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The innate immune response to *Streptococcus pneumoniae* in the lung depends on serotype and host response

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Abstract

Bacteremic pneumonia with some pneumococcal capsular serotypes, including serotype 3 (ST3), has been associated with a higher risk of death, whereas others, such as ST8, are associated with a lower risk. To provide a molecular basis for understanding such differences, we used oligo cDNA microarrays to analyze and compare the gene expression profiles of the lungs of Balb/c mice infected intranasally with either ST3, strain A66.1, or ST8, strain ATCC 6308 (6308). Compared to uninfected controls, infection with either A66.1 or 6308 led to inoculum-dependent expression of IFN- γ inducible CXC chemokine among other pro-inflammatory genes. To investigate the role that IFN- γ inducible chemokines CXCL9, CXCL10 and CXCL11 play in A66.1- and 6308-induced pneumonia, we examined the effect of the absence of their common receptor, CXCR3, on intranasal infection in CXCR3^{-/-} (Balb/c) mice. Compared to wild type (WT) mice, virulence of A66.1 but not 6308 was attenuated in CXCR3^{-/-} mice. A66.1-infected CXCR3^{-/-} mice had fewer lung neutrophils and more alveolar macrophages 48 hours after infection and fewer blood CFU 72 hours after infection. Histopathological examination of lung sections revealed less inflammation among A66.1-infected CXCR3^{-/-} than WT mice. The reduced virulence of A66.1 in CXCR3^{-/-} mice suggests that inhibition of the functional activity of IFN- γ inducible chemokines modulates the host response to A66.1, suggesting a novel approach to improve vaccine-mediated protection against ST3 pneumonia.

Keywords

Serotype 3 pneumococcus; Serotype 8 pneumococcus; IFN- γ inducible chemokines; CXCR3; inflammation; pneumococcal pathogenesis

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Introduction

Despite the use of pneumococcal vaccines, *Streptococcus pneumoniae* (pneumococcus) is the main cause of community-acquired pneumonia in the United States, which is associated with significant morbidity, mortality and cost [1]. There are more than ninety capsular polysaccharide types, or serotypes (STs) of pneumococcus [2], with the prevalence of disease-causing STs varying by geographic location and age [3]. In the pre-pneumococcal conjugate vaccine (PCV) era, ST3 and ST8 caused disease predominantly in adults [3;4]. ST3 and ST8 are included in the 23-valent capsular polysaccharide vaccine that is used in adults, but neither is included in the heptavalent pneumococcal conjugate vaccine (PCV7). Since use of PCV7 began in 2000, ST3 has emerged as a cause of severe pneumonia and empyema [5]. A ST3 moiety is included in the newly introduced PCV-13 [6]; however, to date, PCVs containing ST3 moieties have not protected against ST3 [7].

There are ample data that the severity of pneumococcal disease in humans is ST-specific [4;8;9]. Similarly, data from mouse models demonstrate that virulence is strongly associated with capsule type [10–16]. Among pneumococcal STs for which data are available, those that cause invasive disease in adults also cause invasive disease in mice, whereas those that predominantly cause mucosal disease in children are often not invasive in mice [10]. *In vitro* assays have demonstrated a correlation between capsule type and phagocytosis, with phagocytosis of virulent STs being poorer than less virulent STs [7;9;17;18]. Vaccine failure to protect against ST3 and the inability to develop therapeutic ST3-specific serum in the pre-antibiotic era against this ST has been linked to inhibition of phagocytosis by the ST3 capsular polysaccharide [7;19]. However, ST3 virulence in mice has also been shown to stem from a detrimental lung inflammatory response [11;16].

The ST3 capsule is a polymer comprised of cellobiuronic acid that produces large mucoid colonies on blood agar plates, whereas the ST8 capsule is comprised of cellobiuronic acid with two hexoses that produce smaller, non mucoid colonies [20;21]. In addition, ST8 strains produce a non-hemolytic pneumolysin [12;22]. In an analysis of ST-specific outcomes in bacteremic pneumococcal pneumonia, ST3 was associated with increased, whereas ST8 was associated with reduced risk of death compared to a reference ST [9]. In a recent report, ST8 was the most common cause of invasive pneumococcal disease in Spain [23]. ST8 was also associated with bacteremic, but not non-bacteremic pneumonia in patients with COPD, whereas ST3 was associated with bacteremic and non-bacteremic disease [24]. To seek a molecular basis for ST3- and ST8-induced disease, we compared gene expression profiles of the lungs of ST3 (A66.1)- and ATCC 6308 (6308)-infected mice. Our data show that although both STs induced inoculum-dependent expression of IFN- γ inducible chemokines, A66.1 infection induced more expression in a model with similar lung bacterial burdens and absence of CXCR3, the shared receptor for these chemokines, reduced its lethality in mice.

