ASC and NLRP3 impair host defense during lethal pneumonia caused by highly virulent serotype 3 Streptococcus pneumoniae

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Miriam H.P. van Lieshout 1,2 *
Alex F. de Vos 1,2 *
Mark C. Dessing 3
Alexander P.N.A. de Porto 1,2
Onno J. de Boer 3
Regina de Beer 1,2
Sanne Terpstra 1,2
Sandrine Florquin 3
Cornelis van ’t Veer 1,2
Tom van der Poll 1,2,4

* These authors contributed equally.

Academic Medical Center, University of Amsterdam, the Netherlands:
1Center of Infection and Immunity Amsterdam
2Center for Experimental and Molecular Medicine
3Department of Pathology
4Division of Infectious Diseases
Abstract

*Streptococcus (S.) pneumoniae* is the most common cause of community-acquired pneumonia. The Nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, consisting of NLRP3, ASC (the adaptor apoptosis-associated speck-like protein containing a CARD) and caspase-1, has been implicated in protective immunity in bacterial infections, amongst other during pneumonia induced by high doses of a moderately virulent strain of *S. pneumoniae*. Here we investigated the role of the NLRP3 inflammasome in the host response during airway infection with a low dose of a highly virulent gradually growing serotype 3 *S. pneumoniae* in a model that more closely mimics human disease. Mice were euthanized at predefined endpoints for analysis or observed in survival studies. In additional studies *Tlr2−/−/Tlr4−/−* mice and *Myd88−/−* mice, incapable of Toll-like receptor signaling, were studied. Surprisingly, and in stark contrast with existing literature, both *Nlrp3−/−* and *Asc−/−* mice showed a strongly improved host defense, as reflected by a markedly reduced mortality accompanied by diminished bacterial growth and dissemination. Lung inflammation and pathology were attenuated in *Nlrp3+/* and *Asc+/* mice during the late stages of the infection. Host defense was unaltered in *Tlr2−/−/Tlr4−/−* mice and *Myd88−/−* mice, although *Myd88−/−* mice demonstrated attenuated lung inflammation in the presence of high pneumococcal burdens. These results show that the NLRP3 inflammasome impairs host defense during lethal pneumonia caused by highly virulent serotype 3 *S. pneumoniae*. Our findings in this clinically relevant model of a highly prevalent human infection challenge the current paradigm that proximal innate detection systems, including the NLRP3 inflammasome and Toll-like receptors (TLRs), are indispensable for an adequate host immune response against bacteria.
Introduction

Community-acquired pneumonia is an important cause of mortality world-wide. The most frequently isolated causative pathogen is *Streptococcus (S.) pneumoniae*, a Gram-positive diplococcus of which over 90 serotypes have been identified (1-3). Depending on health care settings, the mortality rate associated with pneumococcal pneumonia ranges from 6 to > 40% (2, 3). The health burden caused by pneumococcal pneumonia is further aggravated by the increasing incidence of antibiotic resistance in *S. pneumoniae* (4).

In recent years, the importance of large multi-protein complexes called “inflammasomes” for the antimicrobial response has become apparent (5, 6). Inflammasomes consist of a cytosolic sensor protein and caspase-1. The NLRP3 (NLR family pyrin domain containing 3) inflammasome uses the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) to recruit and bind caspase-1, which upon activation cleaves immature pro-IL-1β and pro-IL-18 to the mature secreted forms (5, 6). NLRP3-mediated secretion of IL-1β and IL-18 is a tightly regulated process that requires at least two signals (5, 6). In response to bacterial infection, the first signal occurs when pathogen-associated molecular patterns (PAMPs) derived from the invading pathogen stimulate innate immune cells through activation of pattern recognition receptors, most notably members of the Toll-like receptor (TLR) family, resulting in the transcription and translation of the pro-forms of IL-1β and IL-18. The second signal requires the assembly and activation of the NLRP3 inflammasome resulting in the formation of active caspase-1 by an ASC dependent mechanism (5, 6). Activation of the NLRP3 inflammasome can also induce pyroptosis, which, in contrast to caspase-3-mediated apoptotic cell death, is associated with the release of pro-inflammatory mediators (7).

