fulop et al., 2017; Ferrucci and Fabbri, 2018). This increased inflammation extends to the lungs, as healthy individuals >65yrs have elevated levels of IL-6, IL-8 and higher numbers of neutrophils in bronchoalveolar lavage (BAL) samples (Thompson et al., 1992; Meyer et al., 1996). The increased susceptibility to respiratory tract infections has also been linked to the heightened inflammatory status in older people. In a large prospective study of people aged 70-79yrs, being in the highest tertile for systemic IL-6 and TNF levels was associated with 1.6-1.7-fold increased risk for developing CAP (Yende et al., 2005). Inflammation, in particular IL-6 levels, at time of admission to hospital is also associated with CAP disease severity (Glynn et al., 1999; Antunes et al., 2002). Whether this represents more severe disease, or a pre-existing heightened inflammatory state is uncertain. For example, at admission, in a cohort of 22 patients with pneumonia, of which 19 had confirmed *S. pneumoniae*, patients <55yrs had increased levels of IL-6 compared to patients >68yrs (Bruunsgaard et al., 1999). However, at 7 days post admission, pro-inflammatory cytokines TNF and soluble TNF receptor I remained elevated in older adults, while they had returned to baseline in young adults. This increased inflammatory state with age may therefore contribute to dysregulation of immunomodulation of innate immune cells such as neutrophils, cytokines and chemokines (Williams et al., 2015) and ultimately, pneumococcal colonization, increasing the chance of IPD in older individuals.

EPITHELIAL CELL FUNCTION AND AGEING

The nasopharyngeal epithelium provides the first line of defense against respiratory pathogens. An intact physical barrier, together with epithelial secretions of mucus, anti-microbial peptides and proteins, chemokines and cytokines, forms the basis of epithelial-derived innate immunity (**Figure 1**). Hence, age related alterations in epithelial responses would have profound effects on pneumococcal colonization, as summarized in **Table 1**.

Epithelial Cell Activation

S. pneumoniae binds to a variety of epithelial receptors including Keratin 10, laminin receptor and platelet-activating factor receptor (PAFr), the expression of which is altered in older individuals, thus potentially influencing pneumococcal adherence and susceptibility towards disease. For example, levels of Keratin 10, laminin receptor and PAFr are elevated in aged mice, human lung tissue and senescent A549 cells (Hinojosa et al., 2009; Shivshankar et al., 2011) which could contribute to altered outcomes after pneumococcal colonization. Pneumococcal micro-invasion of the epithelium *in vitro* is also associated with epithelial secretion of cytokines and chemokines

TABLE 1 | Epithelial cell changes and ageing in the context of pneumococcal infection.

Molecular changes	Pneumococcal outcome	Impact on epithelial barrier
↑Keratin 10, laminin receptor, PAFr expression	↑ Pneumococcal adherence, micro-invasion and toxin concentrations	↑ Epithelial damage, inflammation and immune cell recruitment
↓ Claudin-5, -7, -10, Occludin, ZO-1, VE-cadherin expression ↑Claudin 2 expression ↓ Vitamin D signalling	↑ Pneumococcal transmigration across the epithelial barrier	↑ Barrier permeability, NFκB activation, inflammation and immune cell recruitment ↓ Transepithelial electrical resistance ↑ Epithelial damage, inflammation and immune cell recruitment
↓ LL-37, β defensin -2, -3, -4, S100A7, -8, -9, Lipocalin and RNase 7 secretion	↑ Pneumococcal load and toxin concentrations	↓Autophagy, NLRP3 inflammasome activation
↑ Or ↓ IL-6 production	↓ Or ↑ Effects on pneumococcal-epithelial associations, micro-invasion and transmigration	↓ Or ↑ Affecting barrier permeability, proliferation and epithelial repair

and a transcriptomic enrichment of innate signaling pathways including Toll receptor cascades, NFκB and MAPK activation (Weight et al., 2019). In the EHPC model, an epithelial transcriptomic signature that was associated with bacterial clearance has been identified, indicating the involvement of epithelial activation in the control of pneumococcal colonization (Weight et al., 2019). Furthermore, *in vitro* epithelial cell models reveal that activation of p65, upregulation of the histone demethylase KDM6B and IL-11 secretion are associated with protection from epithelial damage following pneumococcal infection (Connor et al., 2020). We therefore speculate that in older adults, where pneumococcal micro-invasion and cellular senescence may be enhanced, dysregulation of these pathways and regulatory mechanisms could exacerbate invasive disease.