Materials and Methods

Mice

Wild type (WT) female Balb/c mice (6–8 weeks of age) were obtained from the National Cancer Institute (Bethesda, MD). Mice were housed in a pathogen-free facility at the Albert Einstein College of Medicine (Bronx, NY). CXCR3^{-/-} mice on the Balb/c background, which were generated and provided by Dr. B. Lu (Harvard Medical School, MA) [25], were bred and housed at Albert Einstein College of Medicine (Bronx, NY) in a pathogen-free facility. All animal protocols used in this study were approved by the Institution for Animal Care and Use at Albert Einstein College of Medicine.

Bacteria and Infection of Mice

ST3 (strain A66.1) *Streptococcus pneumoniae* was originally provided by Dr. D. E. Briles (University of Alabama, Birmingham). ST8, ATCC 6308, was purchased from American Type Culture Collection (Rockville, MD). The organisms were grown in tryptic soy broth (TSB) (DIFCO, Sparks, MD) to mid-log phase at 37°C and 5% CO₂ and frozen in TSB with 10% glycerol and stored at -80°C until use. To induce pneumococcal pneumonia, mice were anaesthetized with isoflurane and intranasally (i.n.) inoculated as described previously [11;13;16;26]. To determine the amount of bacteria actually administered, the inoculum was cultured on trypticase soy agar plates with 5% sheep's blood (Becton Dickinson, Sparks, MD) before and after infection of mice.

Determination of LD₁₀₀ and Survival Study

LD₁₀₀ experiments were performed to identify inocula of A66.1 and 6308 that resulted in a similar bacterial burden in the lungs 24 hours post-infection and a similar clinical outcome (death) in a similar timeframe. Death in the intranasal model with each ST is associated with bacteremia [11;12]. WT Balb/c mice were infected i.n. with A66.1 and 6308 and survival was monitored and recorded every day and more frequently when mice were ill. Inocula of 5×10^5 CFU of A66.1 and 5×10^3 CFU of 6308 resulted in the death of 100 percent of infected mice by 72 hours (Fig. 1A) post-infection and comparable lung and blood CFU 24 hours (Fig. 1B). An inoculum of 5×10^3 CFU A66.1 did not cause death by 72 hours post-infection and an inoculum of 1×10^5 CFU 6308 resulted in a survival curve similar to 5×10^3 CFU 6308 (data not shown). Thus, to be able to compare gene expression in the lungs for a similar clinical course/outcome, we used an inoculum for each strain that resulted in comparable lung CFU and death by 72 hours after infection: 5×10^3 CFU 6308 and 5×10^5 CFU A66.1, for expression profiling (see below).

Preparation of total RNA from lungs

Mice infected with the aforementioned inocula (5×10^5 CFU for A66.1 and 5×10^3 CFU for 6308) were killed 24 hours post-infection and lung tissue was removed aseptically, washed in 5ml of sterile PBS and homogenized in 6ml of Trizol (Invitrogen, Carlsbad, CA) as described [26]. Total RNA was extracted using Trizol according to manufacturer's instructions and purified with RNeasy kit (Qiagen, Valencia, CA). Integrity and concentration of total RNA was measured by Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA). Extracted total RNA was kept at -80°C until use.

Oligo cDNA microarrays

Two-color mouse oligo cDNA microarrays were purchased from Agilent Technologies (Santa Clara, CA). Total RNA from lung tissue was isolated with Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Labeling of RNA for hybridization was performed with the Agilent Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Briefly, 1 µg of total RNA was reverse transcribed with oligo dT-promoter primer and MMLV-RT. Next, cDNAs were converted to fluorescent antisense cRNA using T7 RNA polymerase and either Cyanine 5-CTP or Cyanine 3-CTP. Fluorescent antisense cRNA was purified using Qiagen's RNeasy kit (Qiagen, Valencia, CA). The concentration of antisense cRNA was measured and 1.65 µg of cRNA samples was fragmented with 25X fragmentation buffer and 10X blocking agent (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. Samples were hybridized to the array for 17 hours at 65°C. Slides were washed and scanned at 5 µm resolution (Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. Features were extracted from raw images using Agilent Feature Extraction

Software version 10.1.1.1. Further analysis was done using the Linear Models for Microarray Data (LIMMA) software.