Recent investigations have revealed an important protective role for the NLRP3 inflammasome during pneumococcal pneumonia (8-11) as indicated by enhanced bacterial growth in the lungs of NLRP3 deficient (*Nlrp3−/−*) and *Asc−/−* mice after infection with a serotype 2 pneumococcus (D39) (8-10). One study examined the contribution of NLRP3 to host defense during serotype 3 *S. pneumoniae* pneumonia, reporting a more modest protective role (11). *S. pneumoniae* can activate the NLRP3 inflammasome through its crucial virulence factor pneumolysin (10, 12). Besides NLRP3 and ASC, several TLRs have been reported to contribute to the protective immunity during pneumococcal infection. TLR2, that detects lipoteichoic acid, a constituent of the pneumococcal cell wall (13, 14), played a modest role in the cytokine response during pneumococcal pneumonia after infection with a serotype 3 pneumococcus (15), while TLR4 limited the growth of pneumococci during nasopharyngeal colonization (16) and lower respiratory infection (17), at least in part by recognition of pneumolysin (16, 18). Likewise, *Tlr9−/−* mice showed enhanced bacterial growth and dissemination after induction of pneumococcal pneumonia (19). The intracellular adaptor protein myeloid differentiation primary response gene 88 (MyD88) is a critical component in TLR2, TLR4 and TLR9 signaling, and in accordance *Myd88−/−* mice showed a profoundly enhanced bacterial outgrowth and a strongly reduced survival after intranasal infection with serotype 2, 4 or 19F *S. pneumoniae* strains (20, 21).
The serotype of the infecting *S. pneumoniae* is an important risk factor for the occurrence of invasive disease and mortality (22). In humans, infections caused by serotype 3 pneumococci are associated with a complicated course and an increased risk of death (22-25). In conjunction with the implementation of the 7-valent pneumococcal conjugate vaccine (26, 27), the prevalence of invasive pneumococcal disease caused by non-vaccine serotypes, including serotype 3, has increased. Our laboratory has used a highly virulent serotype 3 *S. pneumoniae* strain (6303) in a mouse model of pneumococcal pneumonia to mimic severe infection associated with this serotype (15, 28-30). In contrast to models with the commonly used D39 serotype 2 strain, low dose infection with *S. pneumoniae* 6303 results in a high mortality in immune competent mice caused by a gradual growth and subsequent dissemination of bacteria, thereby more closely mimicking human disease (8, 10, 14, 15, 18, 28-31). Here we infected *Nlrp3*<sup>-/-</sup> and *Asc*<sup>-/-</sup> mice with low dose *S. pneumoniae* 6303 via the airways to investigate the role of the NLRP3 inflammasome in the host response. Much to our surprise we found that both *Nlrp3*<sup>-/-</sup> and *Asc*<sup>-/-</sup> mouse strains were strongly protected against lethality. We also infected *Myd88*<sup>-/-</sup> mice with *S. pneumoniae* 6303 and found that MyD88-dependent signaling does not contribute to protective immunity in this model. Our data report for the first time a detrimental role of the NLRP3 inflammasome during respiratory tract infection in a clinically relevant model of a common human disease.

**Methods**

**Mice**

*Nlrp3*<sup>-/-</sup>, *Asc*<sup>-/-</sup> and *Myd88*<sup>-/-</sup> mice were generated as described (32-34). *Tlr2*<sup>-/-</sup>/*Tlr4*<sup>-/-</sup> mice were generated by intercrossing *Tlr2*<sup>-/-</sup> and *Tlr4*<sup>-/-</sup> mice (35, 36). Mice were backcrossed 6-9 times to a C57Bl/6 genetic background. Age and sex matched wild-type (WT) C57Bl/6 mice were obtained from Harlan Nederland (Horst, the Netherlands). Mice were infected at 9-12 weeks of age. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

**Induction of pneumonia and tissue harvest**

Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands) and inoculated intranasally with ~5x10<sup>4</sup> or ~1 x 10<sup>7</sup> CFU (in 50 µl) of *S. pneumoniae* 6303 (serotype 3; American Type Culture Collection, Manassas, VA) as described (15, 28-30). Mice were followed for a maximum of 7 days for survival studies or euthanized at 6, 24 or 48 hours after infection by heart puncture under ketamine / medetomidine anesthesia. Subsequently lungs and spleen were harvested and processed for the determination of bacterial outgrowth and cytokine levels exactly as described (37, 38).

**Assays**

TNF-α and IL-6 were measured using a cytometric bead array (BD Biosciences, San Jose, CA). IL-1β, CXCL1 and CXCL2 were measured by ELISA (R&D Systems, Minneapolis, MN). Myeloperoxidase (MPO) was measured by ELISA (HyCult Biotechnology, Uden, the Netherlands).
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Histologic examination
For histologic examination, lung sections were stained with hematoxylin and eosin, and lung inflammation was scored exactly as described (37). For analysis of neutrophil influx in the lung, immunohistochemical stainings were prepared using a FITC-labeled anti-mouse Ly-6C/G mAb (BD Biosciences, San Jose, CA) and Ly-6C/G was quantified as described (39). Analysis of γ-H2AX expression in lung tissue was performed in a similar manner using an antibody specific for phospho-H2AX (Ser139; Cell Signaling Technology, Danvers, MA) and peroxidase-conjugated anti-rabbit IgG (Immunologic, Duiven, the Netherlands).