Mucosal Barrier Function: The expression profiles of intercellular junctional proteins such as Claudins, ZO-1 and E-Cadherin, determine the permissiveness of the respiratory epithelium to the passage of microbes across the barrier. Changes in epithelial junctional expression during pneumococcal infection leads to structural reorganization of the barrier. For example, *in vitro* infection of human nasopharyngeal cells with *S. pneumoniae* is initially associated with decreased permeability to 4kDa FITC-dextran, indicating a strengthened barrier (Weight et al., 2019). However, over time (>8 hours post-infection, when bacterial replication and autolysis have also occurred) barrier integrity is altered, demonstrated through decreased expression of Occludin, ZO-1, Claudin-5 and VE-cadherin in the alveoli epithelium and endothelium in younger adults lung explants (Peter et al., 2017). Furthermore, downregulation of Claudin-7 and Claudin-10 in human and murine epithelial cells, led to increased pneumococcal transmigration across the epithelial barrier (Clarke et al., 2011).

The vitamin D receptor (VDR) regulates epithelial barrier function (Chen et al., 2018). In *Salmonella* infected $VDR^{-/-}$ mice, Claudin 2 upregulation was associated with leaky intestinal barrier, increased pathology and upregulation of NFκB (Zhang et al., 2019), a critical regulator of inflammageing (Salminen et al., 2008) and tight junction protein expression (Ward et al., 2015). In the older human intestine, Claudin-2 upregulation has been detected, which was accompanied by decreased transepithelial electrical resistance and increased permeability (Man et al., 2015). Activation of NFκB following pneumococcal infection is widely reported (Malley et al., 2003; Weight et al., 2019), and Vitamin D deficiency is more severe in older generations (Hirani and Primatesta, 2005; Jolliffe et al., 2013), and there is evidence to suggest that supplementation of Vitamin D could be beneficial in boosting immunity and reducing acute respiratory infections (Martineau et al., 2017; Chambers et al., 2021). Whether regulation and junctional protein responses in the URT are altered with age in humans, and how this contributes to control of pneumococcal colonization, remains to be determined.

Antimicrobial Peptides and Proteins (AMPs)

An important factor of epithelial innate immunity includes AMPs that neutralize toxins and eliminate pathogens (Hiemstra, 2001). Infection of human corneal epithelial cells with pneumococcus induced NFκB activation leading to the secretion of LL-37, β defensin -2, -3, -4, S100A7, S100A8, S100A9, Lipocalin and RNase 7 (Sharma et al., 2019). LL-37 plays a role in wound healing, can induce autophagy in a 1,25-dihydroxyvitamin D3 dependent manner, can activate the NLRP3 inflammasome in a model of *P. aeruginosa* and, is bactericidal against *S. pneumoniae* and *Mycobacterium tuberculosis* (Nijnik and Hancock, 2009; Yuk et al., 2009; McHugh et al., 2019; Sharma et al., 2019). Older individuals maintain similar levels of baseline production of cathelicidins and β defensin 2 in serum compared to younger adults (Castaneda-Delgado et al., 2013). However, in aged mice, CRAMP expression, the murine homolog of LL-37, was not upregulated following pneumococcal infection, compared to younger adults (Krone et al., 2013). This suggests a potential dysregulation of AMPs in older individuals and the implications for the control of *S. pneumoniae* at the mucosal surface warrants further investigation.