Histopathological analysis of lung tissue from A66.1- and 6308-infected Balb/c mice

Histopathology was performed on lung sections from WT Balb/c mice infected i.n. with 5×10^5 CFU A66.1 and 5×10^3 and 5×10^5 CFU 6308. These inocula were chosen because we wanted to examine the inflammatory response with each ST in the setting of a similar bacterial burden and clinical endpoint (death) at a similar time after infection. An inoculum of 5×10^5 CFU 6308 was used, because induction of IFN- γ -inducible chemokines was similar between the two STs at this inoculum (see results); an inoculum of 5×10^3 A66.1 was not used because this was not a lethal inoculum. Mice were anesthetized with isoflurane and killed by cervical dislocation 24 hours postinfection. Lungs were inflated with 10% formalin and fixed in formalin for 48 hours. Lung lobes were excised and put in cassettes and immersed in 70% ethanol. Paraffin embedded tissue sections of 5 μ m were cut and stained with H&E by the Albert Einstein College of Medicine Pathology Core facility and viewed with AxioCam MRc Zeiss microscope. Histopathological examination was performed by an experienced veterinary pathologist.

Quantitative polymerase chain reaction analysis

The expression of selected genes identified in the microarray experiments was determined by quantitative real-time PCR. For these experiments, total RNA from lung tissue was prepared from mice infected with different inocula of A66.1 and 6308 as described above, after which 1 μ g of total RNA was reverse transcribed using iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). Next, Power SYBR Green Supermix from Applied Biosystems (Carlsbad, CA) and appropriate forward and reverse primer sets from SA Biosciences (Fredrick, MD) were used to amplify specific genes at 24 hours post-infection.

Determination of the survival and bacterial burden in the lungs and blood of A66.1- and 6308- infected CXCR3^{-/-} mice

Based on the results of the microarray experiments, which revealed induction of IFN- γ inducible CXC chemokines, survival studies were performed with Balb/c CXCR3^{-/-} and WT mice infected i.n. with A66.1 or 6308 as described above. Separate groups of CXCR3^{-/-} and WT mice were used for bacterial burden experiments. At different times post-infection, mice were anaesthetized with isoflurane and terminally bled by cardiac puncture. Blood was immediately diluted in TSB and kept on ice until it was cultured. Lung tissue was excised and homogenized in 1 ml of HBSS. Lung homogenates were diluted in TSB and kept on ice until they were cultured. Then serial dilutions of both blood and lung homogenates were cultured in duplicate on blood agar plates and incubated overnight at 37°C with 5% CO₂. The number of colonies (CFU) on the plates was counted the next day.

Histopathological analysis of A66.1-infected CXCR3^{-/-} versus WT mice

We compared the lung inflammatory responses of A66.1-infected WT and CXCR3^{-/-} mice 24 and 48 hours post-infection histopathologically to evaluate the influence of the functional activity of IFN- γ inducible CXC chemokines on the inflammatory response. Mice were infected with 5×10^4 CFU A66.1, anesthetized with isoflurane, killed at the indicated times, and tissue was processed and evaluated as described above.

Analysis of lung cellular populations in A66.1-infected CXCR3^{-/-} and WT mice by flow cytometry

Mice were infected with A66.1 and killed 24 and 48 hours post-infection as described above. Then, lung tissue was excised from groups of three to six WT and CXCR3^{-/-} mice

and placed in a petri dish containing 10ml of digestion buffer which consists of RPMI complemented with 1mg/ml collagenase A, 30µg/ml DNase I and 10% FBS. Then, lung tissue was chopped into pieces with two razor blades, incubated at 37°C for 30 min, placed on a 70µm cell strainer and teased apart using a 3ml syringe plunger. Cell suspensions were spun at 1200 RPM for 10 minutes. Cells were washed, red blood cells were lysed and cells were again washed, passed through a 70µm cell strainer and counted. 1×10^6 cells per sample tube were incubated with CD16/CD32 for 15 min (BD Pharmingen, San Diego, CA) and stained with a combination of CD8-PE-Cy7 (eBioscience, San Diego, CA), CD49b-APC (BioLegend), F4/80-Alexa 647 (BioLegend, San Diego, CA), CD3-Alexa Fluor 488, CD4-Alexa Fluor 700, CD45-Pacific Blue, CD19-PE-Cy7, CD11b-PE-Cy7, CD11c-PerCP-Cy5.5 and Ly6G-FITC (BD Pharmingen). DAPI was added right before samples were run on the LSR II. Flow-Jo software was used to gate on the leukocyte population on the forward- and side-scatter plots. Next dead cells were excluded by gating out DAPI positive cells. Subsequent gating was done on DAPI⁻CD45⁺ cells to identify the following cell populations neutrophils (Ly6G⁺), alveolar macrophages (F4/80⁺CD11c⁺), tissue macrophages (F4/80⁺), dendritic cells (CD11c⁺), B cells (CD19⁺), NK cells (CD3⁻DX5⁺), CD4 T cells (CD3⁺CD4⁺) and CD8 T cells (CD3⁺CD8⁺).