Statistical analysis
Data are expressed as medians with individual data points (bacterial loads), Kaplan-Meier plots (survival) or box and whisker plots showing the smallest observation, lower quartile, median, upper quartile and largest observation. Data in tables are mean ± SEM. Comparisons between mouse strains were done by log-rank test for survival curves, Fisher’s exact test for proportions of positive blood cultures and Mann Whitney U test for other outcomes. These analyses were done using GraphPad Prism (San Diego, CA), \( p < 0.05 \) was considered statistically significant.

Results

\textit{Nlrp3}\textsuperscript{-/-} and \textit{Asc}\textsuperscript{-/-} mice display a strongly improved host defense during lethal pneumonia caused by highly virulent \textit{S. pneumoniae}

We studied mortality and bacterial growth and dissemination in lethal pneumonia caused by low dose infection (5 x 10^{4} CFU) with a virulent serotype 3 \textit{S. pneumoniae} strain (6303). For this we performed two separate survival studies, comparing \textit{Nlrp3}\textsuperscript{-/-} or \textit{Asc}\textsuperscript{-/-} mice with normal WT mice. As expected, in both experiments WT mice showed massive mortality between 48 and 72 hours; the last WT mice died just after 3 days of infection (Figure 1A-B). Surprisingly, both \textit{Nlrp3}\textsuperscript{-/-} and \textit{Asc}\textsuperscript{-/-} mice displayed a strongly reduced mortality; at the end of the 7-day observation period 9/15 \textit{Nlrp3}\textsuperscript{-/-} and 7/16 \textit{Asc}\textsuperscript{-/-} mice were still alive (both \( p < 0.001 \) versus WT mice).

To study antibacterial defense, we infected mice with the same bacterial dose in separate experiments and subsequently euthanized them after 6, 24 or 48 hours for quantitative cultures of lungs (the primary site of infection), blood and spleen (to obtain insight into dissemination to distant organs). After 6 hours, bacterial loads in the lungs of \textit{Nlrp3}\textsuperscript{-/-} and \textit{Asc}\textsuperscript{-/-} mice were similar to those in lungs of WT mice (Figure 1C-D). At later time points, however, \textit{Nlrp3}\textsuperscript{-/-} or \textit{Asc}\textsuperscript{-/-} mice showed strongly reduced bacterial burdens in their lungs (\( p < 0.01 - < 0.001 \) versus WT mice). Strikingly, while in all WT mice the infection disseminated to blood and spleen from 24 hours after infection onward (Figure 1E-H), blood and spleen cultures remained sterile in the vast majority of \textit{Nlrp3}\textsuperscript{-/-} and \textit{Asc}\textsuperscript{-/-} mice (\( p < 0.01 - < 0.001 \) versus WT mice). Together these data suggest that \textit{Nlrp3}\textsuperscript{-/-} and \textit{Asc}\textsuperscript{-/-} mice are strongly protected against lethality during pneumonia caused by highly virulent \textit{S. pneumoniae} by a robust reduction of bacterial growth and dissemination.
Figure 1: NLRP3 and ASC contribute to mortality and enhance bacterial growth of *S. pneumoniae*. WT, *Nlrp3*<sup>−/−</sup> and *Asc*<sup>−/−</sup> mice were inoculated with ~5 x 10<sup>4</sup> CFU *S. pneumoniae* and monitored for survival or sacrificed at designated time-points. Survival of WT (closed squares), *Nlrp3*<sup>−/−</sup> (white triangles) and *Asc*<sup>−/−</sup> mice (open circles) (n=15-16 per group) expressed as Kaplan-Meier plot (A, B). Bacterial loads in lung (C,D), blood (E,F) and spleen (G,H) 6, 24 and 48 hours after infection in WT (closed squares), *Nlrp3*<sup>−/−</sup> (white triangles) and *Asc*<sup>−/−</sup> mice (open circles) (n=7-8 per group). Each symbol represents an individual mouse, with horizontal lines showing medians. ### p < 0.001 for the comparison between *Asc*<sup>−/−</sup> and WT mice, and between *Nlrp3*<sup>−/−</sup> and WT mice as determined by Log-Rank test. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to WT mice determined with Mann-Whitney U test or Fisher’s exact test for the proportion of positive blood and spleen cultures. ND= not done.
**Nlrp3**−/− and **Asc**−/− mice demonstrate increased pulmonary cytokine and chemokine concentrations early after infection

