IL-12, IL-18 and IFN-gamma in the immune response to bacterial infection

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Chapter 6

IL-18 has a protective role in the early antimicrobial host response to pneumococcal pneumonia

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Abstract

To determine the role of endogenous IL-18 during pneumonia, IL-18 gene deficient (IL-18"⁻⁻") mice and wild-type (WT) mice were intranasally inoculated with *Streptococcus pneumoniae*, the most common causative agent of community-acquired pneumonia. Infection with *S. pneumoniae* increased the expression of IL-18 mRNA, and was associated with elevated concentrations of both precursor and mature IL-18 protein within the lungs. Although survival did not differ between IL-18"⁻⁻" and WT mice, IL-18"⁻⁻" mice had significantly more bacteria in their lungs and were more susceptible for progressing to systemic infection at 24 h and 48 h post inoculation. The increased bacterial outgrowth in IL-18"⁻⁻" mice did not result from a reduced production of cytokines reported to have a protective role during pneumonia, nor from overproduction of anti-inflammatory cytokines. In addition, the release of chemokines, influx of granulocytes and the formation of an inflammatory response in the lung in response to *S. pneumoniae* were enhanced, rather than reduced in IL-18"⁻⁻" mice. Anti-IL-12 did not influence bacterial clearance in either IL-18"⁻⁻" or WT mice. These data suggest that endogenous IL-18 plays an important role in the early antibacterial host response during pneumococcal pneumonia.
Introduction

*Streptococcus pneumoniae* is the most common causative microorganism in community-acquired pneumonia (1, 2). Despite the availability of potent antimicrobial agents, pneumococcal pneumonia remains an important cause of hospitalization and death. In addition, emergence and spread of penicillin-resistant *S. pneumoniae* have become a worldwide problem (3). Because of the high incidence of pneumococcal pneumonia and the increasing occurrence of resistance of *S. pneumoniae* to penicillin and other antimicrobial agents, it is important to obtain insight into the pathogenesis of pneumococcal pneumonia.

Antibacterial host defense in the pulmonary compartment is regulated by a complex interaction between immune competent cells and a network of cytokines and chemokines (4). Proinflammatory cytokines, like TNF (5, 6), IL-6 (7) and IFN-γ (8), produced in the lung during murine pneumococcal pneumonia, have been found important for clearance of bacteria from the respiratory tract, while anti-inflammatory cytokines, including IL-10 (9), impair this process. IL-18 is a proinflammatory cytokine which was originally identified in mice during endotoxin shock as a co-stimulatory factor for the production of IFN-γ (10-12). IL-18 is mainly produced by activated macrophages, and is first synthesized as a precursor protein (pro-IL-18, 24 kD), which requires splicing by IL-1β-converting enzyme (ICE) to liberate the 18 kD mature active protein (13, 14). Although IL-18 alone is not a potent stimulator of IFN-γ production, it synergistically enhances IL-12-induced IFN-γ production (15). Besides its IFN-γ-inducing effect, IL-18 has many proinflammatory effects on T and natural killer (NK) cells, enhancing proliferation and cytotoxicity, and stimulating the production of cytokines, including TNF, IL-2 and GM-CSF (15-18). In addition, IL-18 enhances Fas ligand-mediated cytotoxicity of NK and T cells and possesses potent antitumor activity (19-21).

Recent studies have investigated the role of IL-18 in the host response to infection. During experimental endotoxemia in mice, neutralization of IL-18 protected against LPS-induced liver injury (22). In contrast, IL-18 was protective during infections with *Yersinia enterocolitica*, and intracellular pathogens like *Leishmania major* and *Salmonella typhimurium* (23-25). The role of IL-18 in the pathogenesis of bacterial pneumonia is unknown. Therefore, in the present study we sought to determine the importance of IL-18 in host defense against pneumonia caused by *S. pneumoniae*. For this purpose, we compared survival and several components of the host response in IL-18 gene deficient (IL-18⁻⁻) and wild type (WT) mice. In addition, the possible interaction between endogenous IL-12 and IL-18 during pneumococcal pneumonia was studied by treatment of IL-18⁻⁻ and WT mice with an anti-IL-12 antibody.
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Materials and methods

Mice
All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center. IL-18−/− mice were generated as described previously (17). IL-18−/− mice were on the C57BL/6 background. Normal C57BL/6 WT mice were obtained from Harlan Spague Dawley Inc. (Horst, the Netherlands). Sex- and age-matched (8-10 weeks old mice) were used in all experiments.