Statistical analysis

The results of survival studies were tested for statistical significance using the Log-rank test (GraphPad Prism, San Diego, CA). Unpaired student t-test was used for normally distributed data, including real-time polymerase chain reaction and flow cytometry data. The Mann-Whitney test was used for non-normally distributed data, including blood and lung CFU studies and cytokine assays. For microarray data p-values were adjusted for multiple testing by the method of Benjamini & Hochberg. Gene Ontology analysis was performed using the *topGO* package and results were ranked using the Kolmogorov-Smirnov test via the *topGO* classic method.

Results

Survival studies with A66.1 and 6308 in Balb/c mice

The inocula for each ST that resulted in a comparable bacterial burden in the lungs 24 hours post-infection and a lethal outcome by 72 hours post-infection were 5×10^5 CFU for A66.1 and 5×10^3 CFU for 6308 (Fig. 1A and 1B). These inocula resulted in comparable lung and blood CFU ($p=0.09$ and $p=0.41$, respectively, Mann Whitney test, Fig. 1B) with no statistically difference in survival ($p=0.063$, Log-rank test, Fig. 1A). Histopathological examination of lung sections performed 24 hours post-infection with these inocula revealed a more robust inflammatory response with more perivascular inflammation, edema, suppuration and neutrophil degeneration in the lungs of A66.1 than 6308-infected mice; whereas sections from mice infected with 5×10^5 CFU 6308 revealed an inflammatory response similar to 5×10^5 CFU A66.1 (Fig. 1C).

Lung gene expression profiles after infection with A66.1 and 6308

Inocula of 5×10^5 CFU A66.1 and 5×10^3 CFU 6308 were selected for use in the microarray experiments to enable us to compare the response to each ST in mice with a similar clinical course. Twenty-four hours post-infection, comparison of the gene expression profiles of naïve, uninfected mice to mice receiving the lethal inocula of either A66.1 (5×10^5 CFU) or 6308 (5×10^3 CFU) revealed that A66.1 induced more expression of numerous pro-inflammatory cytokines and chemokines, including IFN- γ , CXC, and CC chemokines, and other pro-inflammatory cytokines (Table 1). Compared to uninfected mice, A66.1 induced a 24-fold increase in IFN- γ gene expression 24 hours post-infection, whereas 6308 induced a 2-fold increase ($p = 5.16 \times 10^{-7}$). The IFN- γ inducible chemokines CXCL9, CXCL10, and

CXCL11 were among the genes most up-regulated by A66.1: compared to uninfected mice, expression of CXCL9, CXCL10 and CXCL11 were 29-, 501-, and 69-fold higher, respectively, in A66.1-infected mice, whereas these chemokines were 6-, 16-, and 17-fold higher, respectively in 6308-infected mice.

Effect of inoculum on gene expression

Given that the lethal inocula of A66.1 and 6308 differed by two logs, we compared the expression of the most up-regulated genes from the microarray, the IFN- γ -inducible chemokines CXCL9, CXCL10 and CXCL11, at other inocula by quantitative real-time PCR to assess the effect of inoculum on gene expression. Comparing expression of these genes in uninfected mice to mice infected with either 5×10^3 or 5×10^5 CFU of A66.1 or 6308 revealed that gene expression was inoculum-dependent for both STs (Fig. 2A and 2B). However, mice infected with 5×10^3 CFU, a lethal inoculum for 6308 but not A66.1, demonstrated a trend towards more expression of CXCL 9 and CXCL11 and significantly more expression of CXCL10 than A66.1-infected mice at this inoculum (Fig. 2A). On the other hand, when mice were infected with 5×10^5 CFU, a lethal inoculum for both STs, there was a similar increase in IFN- γ inducible chemokine gene expression (Fig. 2B).