For an adequate defense against uncontrolled bacterial multiplication early induction of the immune response to *S. pneumoniae* in the lower airways is of utmost importance (2, 40). The virulent *S. pneumoniae* strain used here logarithmically proliferates in the lungs during the early stages of the infection without causing a clear inflammatory response (15, 15, 28, 29). Indeed, WT mice showed a relatively modest increase in levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (CXCL1, CXCL2) in whole lung homogenates harvested 6 hours post infection compared to values of uninfected mice (Table 1A, B, C). Remarkably, at this early time point the lung levels of all proinflammatory mediators were higher in *Nlrp3*−/− and *Asc*−/− mice than in their respective WT groups, significantly so for all mediators in *Asc*−/− mice and for IL-1β in *Nlrp3*−/− mice (Table 1B, C). The early enhanced cytokine and chemokine response in lungs of *Nlrp3*−/− and *Asc*−/− mice did not result in an altered recruitment of neutrophils into lungs, as reflected by similarly low MPO concentrations in whole lung homogenates (Figure 2A-B) and similarly low number of Ly-6C/G+ cells in lung tissue slides when compared with WT mice (Figure 2C-F). Likewise, the extent of lung pathology was similar in all mouse strains at this early time point (Figure 2G-J).

**Nlrp3**−/− and **Asc**−/− mice show strongly attenuated lung inflammation during late stages of the infection

In accordance with previous reports (15, 28-30), WT mice displayed profound lung pathology during late stages of the infection (Figure 2G-J) associated with accumulation of neutrophils in lung tissue (Figure 2A-F). These changes were strongly reduced in *Nlrp3*−/− and *Asc*−/− mice: histopathology scores were much lower in inflammasome deficient mice, especially at 48 hours after infection, i.e., shortly before the first WT mice started dying (Figure 2G-J, both *p* < 0.01 versus WT mice), as were pulmonary MPO levels (Figure 2A,B, both *p* < 0.05 versus WT mice) and the number of Ly-6C/G+ cells in lung tissue (Figure 2C-F, both *p* < 0.05 versus WT mice). In addition, the lung levels of TNFα, IL-1β, IL-6, CXCL1 and CXCL2 were all significantly lower in *Nlrp3*−/− or *Asc*−/− mice than in WT mice at 24 and 48 hours after infection (Table 1).
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Table 1 Lung cytokine and chemokine levels in Nlrp3−/− and Asc−/− mice before and after pulmonary infection with S. pneumoniae

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<th>Asc−/−</th>
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<td>IL-1β</td>
<td>bd</td>
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<td>TNF-α</td>
<td>bd</td>
<td>bd</td>
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<td>27 (12)</td>
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<td>140 (15)</td>
<td>229 (29) *</td>
<td>327 (100)</td>
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<td>7 (2)</td>
<td>13 (4)</td>
<td>247 (56)</td>
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<td>15 (9)</td>
<td>25 (10)</td>
<td>1721 (623)</td>
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<td>276 (53)</td>
<td>411 (54)</td>
<td>9169 (2007)</td>
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<td>CXCL-2</td>
<td>2223 (266)</td>
<td>2447 (231)</td>
<td>5642 (562)</td>
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Uninfected mice (A) were sacrificed. WT, Nlrp3<sup>-/-</sup> (B) and Asc<sup>-/-</sup> (C) mice were inoculated with ~5 x 10<sup>4</sup> CFU S. pneumoniae and sacrificed after 6, 24 or 48 hours. Cytokine and chemokine levels are presented in pg/ml lung homogenate. Data are mean (SEM) of 7-8 mice per group.* p < 0.05, ** p < 0.01, *** p < 0.001 vs WT mice determined with Mann-Whitney U test. bd = below detection.

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<td>WT</td>
<td>Asc&lt;sup&gt;-/-&lt;/sup&gt;</td>
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<tr>
<td>IL-1β</td>
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<td>200 (9)*</td>
<td>347 (115)</td>
<td>176 (30)</td>
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<td>35 (4)*</td>
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<td>40 (21)**</td>
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<td>3351 (47)**</td>
<td>4583 (905)</td>
<td>5720 (475)</td>
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**Improved host defense in Nlrp3<sup>-/-</sup> and Asc<sup>-/-</sup> mice is associated with diminished cell death during late stages of the infection**