Cytokines and Chemokines

AMPs also induce the secretion of cytokines and chemokines like IL-6 and IL-8 from nasal epithelial cells, in an NFκB dependent manner (Pistolic et al., 2009). One might predict that given elevated levels of cytokines such as IL-6 and TNFα in older individuals (Yende et al., 2005; Man et al., 2015), epithelial cell responses may also differ in the response to pneumococcal carriage. IL-6 is a pleiotropic cytokine and so elevated baseline secretion in older individuals may either enhance or weaken barrier integrity upon pathogenic challenge. For example, IL-6 regulates the expression of tight junction proteins such as Claudin 2 and increases intestinal barrier permeability (Suzuki et al., 2011; Man et al., 2015). This could also occur in the respiratory setting, which may increase pneumococcal transmigration across the epithelial barrier. Alternatively, IL-6 is also known to confer epithelial repair and promote proliferation (Kuhn et al., 2014), which may inhibit pneumococcal adherence to the epithelium. For example, co-infection with Influenza A increases susceptibility to *S. pneumoniae* in both adult and older mice and in younger adults, characterized by increased bacterial burden in the URT (Mina et al., 2014; Jochems et al., 2018; Gou et al., 2019). In the murine study, IL-6 production was required to maintain barrier function and macrophage phagocytic function, which played a role in pneumococcal control and clearance (Gou et al., 2019). Although secreted by infected human nasopharyngeal cells *in vitro* (Weight et al., 2019), levels of epithelial IL-6 secretion *in vivo* have not been directly investigated in adults or older individuals.

INNATE IMMUNITY AND AGEING

The degree of inflammageing likely influences the functional responses of monocytes/macrophages, neutrophils and *Mucosal-associated invariant T (*MAIT) cells to *S. pneumoniae*, which in turn, may be detrimental in controlling the outcome of

nasopharyngeal pneumococcal colonization in older people, as summarized in **Table 2**.

Monocytes and Macrophages

TLR1 levels are reduced on monocytes from older adults and TLR1/2 specific stimulation using Pam2SCK4 is associated with reduced responses in monocytes from older people (van Duin et al., 2007). Indeed, cytokine responses to pneumococcal or relevant ligands also appear decreased with age. Frail older individuals have increased baseline production of TNF by intermediate monocytes in particular, but show an impaired induction upon TLR1/2 or TLR4 stimulation (Hearps et al., 2012; Verschoor et al., 2014b). Upon heat-killed pneumococcal stimulation however, monocyte-derived macrophages in frail older people produce less TNF, IL-6, IL-1β and IL-8 and have a reduced capacity to kill *S. pneumoniae*. This is possibly related to defective PI3K-AKT signaling (Verschoor et al., 2014a), and/or insufficient activation of the NLRP3 inflammasome, as demonstrated in bone marrow derived macrophages from aged mice (Cho et al., 2018).

In younger adults, infiltration of classical monocytes into the nasal mucosa in the EHPC model coincides with initiation of pneumococcal clearance, while nasal myeloid cytokines correlate with clearance of colonization (Jochems et al., 2018). The impact of the respiratory monocyte/macrophage dysfunction described above on pneumococcal control in older people is not fully understood. However, altered monocyte subsets are an important contributor to a reduced ability to prevent pneumococcal infection in older people (Puchta et al., 2016). For example, reduced cytokine production to TLR1/2 agonists seem to be mediated by changes in CD14++ CD16+ intermediate and CD14+ CD16+ non-classical monocytes (Nyugen et al., 2010). In addition, alveolar macrophage numbers are higher in BAL samples from healthy older adults compared to younger adults (Thompson et al., 1992; Meyer et al., 1996), although how this affects innate immune responses to *S. pneumoniae* is unknown.