Induction of pneumonia.
Pneumonia was induced as described before (5, 7, 9). *S. pneumoniae* serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD). Pneumococci were cultured for 16 h at 37°C in 5% CO₂ in Todd-Hewitt broth. This suspension was diluted 1:100 in fresh medium and grown for 5 h to midlogarithmic phase. Pneumococci were harvested by centrifugation at 1500 x g for 15 min. and washed twice in sterile 0.9% saline. Bacteria were resuspended in saline at different concentrations (see results), as determined by plating 10-fold dilutions of the suspensions on blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, the Netherlands), and 50 μl of bacterial suspension was inoculated intranasally. Control mice received 50 μl saline.

Reverse transcription PCR (RT-PCR) for cytokine message
Lungs were harvested at 24 h and 48 h after inoculation with *S. pneumoniae* and 24 h after inoculation with saline, snap-frozen in liquid nitrogen and stored at -70°C. To extract total cellular RNA, lungs from 3 mice per time point were pooled and homogenised in 1 ml of Trizol Reagent (GibcoBRL, Life Technologies, Grand Island, NY). Then, total RNA was isolated using chloroform extraction and isopropanol precipitation. The RNA pellet was dissolved in 100 μl diethylpyrocarbonate (DEPC)-treated water and quantified by spectrophotometry. Reverse transcription (RT) was performed by mixing 2 μg of total cellular RNA with 0.5 μg oligo(dT) (GibcoBRL) in a total volume of 12 μl. The mixture was incubated at 72°C for 10 min. Thereafter, 8 μl of a solution containing 4 μl 5x First Strand Buffer (GibcoBRL), 10 mM dithiothreitol (DTT; GibcoBRL), 1.25 mM dNTP’s (Amersham Pharmacia, Biotech, UK), and 100 U Superscript Reverse Transcriptase (GibcoBRL), was added, and incubated at 42°C for 1 h. Finally, the tubes were heated to 72°C for 10 min. after which 180 μl H₂O was added to the reaction mixture. Samples were stored at -20°C until further use. For PCR, 5 μl of cDNA solution was mixed with 20 μl of a solution containing, 1x PCR buffer (67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 0.67 μg EDTA, 16.6 mM (NH₄)₂SO₄), 2% DMSO (Merck, München,
Germany), 1.25 μg BSA (Biolabs Inc., New England), 0.5 U AmpliTaq DNA polymerase (Perkin Elmer Corp., Branchburg, NJ, USA) and 75 ng sense- and anti-sense oligonucleotide primers specific for IL-18 and β-actin (internal standard). The PCR reactions were performed in a thermocycler (Gene Amp. PCR System 9700, Perkin-Elmer Corp.) using the following conditions: 94°C for 5 min (1 cycle), followed immediately by 95°C for 1 min, 58°C for 1 min, 72°C for 1 min (with variable numbers of cycles) and a final extension phase of 72°C for 10 min. For semiquantitative assessment of IL-18 mRNA, variable numbers of cycles were used to ensure that amplification occurred in the linear phase. To exclude the possibility of finding differences between tubes due to unequal concentrations of cDNA in the PCR-reaction, a PCR using β-actin as the internal standard was performed on each sample. β-actin was found to be linear at 27 amplification cycles, IL-18 at 29 amplification cycles. The primers used for IL-18 (433 bp) were 5’-ACTGTACAACCGCAGTAATACGG-3’ (sense) and 5’-AGTGAACATTACAGATTTATCCC-3’ (anti-sense), and for β-actin (617 bp) 5’-GTCAGAAGGACTCCTATGTG-3’ (sense) and 5’-GCTCGTTGCCAATAGTGATG-3’ (anti-sense). PCR products were visualized by agarose gel electrophoresis.