Besides processing of IL-1β and IL-18, activation of the NLRP3 inflammasome may trigger pyroptotic cell death (7, 41, 42). Since pneumococcal pneumonia in Nlrp3<sup>-/-</sup> and Asc<sup>-/-</sup> mice was characterized by reduced bacterial dissemination and reduced lung pathology, we wanted to determine whether the improved host defense response in these inflammasome-deficient mice resulted from diminished cell death early during infection. Since specific markers for pyroptosis detection *in situ* have not been described yet, we analyzed the presence of phosphorylated histone H2AX (γ-H2AX) in the lung. H2AX is modified to γ-H2AX immediately following DNA double-strand breaks by various means including apoptosis and necroptosis (43, 44). Immunohistological staining of γ-H2AX in lung sections showed only few strongly positive cells 6 and 24 h after infection and revealed no differences between WT and Nlrp3<sup>-/-</sup> or Asc<sup>-/-</sup> mice (Fig 3A-D). During late stages of the infection, strongly γ-H2AX-positive cells were detected in areas with neutrophil accumulation and cells along the pleura in the lung tissue of WT mice, but not in Nlrp3<sup>-/-</sup> and Asc<sup>-/-</sup> mice (Fig 3A-D). These data suggest that NLRP3-inflammasome activation does not evoke cell death in the lung early during *S. pneumonia* 6303-induced pneumonia, but is associated with increased cell death during late stages of the infection.
Figure 3: Reduced numbers of γ-H2AX-positive cells in the lung of Nlrp3⁻/⁻ and Asc⁻/⁻ mice during late stages of pneumococcal pneumonia. WT, Nlrp3⁻/⁻ and Asc⁻/⁻ mice were inoculated with ~ 5 x 10⁴ CFU S. pneumoniae and sacrificed at designated time-points. Expression of γ-H2AX in lung sections was quantified by digital image analysis as described in the Methods; the amount of γ-H2AX positivity was expressed as a percentage of the total lung surface area (A, B). Representative images of γ-H2AX staining on lung slides from WT, Nlrp3⁻/⁻ and Asc⁻/⁻ mice (C,D), original magnification 20x. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. * p < 0.05, ** p < 0.01 compared to WT mice determined with Mann-Whitney U test.

MyD88 is not involved in protective immunity against lethal pneumococcal pneumonia

In a previous study, we found that protective immunity during respiratory tract infection caused by S. pneumoniae D39 is strongly dependent on MyD88 signaling, as reflected by increased bacterial growth and lethality in Myd88⁻/⁻ mice (21), which is in full accordance with a previous investigation using serotype 4 or 19F S. pneumoniae (20). Considering the unexpected role of NLRP3/ASC in airway infection caused by S. pneumoniae 6303 reported here, we also evaluated the role of MyD88 in host defense against this virulent strain. Myd88⁻/⁻ and WT mice were infected with 5 x 10⁴ CFU S. pneumoniae 6303 and followed for 5 days. Remarkably, lethality progressed in Myd88⁻/⁻ mice at the same pace as in WT mice and all animals had succumbed at the end of the observation period (Figure 4A). In separate experiments we euthanized Myd88⁻/⁻ and WT mice at 6 or 48 hours post infection to obtain insight in the role of MyD88 in controlling bacterial growth and
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Figure 4: MyD88-dependent signaling is not important for survival and does not restrict bacterial defense, nor do TLR2 and TLR4 contribute to antibacterial defense. WT, Myd88−/− and Tlr2−/−/Tlr4−/− mice were inoculated with ~5 x 10⁴ CFU S. pneumoniae and monitored for survival or sacrificed 6 or 48 hours after infection. Survival of WT (closed squares) and Myd88−/− mice (open squares) (n=12 per group) expressed as Kaplan-Meier plot (A). Bacterial loads in lung (B, D) and blood (C) 6 and 48 hours after infection in WT (closed squares) and Myd88−/− (open squares) and Tlr2−/−/Tlr4−/− mice (diamonds) (n=7-8 per group). Each symbol represents an individual mouse, with horizontal lines showing medians. BC+= ratio of positive blood cultures in Tlr2−/−/Tlr4−/− mice.

dissemination. In accordance with the unaltered lethality, Myd88−/− mice displayed similar pneumococcal loads in the lung as WT mice at both time points (Figure 4B). In addition, analysis of dissemination of pneumococci to the circulation revealed no differences between the number of WT and Myd88−/− mice with positive blood cultures or bacterial loads in blood (Figure 4C). To obtain further proof for an absent role for MyD88 in host defense against pneumonia caused by S. pneumoniae 6303, we also infected Tlr2−/−/Tlr4−/− and WT mice with 5 x 10⁴ CFU of this strain and determined bacterial loads in lungs at 6 and 48 hours after infection; no differences were found between mouse strains (Figure 4D). Together these data suggest that MyD88-dependent signaling does not contribute to protective immunity during lethal pneumonia caused by S. pneumoniae 6303.
MyD88 is not involved in the early inflammatory response in the lung during pneumonia caused by *S. pneumoniae* 6303