CXCR3^{-/-} mice are more resistant to A66.1 than WT Balb/c mice, but virulence of 6308 is not altered

The interferon- γ -inducible chemokines, CXCL9, CXCL10 and CXCL11 bind a common receptor, CXCR3 [27;28]. Given that CXCL9, CXCL10 and CXCL11 were among the most highly expressed genes in the lung microarrays and expression was higher in A66.1- than 6308-infected mice with similarly lethal outcomes (see above), we determined the effect of a loss of their function on the outcome of infection in CXCR3^{-/-} mice. CXCR3^{-/-} mice infected with 5×10^4 or 5×10^5 CFU A66.1 survived significantly longer than WT mice ($p=0.01$ for 5×10^4 CFU, Fig. 3A; $p=0.004$ for 5×10^5 CFU, Fig. 3B). A similar trend that was not statistically significant was demonstrated in C57BL/6 CXCR3^{-/-} mice for the 5×10^5 CFU dose (data not shown). There was no difference in survival of 6308-infected CXCR3^{-/-} and WT mice infected with 5×10^3 CFU (Fig. 3C). This result was confirmed at other inocula (data not shown).

A66.1-infected CXCR3^{-/-} mice develop less bacterial bloodstream invasion than WT mice

Lung CFU of A66.1-infected WT and CXCR3^{-/-} mice were comparable at all times tested post-infection (Fig. 4A). Blood CFU were higher in A66.1-infected WT than CXCR3^{-/-} mice 72 hours post-infection ($p = 0.03$) (Fig. 4B).

A66.1-infected CXCR3^{-/-} mice recruit fewer lung neutrophils and more alveolar macrophages to the lungs than WT mice

The phenotypes of cells in the lungs of mice in the naïve state and 24 and 48 hours post-infection with 5×10^4 CFU of A66.1 revealed that neutrophil recruitment into the lungs of WT and CXCR3^{-/-} mice reached a peak 24 hours post-infection ($p < 0.05$) (Fig. 4C). WT mice had significantly more neutrophils in the lungs 24 and 48 hours post-infection than in the naïve state. On the other hand, CXCR3^{-/-} mice had a comparable level of neutrophils at 48 hours post-infection and in the naïve state. By 48 hours post-infection, WT mice had significantly more neutrophils than CXCR3^{-/-} mice, whereas CXCR3^{-/-} mice had significantly more alveolar macrophages than WT mice. The higher level of alveolar macrophages observed by cell count was also observed for the percentage of alveolar macrophages (out of total CD45⁺ cells) (Fig. 4C). CXCR3^{-/-} mice also exhibited significantly higher dendritic cell counts in their lungs 48 hours post-infection than WT mice (Fig. 4C). There was no statistical difference between A66.1-infected WT and CXCR3^{-/-}

mice in the percentage or total cell count of lung B cells, NK cells, CD4 T cells and CD8 T cells (Supplementary figure 1).

There was no significant difference between WT and CXCR3^{-/-} mice in the expression of several pro-inflammatory cytokines and chemokines known to be up-regulated during pneumococcal infection (Supplementary figure 2). However, within group differences were observed. For WT mice, there was continued production of IL-6 and the neutrophil chemoattractant MIP-2 at each time examined, with statistical significance at 72 compared to 24 hours post-infection ($p < 0.05$ and $p < 0.001$, respectively). CXCR3^{-/-} mice also had significantly more MIP-2 at 72 than 24 hours post-infection ($p < 0.05$).

The lungs of WT mice exhibit more inflammation and tissue damage than those of CXCR3^{-/-} mice

Histopathological examination of lung sections from WT and CXCR3^{-/-} mice 24 hours post-infection revealed that WT mice had mild perivascular inflammation with minimal airway inflammation, whereas CXCR3^{-/-} mice had less perivascular, but more airway inflammation (Fig. 5A and B). At 48 hours post-infection, WT mice still had perivascular inflammation but CXCR3^{-/-} mice had virtually no inflammation (Fig. 5E and F). Additionally, minimal to mild suppurative pleuritis was observed in WT, but not CXCR3^{-/-} mice. Hence, A66.1-infected CXCR3^{-/-} mice exhibited resolution of inflammation.