Murine studies have also identified age-related functional differences in monocyte/macrophage interactions with *S. pneumoniae* that may be relevant for disease pathogenesis. Puchta et al. demonstrated that TNF is a crucial mediator of the susceptibility to pneumococcal infections in inflammageing, as well as the alterations in monocyte subsets (Puchta et al., 2016). Increasing TNF with age led to premature egress of pro-inflammatory monocytes from bone marrow and increased levels of intermediate CD14++ CD16+ monocytes. Specific depletion of these monocytes or reduction in TNF levels enhanced immunity to pneumococcal infection and increased clearance in old mice (Puchta et al., 2016). Koppe found that following sensing of pneumococcal dsDNA by murine macrophages, STING ("stimulator of IFN genes") binds to TBK1 (TANK-binding Kinase 1), leading to IRF3 (Interferon Regulatory Transcription Factor 3) activation and production of IFNβ, which assists pneumococcal clearance (Koppe et al., 2012). In aged mice, there is less STING/TBK1/IRF3 mRNA and protein expression compared to young mice infected with *S. pneumoniae* (Mitzel et al., 2014). This was associated with lower levels of IFNβ and higher bacterial burden in the lung, thought to be due to age-associated stress of the endoplasmic reticulum, resulting in increased autophagy-related protein 9a-STING complex formation (Mitzel et al., 2014), preventing STING complex formation with TBK1 (Saitoh et al., 2009).

Neutrophils

There is a large body of evidence from both murine and human studies showing the importance of neutrophils in protecting against pneumococcal colonization of the nasopharynx (Lu et al., 2008; Weinberger et al., 2009; Jochems et al., 2018; Nikolaou et al., 2018). Impaired functional responses of neutrophils in older individuals may therefore also be detrimental during pneumococcal infection.

In the nose and lungs of healthy older people, neutrophils are highly abundant (Thompson et al., 1992; Meyer et al., 1996; Reiné et al., 2019). Excessive neutrophil recruitment to the lung following infection can mediate tissue damage and may exacerbate inflammation in older people (Menter et al., 2014). In frail older individuals, neutrophils have an immature profile with increased levels of intracellular reactive oxygen species (ROS) and cell surface expression of proinflammatory markers like CD11b (Verschoor et al., 2015). They have an impaired capacity to produce neutrophil extracellular traps (Hazeldine et al., 2014) and altered chemotaxis responses to respiratory infection, which leads to prolonged production of proteinase and a pro-inflammatory milieu (Sapey et al., 2014; Sapey et al., 2017). Neutrophils in older individuals may also be impaired in their opsonophagocytotic ability to several bacterial pathogens including *S. pneumoniae* and *S. aureus* (Wenisch et al., 2000; Butcher et al., 2001; Simell et al., 2011). Interestingly, vitamin E supplementation in aged mice prevented neutrophil migration and mortality during pneumococcal pneumonia (Ghanem et al., 2015), suggesting that regulating neutrophil activity in older individuals could be beneficial to the host.

TABLE 2 | Changes in Innate Immunity with age.

Changes with age	Monocytes/Macrophage	Neutrophils	MAIT cells
Prevalence	↑ Alveolar macrophages ↑ CD14++CD16+ monocytes	↑ Nose and lungs	↓ Blood ↓Total CD8+ T cells in nasal mucosa
Cellular changes	↓ TLR1/2/4 ↓STING/TBK1/IRF3 ↓ Inflammasome activation	↑ CD11b ↓Extracellular traps, migration and opsonophagocytosis	↑ Clonal expansion
Cytokine responses	↑ TNF baseline ↓TNF, IL-6, IL-1β, IL-8, IFNβ	↑ ROS, Proteinase	