*Myd88*-/- and WT mice had similar (relatively low) lung levels of proinflammatory cytokines (TNFα, IL-1β, IL-6) and chemokines (CXCL1, CXCL2) at 6 hours after infection (Table 2A, B). Likewise, early neutrophil recruitment was comparable in both mouse strains, as indicated by lung MPO concentrations (Figure 5A) and the number of Ly-6C/G+ cells in lung tissue slides (Figure 5B,C), as was the extent of lung pathology (Figure 5D,E). Hence, these results indicate that MyD88 is not involved in the induction of the inflammatory response at the primary site of infection after intrapulmonary delivery of *S. pneumoniae* 6303.

*Myd88*-/- mice show reduced pulmonary cytokine levels during late stages of the infection

Cytokine and chemokine levels measured in whole lung homogenates harvested 48 hours after infection, i.e. just before the first mice started dying, were lower in *Myd88*-/- than in WT mice, significantly so for IL-1β, CXCL1 and CXCL2 (Table 2). Similarly, lung MPO levels were lower in *Myd88*-/- mice at this late time point (Figure 5A). The number of Ly-6C/G+ cells in lung tissue (Figure 5B,C) and the extent of lung histopathology (Figure 5D,E) were similar in both mouse strains at 48 hours post infection.

MyD88 mediates the early inflammatory response during pneumonia caused by high dose *S. pneumoniae* 6303

An important difference in pneumonia models evoked by different pneumococcal serotypes is the number of bacteria used to induce disease with approximately similar severity: typically the infectious dose of *S. pneumoniae* 6303 is 100-1000-fold lower (~10⁴ CFU) than the dose used in investigations using *S. pneumoniae* D39 (~10⁶ – 10⁷) (8, 10, 15, 18, 28-31, 45). The finding that MyD88 did not contribute to lung inflammation early after infection with *S. pneumoniae* 6303, but that differences in cytokine production became evident when bacterial loads had become much higher, prompted us to infect *Myd88*-/- and WT mice with 1 x 10⁷ CFU *S. pneumoniae* 6303 and evaluate bacterial loads and the extent of lung inflammation at 6 and 24 hours post infection. In contrast to pneumonia evoked by 5 x 10⁴ CFU *S. pneumoniae* 6303 (see above), 1 x 10⁷ CFU *S. pneumoniae* 6303 caused an early severe inflammatory response in the lungs of WT mice with significant cytokine and chemokine release and neutrophil influx at 6 hours after inoculation (Table 3). Importantly, *Myd88*-/- and WT mice displayed similar pulmonary bacterial loads at both 6 and 24 hours in this high dose infection model; in addition, the number of positive blood cultures did not differ between strains (Figure 6A,B). Moreover, the extent of lung injury, that was remarkably more severe 6 hours after infection than after infection with the lower infectious dose, was not different between *Myd88*-/-
Table 2: Lung cytokine and chemokine levels in MyD88\(^{-/-}\) mice after pulmonary infection with S. pneumoniae

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<td>Lung</td>
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<td>IL-6</td>
<td>bd</td>
<td>bd</td>
</tr>
<tr>
<td>CXCL-1</td>
<td>1056 (358)</td>
<td>371 (71)</td>
</tr>
<tr>
<td>CXCL-2</td>
<td>665 (169)</td>
<td>443 (63)</td>
</tr>
</tbody>
</table>

WT and MyD88\(^{-/-}\) mice were inoculated with ~5 x 10\(^4\) CFU S. pneumoniae and sacrificed after 6 or 48 hours. Cytokine and chemokine levels are presented in pg/ml lung homogenate. Data are mean (SEM) of 7-8 mice per group.* \(p < 0.05\) vs WT mice determined with Mann-Whitney \(U\) test. bd = below detection.
Figure 5: Reduced neutrophil activation in Myd88<sup>−/−</sup> mice during late stage infection, while lung inflammation is not determined by MyD88-dependent signaling. MPO levels (A) and Ly-6C/G lung surface positivity (B) as a reflection of neutrophil influx in the lung at 6 and 48 hours after infection in WT (dark grey) and Myd88<sup>−/−</sup> mice (dotted white) (n=7-8 per group). Representative images of Ly-6C/G staining on lung slides from WT and Myd88<sup>−/−</sup> mice, original magnification 10 x (C). Histological scores were determined as described in the Methods section (D). In panel E representative lung histology of WT and Myd88<sup>−/−</sup> mice are shown, H&E staining, original magnification 10x. Data are expressed as box and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation.* p < 0.05 compared to WT mice determined with Mann-Whitney U test.
Table 3: Lung cytokine and chemokine levels in MyD88<sup>−/−</sup> mice after high dose pulmonary infection with S. pneumoniae