Discussion

The lung gene expression profiles presented herein demonstrate that a ST3 (A66.1) and a ST8 (6308) strain of *S. pneumoniae* each induced IFN- γ -inducible chemokine gene expression in the lungs of Balb/c mice in an inoculum dependent manner. However, gene expression was higher among A66.1- than 6308-infected mice for inocula that resulted in a similar lung bacterial burden and clinical picture, e.g. death at a similar time after infection. These findings illustrate that given the same clinical picture; the host response to different STs can be very different. This point is relevant to human disease in that some patients with pneumococcal pneumonia respond to antibiotics and improve, whereas others do not. At the inocula used in our model, 6308 invasiveness was not preceded by significant pulmonary inflammation and was accompanied by less IFN- γ inducible CXC chemokine gene expression than infection with A66.1. Other groups have also used microarray-based gene expression profiling to examine the murine lung inflammatory response to pneumococcal pneumonia [29–32]. Our study confirms and extends to ST8 (6308) data showing that type I and II interferon gene expression was increased by ST3 (NCTC7978 (A66)) pulmonary infection in C57Bl/6 and ST2 (D39) in a colonization model in Balb/c mice [29;30].

Lung CFU were comparable statistically 24 hours post-infection in mice infected with 5×10^5 CFU A66.1 or 5×10^3 CFU 6308 with no significant difference in survival. However, there was a trend towards earlier death in 6308-infected mice even though they received two logs less bacteria and had a less extensive inflammatory response at this inoculum. Hence, our data illustrate that the lung inflammatory response does not predict clinical outcome for 6308. The propensity of ST8 to invade the bloodstream in mice mirrors clinical data from a recent study showing that the most common ST causing invasive pneumococcal disease was ST8 [23]. The invasiveness of 6308 in mice [12;26] and ST8 strains in humans could be an effect of the non-hemolytic pneumolysin expressed by 6308 and other ST8 strains [12;33]. Hemolytic pneumolysin was required for a protective host response to pneumococcus in mice [33;16], but its absence increased lethality of A66.1 in one model [16].

Microarray data showed that IFN- γ and IFN- γ associated genes, including CXCL9, CXCL10 and CXCL11, were among the most up-regulated genes by both STs. Gene expression was

inoculum-dependent, but higher in A66.1- than 6308-infected mice at inocula that resulted in similar lung and blood CFU. CXCL9, CXCL10 and CXCL11 recruit CXCR3-expressing leukocytes, and are expressed by macrophages, neutrophils, and epithelial and endothelial cells in the lungs [34]. CXCR3 is expressed on Th1 polarized activated T-cells, NK cells, peripheral blood B cells and plasmacytoid and myeloid dendritic cells [35–38]. Neutrophils produce IFN- γ inducible chemokines in response to IFN- γ [39] and CXCL10 has been implicated in neutrophil recruitment and acute lung injury [40]. Previous reports have shown that ST3 (NCTC7978 (A66.1)) induced type I and type II IFN-associated, including IFN- γ inducible chemokine genes in C57Bl/6 mice [30] and that ST4-induced CXCL9, with human and murine CXCL9 exhibiting an ability to kill pneumococcus *in vitro* [41]. However, CXCL9 knockout mice were not more susceptible to pneumococcal infection, suggesting a redundant role for CXCL9 with CXCL10 and CXCL11 [41].

To evaluate the aggregate role of CXCL9, CXCL10, and CXCL11 in the response to A66.1 and 6308, we used CXCR3^{-/-} mice, which lack CXCR3, their common receptor [27;28]. Lethality of A66.1, but not 6308, was reduced in these mice. This finding was paralleled by histopathology demonstrating less lung inflammation in A66.1-infected CXCR3^{-/-} than WT mice 48 hours post-infection and a shift from a neutrophil (24 hours post-infection) to a macrophage predominant (48 hours post-infection) cellular profile (by percent and cell number) in the lungs of CXCR3^{-/-}, but not WT mice. Macrophages were required for clearance of colonization with ST23F [42] and had a protective, immunomodulatory role in an i.n. model of ST3 [43]. In contrast, continued neutrophil recruitment, such as that which we observed in WT mice, is associated with lethality in murine pneumococcal pneumonia models [11;12;44;45]. A66.1-infected CXCR3^{-/-} mice had less lung inflammation than WT mice, but their inflammatory responses were different, as CXCR3^{-/-} mice had an alveolar, and WT mice had a perivascular pattern of inflammation. These findings suggest that alveolar inflammation might actually contain or delay bacteria in the lungs, with perivascular inflammation leading to invasion of the bloodstream. Consistent with the latter, WT mice had more blood CFU and evidence of pleuritis than CXCR3^{-/-} mice 72 hours post-infection.