MAIT cells

Unconventional, innate-like T cells called MAIT cells, play important roles in the defense against bacterial and viral infections. Recently, studies with the EHPC model demonstrated that blood and nasal MAIT cells were associated with protection from pneumococcal colonization (Jochems et al., 2019b). They can be activated by conserved bacterial ligands derived from vitamin B (riboflavin) biosynthesis or indirectly *via* cytokines (Godfrey et al., 2019; Toubal et al., 2019). MAIT cells recognize precursors of the riboflavin synthesis pathway after presentation *via* MHC class I–related protein 1 (MR-1). This pathway is highly conserved in the pneumococcal genome, and MAIT cells can respond to pneumococcal isolates in both MR-1 dependent and independent manners (Kurioka et al., 2018). MAIT cells are depleted from blood in old age, with levels progressively dropping from 3-5% of T cells in young adulthood to below 1% in older adults (Novak et al., 2014; Walker et al., 2014; Loh et al., 2020). Remaining MAIT cells in this population show clonal expansion, similar to conventional CD8 T cells, and increased basal inflammation, although they retain potent anti-microbial function (Loh et al., 2020). There appears to be a substantial depletion of total CD8+ T cells from the nasal mucosa in older adults (Reiné et al., 2019) and the ratio of CD4/CD8 T cells increases in the lung in middle age (Meyer et al., 1996). Therefore, as MAIT cells are predominantly found within the CD8+ T cell compartment, we postulate that there is loss of MAIT cells at the mucosal surface in older individuals which negatively impacts on the control of pneumococcal colonization. This warrants further investigation.

Th17 and T Regulatory Cells (Tregs)

In murine models, a Th17-mediated recruitment of monocytes and neutrophils leads to clearance of *S. pneumoniae* colonization (Lu et al., 2008; Zhang et al., 2009). Some human studies also suggest a role for Th17 cells in protection against colonization showing increased ratios of pneumococcal-specific Th17/Tregs with increasing age as carriage rates decrease (Mubarak et al., 2016) and a SNP in the IL17A gene associated with ~2-fold increased risk of pneumococcal colonization in children in the first year of life (Vuononvirta et al., 2015). However, a protective role for Th17 cells in humans has not been fully substantiated. We have shown acquisition of pneumococcal antigen-specific tonsillar Th1 T cells but not Th17 cells with age (Pido-Lopez et al., 2011). In the EHPC model, Th17 cells were found in the lung after colonization, which associated with increased bacterial killing in macrophages (Wright et al., 2013), but were not identified in the nasopharynx. Higher Th17 responses were found in children from Bangladesh with high carriage rates, compared to children from Sweden with lower carriage rates (Lundgren et al., 2012). In HIV^+ individuals in Malawi where carriage rates are high, there was no evidence of a Th17 protective phenotype (Glennie et al., 2013). Furthermore, colonization by *S. pneumoniae* has been associated with decreased Th17/Treg ratios in children, possibly mediated by TGF-β induction leading to regulatory responses (Zhang et al., 2011; Neill et al., 2014; Jiang et al., 2015). Stimulation of PBMCs from individuals of different ages with three different pneumococcal proteins revealed a non-significant decrease in responses of older adults, although polyfunctionality (co-production of IFNγ by same donor) was decreased (Schmid et al., 2011). In older adults, total Th17 numbers in blood are decreased and total Treg numbers increased (van der Geest et al., 2014). Together, these observations highlight that a role of Th17 cells in conferring protection against colonization in humans remains unclear. Further investigations of mucosal Th17 and Tregs responses in older people using longitudinal studies and the EHPC model are required.

PNEUMOCOCCAL VACCINES IN OLDER PEOPLE

With the high risk of *S. pneumoniae* infection in the older population and an ageing population, there is an urgent need for effective vaccine approaches to protect this vulnerable group. The 23-valent pneumococcal polysaccharide vaccine (PPV23) is widely used in richer countries to prevent pneumococcal disease in older people and often administered alongside the influenza vaccine. Observational studies have suggested that PPV23 reduces the incidence of pneumococcal pneumonia and vaccine-serotype IPD and mortality in older individuals by 29% - 57% (Christenson et al., 2001; Andrews et al., 2004; Spindler et al., 2008; Suzuki et al., 2017). However, meta-analyses have suggested that PPV23 may only be beneficial against IPD, with no effect against the far more common non-bacteremic pneumonia (Moberley et al., 2013; Latifi-Navid et al., 2018). Along with its disputed efficacy against pneumonia, PPV23 has no protective effect against pneumococcal colonization (Adler et al., 2020).