<table>
<thead>
<tr>
<th>Lung</th>
<th>T=6</th>
<th>T=24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-1β</td>
<td>554 (47)</td>
<td>503 (111)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>481 (53)</td>
<td>86 (54)**</td>
</tr>
<tr>
<td>IL-6</td>
<td>1087 (117)</td>
<td>291 (178)*</td>
</tr>
<tr>
<td>CXCL-1</td>
<td>111234 (17635)</td>
<td>14720 (10648)**</td>
</tr>
<tr>
<td>CXCL-2</td>
<td>4560 (197)</td>
<td>1489 (688)*</td>
</tr>
</tbody>
</table>

WT and MyD88<sup>−/−</sup> mice were inoculated with ~1 x 10<sup>7</sup> CFU S. pneumoniae and sacrificed after 6 or 24 hours. Cytokine and chemokine levels are presented in pg/ml lung homogenate. Data are mean (SEM) of 7-8 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001 vs WT mice determined with Mann-Whitney U test.
and WT mice (Figure 6C). However, neutrophil influx, as reflected by MPO levels in whole lung homogenates (Figure 6D), and lung cytokine (TNFα, IL-1β, IL-6) and chemokine (CXCL1 and CXCL2) concentrations were lower in *Myd88*<sup>−/−</sup> mice at 6 and 24 hours after challenge (Table 3; not significant for IL-1β at 6 hours and IL-6 at 24 hours). These data further indicate that *S. pneumoniae* 6303 is recognized by TLRs when present in high numbers and that the virulence of this strain in part might be related to the fact that it can multiply in the airways without being noticed by TLRs during the early phase of eventually lethal infection.

Figure 6: MyD88-dependent signaling is involved in the inflammatory response after infection with a supralethal dose of *S. pneumoniae*. WT and *Myd88*<sup>−/−</sup> mice were inoculated with ~ 1 x 10<sup>7</sup> CFU *S. pneumoniae* and sacrificed 6 and 24 hours after infection. Bacterial loads in lung (A), blood (B) 6 and 24 hours after infection in WT (closed squares), *Myd88*<sup>−/−</sup> (open squares) (n=7-8 per group); each symbol represents an individual mouse, with horizontal lines showing medians. MPO levels as a reflection of neutrophil influx in whole lung homogenate 6 and 48 hours after infection in WT (dark grey) and *Myd88*<sup>−/−</sup> mice (dotted white) (n=7-8 per group) (C). Histological scores (D) were determined as described in the Methods section. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile, and largest observation. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to WT mice determined with Mann-Whitney U test.
Discussion

*S. pneumoniae* is the most frequent causative pathogen in community-acquired pneumonia, a leading infectious disease globally (1-3). In recent years, it was concluded that the inflammasome components NLRP3 and/or ASC are crucial for an effective host defense response during pneumococcal pneumonia from studies using *S. pneumoniae* strains with a relatively low virulence, in particular the commonly used serotype 2 D39 strain (8-11). In the current study, we set out to evaluate their roles in a respiratory tract infection model evoked by a low dose of a gradually growing, highly virulent serotype 3 pneumococcal strain and found in stark contrast with these earlier studies that both NLRP3 and ASC strongly impair host defense. In addition, we show that MyD88, the common TLR adapter previously revealed as a major protective mediator during pneumonia caused by relatively modestly virulent *S. pneumoniae* strains (20, 21), does not contribute to an effective innate immune response in this model of severe pneumococcal pneumonia.

Inflammasome activation results in the processing of pro-IL-1β and pro-IL-18 into the mature secreted forms of these proinflammatory cytokines by caspase-1 (5, 6). In accordance, the NLRP3 inflammasome contributed to IL-1β and IL-18 release induced by pneumococci *in vitro* and during pneumonia *in vivo* (8, 10, 11, 46). Importantly, however, previous work from our laboratory suggests that the protective phenotype of *Asc−/−* and *Nlrp3−/−* mice in this model of pneumonia caused by *S. pneumoniae* 6303 is not likely explained by the role of the NLRP3 inflammasome in IL-1β and IL-18 maturation, since both IL-1R1−/− and IL-18−/− mice demonstrated a transient increase in bacterial lung counts with an unaltered survival (30, 47). Accordingly, treatment with recombinant IL-1 receptor antagonist resulted in transiently increased bacterial loads in the lung without influencing survival in pneumonia caused by *S. pneumoniae* 6303 (48). Another group reported that IL-1β was important for limitation of bacterial growth and survival after airway infection with the serotype 3 strain WU2 (49). Hence, these data strongly argue against a role for attenuated IL-1β and/or IL-18 maturation in the improved outcome of *Nlrp3−/−* and *Asc−/−* mice. The finding that *Nlrp3−/−* and *Asc−/−* mice had attenuated lung inflammation during the late phase of the infection most likely was the consequence of the much lower bacterial loads in these mice.