Our data provide new insight into why antibiotics might fail to ameliorate some cases of pneumococcal pneumonia in the setting of chemokine-driven inflammation, underscoring the need for improved vaccine-mediated protection against pneumonia. However, the inability of currently used adult pneumococcal vaccines to prevent pneumonia poses a significant challenge [46;47]. Although work is underway to improve vaccine efficacy and identify new vaccine targets [46], our data suggest that inhibition of IFN- γ inducible chemokines could have a beneficial effect in modulating the inflammatory response to pneumococcal pneumonia. While such inhibition might enhance vaccine-mediated protection in immunized individuals, its benefit is likely be ST-specific, given that absence of CXCR3 did not attenuate 6308 virulence. Increasing recognition that the inflammatory response to pneumococcus can be deleterious (11;12;16;48) has led to the idea that prevention of the inflammatory manifestations of the host response to pneumococcal pneumonia might require immunomodulation. Our data support this idea, with the caveat that identifying the responsible ST will be needed to identify the patients in whom adjunctive inflammatory inhibitors should be considered for treatment of pneumococcal pneumonia.

Highlights

- To gain insight into differences in the serotype-specific host response to pneumococcal infection in the lungs, we compared the effect of intranasal

infection with a serotype 3 and a serotype 8 strain of pneumococcus on gene expression in the lungs of mice

- Both strains induced expression of interferon-gamma-inducible genes in an inoculum dependent manner
- In a model in which the lung bacterial burden was similar and death occurred by 72 hours after infection, serotype 3-infected mice exhibited a more exuberant inflammatory response and higher levels of interferon-gamma-inducible gene expression than serotype 8-infected mice
- The virulence of serotype 3, but not serotype 8 was reduced in CXCR3-deficient mice
- Serotype 3-infected CXCR3-deficient mice recruited more macrophages to their lungs and had a less exuberant lung inflammatory response than wild type mice

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

i.n.	intranasal
PCV	pneumococcal conjugate vaccine
ST	serotype
TSB	tryptic soy broth
WT	wild type

A **B**

Figure 1. Survival, lung and blood CFU and lung histopathology of WT Balb/c mice following i.n. infection with A66.1 and 6308 *S. pneumoniae*

(A) Percent survival of mice infected with either 5×10^5 CFU of A66.1 (●) or 5×10^3 CFU of 6308 (▲). N = 5–8 mice per group. (B) Lung and blood CFU of mice 24 hours post-infection with 5×10^5 CFU of A66.1 or 5×10^3 CFU of 6308. N = 6–9 mice per group. $p = 0.09$ for lung CFU and $p = 0.41$ for blood CFU, Mann Whitney test. (C) Lung histopathology of mice infected with either 5×10^5 CFU of A66.1 or 5×10^3 or 5×10^5 CFU of 6308 24 hours post-infection. Lung sections were stained with H&E and imaged at 10× and 40× magnifications. Scale bars indicate 100μm at 10× magnification and 25μm at 40× magnification unless indicated otherwise. Lungs of A66.1 infected mice reveal greater perivascular suppurative and necrotizing inflammation and suppurative pneumonia with degenerating neutrophils than 5×10^3 CFU 6308 infected mice. Lungs of mice infected with 5×10^5 CFU of either ST reveal similar histopathological findings.

A **B**

Figure 2. Expression of IFN- γ inducible chemokines by A66.1- or 6308-infected mice

Expression of IFN- γ inducible chemokines in the lungs of infected mice was determined by quantitative real-time PCR. (A) Expression of indicated chemokines at 24 hours post-infection with 5×10^3 CFU of A66.1 or 6308. (B) Expression of indicated chemokines at 24 hours post-infection with 5×10^5 CFU of A66.1 or 6308. N = 3–5 mice per group. * $p < 0.05$, unpaired student t-test

A

B

C

Figure 3. Survival of WT and CXCR3^{-/-} mice infected with A66.1 or 6308

(A) Percent survival of WT (●) and CXCR3^{-/-} (■) mice after i.n. infection with 5×10^4 CFU and (B) 5×10^5 CFU of A66.1. N = 5–13 mice per group. * $p < 0.05$, log rank test. (C) Percent survival of WT (★) and CXCR3^{-/-} (⊙) mice infected with 5×10^3 CFU of 6308. N = 8 mice per group.

A **B**

C

Figure 4. Bacterial burden and lung cellular phenotypes of WT and CXCR3^{-/-} mice
 CFU in (A) Lung homogenates and (B) blood of WT (●) and CXCR3^{-/-} (■) mice after i.n. infection with 5×10^4 CFU of A66.1 at the times indicated on the X axis. The horizontal bars indicate the median values for each dataset. * $p < 0.05$, Mann-Whitney test. $N = 4-9$ mice per group. Cellular infiltration (C) into the lungs in the naïve state and 24 and 48 hours post-infection with 5×10^4 CFU of A66.1 in WT (solid bars) and CXCR3^{-/-} (open bars) mice. The denominator for determining the percents shown on the Y axis was the total number of CD45⁺ cells. Cell count represents absolute number of cells. Error bars represent the standard error of mean. * $p < 0.05$ comparing different times between naïve and infected mice

within the same mouse strain, + $p < 0.05$ comparing the same time across mouse strains; N = 3–6 mice per group.