PPV23 induces the production of anti-capsular antibodies *via* a T-cell-independent mechanism. Pneumococcal conjugate vaccines (PCV) induce higher antibody levels and longer-term immune memory *via* carrier protein mediated T-cell-dependent mechanisms. In controlled infection studies of young adults, PCV13 reduced pneumococcal colonization (Collins et al., 2015; German et al., 2019). Together with routine vaccination of children with PCV, which reduces carriage and transmission, they protect older people from *S. pneumoniae* infections through herd immunity. However, as serotype replacement threatens the efficacy of the vaccine (Lewnard and Hanage, 2019), new strategies to protect older individuals are required.

Clinical trials have confirmed that PCV13 can reduce the incidence of vaccine-serotype colonization in older adults, though the effect did not persist beyond six months (van Deursen et al., 2018). This impact of PCV13 on pneumococcal colonization may therefore be expected to overcome some of the deficiencies of PPV23 in its activity against pneumonia and, in adults >65yrs, PCV13 indeed demonstrated a 45% efficacy against non-bacteremic CAP caused by the vaccine serotypes, along with a 75% efficacy against IPD (Bonten et al., 2015). PCV20 represents a potential further advance due to the increase in serotype coverage, and results of phase 3 trials in older

individuals are currently awaited (clinical trial NCT03835975) (Pfizer, 2019).

The process of inflammageing in older individuals which includes physical airway alterations, shifts in the microbiome coupled with effects on the epithelium and innate immunity, will likely contribute to a decreased efficacy of the pneumococcal vaccines. Therefore, understanding in more detail the underlying molecular and cellular mechanisms could help identify interventions to enhance immune responses in this population group. Novel vaccine approaches such as targeting pneumococcal proteins, using whole cell inactivated or attenuated strains, and with new adjuvants or immunomodulating agents to overcome the effects of inflammageing, may enhance protection against IPD in older individuals (Feldman and Anderson, 2014; Ramos-Sevillano et al., 2020; Wagner and Weinberger, 2020). Potential novel mucosal immunomodulating interventions include statins, vitamin supplementation and changes to the nasal microbiome to boost mucosal immunity, but these need to be first supported by high quality clinical trial data. In the meantime, high levels of pneumococcal vaccine uptake by adults alongside PCV vaccination of younger individuals to generate herd immunity will have the greatest effect on the morbidity and mortality associated with *S. pneumoniae* in older individuals. Additional important preventative strategies include annual health checks and smoking cessation as heart disease, diabetes mellitus and smoking increase the incidence of CAP (Torres et al., 2015).

DISCUSSION

The immune dysregulation associated with inflammageing has wide ranging effects on the control of pneumococcal colonization and the transition to invasive disease. To better define these processes, in parallel with murine models and *in vitro* culture systems, the EHPC model provides a unique and safe opportunity to investigate in detail the cellular and molecular changes involved. The model also enables investigation of pneumococcal-epithelial-innate immune cell interactions and activation. For example, the model can be used to assess changes in pneumococcal micro-invasion of the epithelium and characterize subsequent alterations in epithelial-derived innate immune responses following pneumococcal infection in older individuals. Transcriptomic and metabolomic approaches applied to these systems will lead to further understanding of molecular changes that occur during inflammageing and how they influence pneumococcal infection (Valdes et al., 2013; Giamarellos-Bourboulis et al., 2020).

In the last 128 years since William Osler's observations on pneumonia, advances in the understanding of mucosal immune protection against pneumococcal disease in older individuals has progressed considerably. However, it is evident that there is still much more that needs to be discovered if we are to reduce the burden of pneumococcal disease in this vulnerable population.

AUTHOR CONTRIBUTIONS

CW and RH conceptualized the review. CW planned, wrote and revised the manuscript. SJ and HA wrote and revised the manuscript. JB, DF, and RH critically read and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

CW and RH are supported by the Medical Research Council, Grant Ref: MR/T016329/1. RH is a National Institute for Health Research (NIHR) Senior Investigator. The views expressed in this article are those of the authors and not necessarily those of the NIHR, or the Department of Health and Social Care.

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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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