Another hall-mark feature of inflammasome activation is the occurrence of caspase-1-dependent pyroptosis, a form of programmed cell death considered to be part of a protective host response to intracellular bacteria (50). In the present study we did not find proof for inflammasome-mediated cell death in the lung early during pneumococcal pneumonia according to analysis of γ-H2AX expression. Previous work has indicated that pyroptosis does occur upon exposure to *S. pneumoniae in vitro* in an ASC- and caspase-1-dependent way, triggered by pneumolysin (8). Although the role of pyroptosis during infections *in vivo* is not well established, the general assumption is that pyroptosis serves an antibacterial function (50, 51). However, a recent publication provided evidence for an opposite role of caspase-1-dependent pyroptosis during pneumonia caused by the same *S. pneumoniae* strain used here (52). This supports our observation that deficiency of inflammasome
components can be beneficial during pneumococcal infection and we hypothesize that *S. pneumoniae* 6303 benefits from preemptive inflammasome-induced cell-death by facilitating bacterial growth and dissemination and possibly downregulation of early cytokine production in an inflammasome-dependent manner, as suggested by our data. Further studies are required to provide more insight in the process of pyroptotic cell death in this model of severe pneumococcal pneumonia.

The differential contribution of innate immune sensors to control the growth and dissemination of *S. pneumoniae* 6303 and D39 is further illustrated by the fact that Myd88−/− mice displayed an unaltered capacity to control bacterial proliferation during pneumonia caused by *S. pneumoniae* 6303, while these mice have a significantly reduced capacity to limit the growth of *S. pneumoniae* D39 (21). In our current experiments, MyD88 deficiency only influenced the induction of proinflammatory mediators in the lung in the presence of high burdens of *S. pneumoniae* 6303, i.e., either during late phase infection after inoculation with a low infectious dose or early after inoculation with a supralethal dose, suggesting that *S. pneumoniae* 6303 is not sensed by the TLR system when present in low yet eventually lethal quantities. Less virulent strains, such as *S. pneumoniae* D39, only cause significant disease when administered in relatively high infectious doses; these strains are then readily sensed by multiple innate recognition systems, including the NLRP3 inflammasome and TLRs, which in this case are important for protective immunity. It is tempting to speculate that the different outcomes of Asc−/− and Nlrp3−/− mice after infection with *S. pneumoniae* 6303 and D39 at least in part depend on the net balance between the seemingly opposite roles of ASC and NLRP3 in key antibacterial responses, i.e. their ability to induce bacteria-induced pyroptosis (inhibiting bacterial killing) versus their ability to produce proinflammatory cytokines (favoring bacterial clearance), and that these roles are influenced by the bacterial load and the consequent capacity of innate sensing systems to detect *S. pneumoniae*.

Multiple reports have pointed to a key role for ASC and/or NLRP3 in protective innate immunity during infectious diseases (5, 6), including pneumococcal pneumonia (8, 10, 11). We here demonstrate that ASC and NLRP3 strongly impair host defense during lower airway infection with a highly virulent serotype 3 *S. pneumoniae* strain in a model characterized during the early phase after infection by bacterial multiplication without noticeable TLR sensing. These data are of clinical relevance, considering the capacity of serotype 3 pneumococcal strains to cause severe disease in humans (22-25, 53) and considering that in real life lower respiratory tract infections are unlikely to be initiated by the high infectious doses used in most murine models of pneumococcal pneumonia. The current results are the first to expose a detrimental role of ASC and NLRP3 in antibacterial defense during a clinically relevant model of community-acquired pneumonia.

**Acknowledgments**

We are indebted to Fayaz S. Sutterwala and Richard A. Flavell for kindly providing us with Nlrp3−/− and Asc−/− mice, and to Shizuo Akira for kindly providing us with Myd88−/−, Tlr2−/− and Tlr4−/− mice.
ASC and NLRP3 impair host defense during lethal pneumonia caused by highly virulent serotype 3 Streptococcus pneumoniae

References


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