Figure 5. Lung histopathology after i.n. infection with 5×10^4 CFU of A66.1

Mice were infected with 5×10^4 CFU of A66.1 and sacrificed 24 hours (A–D) and 48 hours (E–H) post-infection. Lung sections were stained with H&E and imaged at 10 $\times$ (A, B, E, F) and 40 $\times$ (C, D, G, H) magnifications. Scale bars indicate 100 μ m at 10 $\times$ magnification and 25 μ m at 40 $\times$ magnification. Inflammation is localized to the perivascular region of the lung in WT mice (A); higher magnification reveals that the inflammation is neutrophilic and necrotizing (C). While inflammation in CXCR3^{-/-} mice is also perivascular, these mice also have evidence of a neutrophilic airway inflammation (B, F) which was generally not present in the WT mice. Inflammation was nearly non-existent at 48 hours in CXCR3^{-/-} mice as compared to WT mice.

Table 1
Gene expression of mice intranasally infected with A66.1or 6308

Three mice per group were infected with 5×10^5 CFU of A66.1 or 5×10^3 CFU of 6308 and at 24 hours post-infection their gene expression was compared to pooled total RNA from uninfected control mice. The logFc values represent the exponent for the log base 2 expression values that compare gene expression of infected mice to uninfected control mice. The fold difference demonstrates the gene expression difference between infected and uninfected mice. This table presents selected genes that are involved in the host cytokine and chemokine response.

			Log Fc Values		Fold Change		
Accession	Symbol	Name	A66.1	6308	A66.1	6308	P.Value
NM_021274	Cxcl10	chemokine (C-X-C motif) ligand 10	9.0	4.0	501	16	9.23E-09
NM_008176	Cxcl1	chemokine (C-X-C motif) ligand 1	6.4	3.8	83	14	4.18E-07
NM_013652	Ccl4	chemokine (C-C motif) ligand 4	6.1	2.4	70	5	4.89E-06
NM_019494	Cxcl11	chemokine (C-X-C motif) ligand 11	6.1	4.1	69	17	5.43E-08
NM_009141	Cxcl5	chemokine (C-X-C motif) ligand 5	5.9	3.5	59	11	2.38E-06
NM_031168	Il6	interleukin 6	5.8	3.3	57	10	2.67E-06
NM_013654	Ccl7	chemokine (C-C motif) ligand 7	5.6	3.1	48	9	1.14E-07
NM_009140	Cxcl2	chemokine (C-X-C motif) ligand 2	5.4	3.1	43	9	2.10E-05
NM_016960	Ccl20	chemokine (C-C motif) ligand 20	5.2	2.1	36	4	1.72E-07
NM_203320	Cxcl3	chemokine (C-X-C motif) ligand 3	5.0	4.3	33	19	1.79E-05
NM_008599	Cxcl9	chemokine (C-X-C motif) ligand 9	4.9	2.6	29	6	2.27E-07
NM_011333	Ccl2	chemokine (C-C motif) ligand 2	4.8	3.0	27	8	9.37E-06
NM_008337	Ifng	interferon gamma	4.6	1.3	24	2	5.16E-07
NM_010555	Il1r2	interleukin 1 receptor, type II	4.4	1.2	21	2	1.79E-06
NM_008361	Il1b	interleukin 1 beta	3.7	1.8	13	4	6.06E-05
NM_009971	Csf3	colony stimulating factor 3 (granulocyte)	3.4	0.6	11	2	2.32E-06
NM_013693	Tnf	tumor necrosis factor	3.3	1.2	10	2	3.03E-05
NM_009969	Csf2	colony stimulating factor 2 (granulocyte-macrophage)	2.8	1.3	7	2	1.72E-06
NM_010554	Il1a	interleukin 1 alpha	2.5	1.7	6	3	4.03E-06
NM_013653	Ccl5	chemokine (C-C motif) ligand 5	2.0	0.4	4	4	3.40E-07
NM_009912	CCR1	chemokine (C-C motit) receptor 1	0.7	-1.0	2	1	1.52E-02