**Determination of bacterial outgrowth**

At 24 h and 48 h after infection, mice were anesthetized by FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H2O; of this mixture 7.0 ml/kg intraperitoneally), and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Serial 10-fold dilutions were made in sterile saline and 50 μl volumes were plated onto blood agar plates. In addition, 20 μl volumes of blood were plated. Plates were incubated at 37°C at 5% CO2, and CFUs were counted after 16 h.

**Preparation of lung tissue for cytokine measurements and Western blot analysis**

Lung homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500 x g at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed.

**Electrophoresis and Western blotting**

For Western blots, 5 μg total protein was reduced with SDS sample buffer containing 20% β-mercaptoethanol and denatured for 5 min at 95°C. SDS-polyacrylamide gel electrophoresis using a 15% polyacrylamide gel was done according to Laemmli (26) at a
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constant voltage of 200 V. The proteins were transferred to Immobilon membrane using Tris-glycine buffer containing 20% methanol. The transfer was performed at a constant amperage of 0.33 A for 60 min. Non-specific binding sites on the membrane were blocked by incubation in PBST buffer (PBS with 0.05% Tween 20 (v/v) containing 2% nonfat dry milk (w/v) at 4°C overnight followed by incubation with primary antibody, i.e. 3 µg of purified rat anti-mouse IL-18 mAb (R&D Systems, Abingdon, United Kingdom) for 1 hour at room temperature. After three washes with PBST buffer containing 0.2% nonfat dry milk (w/v), the membrane was incubated with peroxidase-conjugated rabbit anti-rat IgG Abs (P0450, DAKO, Glostrup, Denmark) in a 1:2000 dilution at room temperature. After washing, the IL-18 bands were visualized using the enhanced chemiluminescence (ECL) Western blotting detection system (Boehringer Ingelheim GmbH, Germany). Recombinant mouse (rm) pro-IL-18 and mature IL-18 (both 2 µg) were used as standards. RmIL-18 was obtained from R&D Systems; rmpro-IL-18 was kindly provided by Dr. C. A. Dinarello (University of Colorado Health Sciences Center, Denver, CO).

Bronchoalveolar lavage

The trachea was exposed through a midline incision and canulated with a sterile 22-gauge Abbocath-T catheter (Abott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling 0.5 ml aliquots of sterile saline. Approximately 1 ml of lavage fluid (BALF) was retrieved per mouse. Total cell numbers were counted from each sample, and BALF differential cell counts were done on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL).

Histologic examination

Lungs for histologic examination were harvested at 24 h and 48 h after infection, fixed in 4% formaline and embedded in paraffin. 4 µm sections were stained with hematoxylin and eosin, and analyzed by a pathologist who was blinded for groups.

Reagents

Polyclonal sheep anti-murine IL-12 was administered at a dose of 200 µg intraperitoneally 1 h prior to infection with S. pneumoniae. Anti-IL-12 was prepared as described previously (27), and was kindly supplied by the Bioanalytical Sciences Department of Genetics Institute, Inc. (Cambridge, Mass.). Sheep IgG (Sigma, St. Louis, MO) was used as a control.
Role of IL-18 in pneumococcal pneumonia

Assays
Cytokine and chemokine levels in lung homogenates were measured by ELISA according to the instructions of the manufacturers (with detection limits): TNF (14 pg/ml), IL-12, IFN-γ, macrophage inflammatory protein-2 (MIP-2) (detection limits all 32 pg/ml) and KC (15 pg/ml) were all obtained from R&D Systems; IL-6 (20 pg/ml) and IL-10 (14 pg/ml) were from PharMingen (San Diego, CA).

Statistical analysis
All data are expressed as mean ± SE. Differences between groups were analyzed by Mann-Whitney U test. Survival was analyzed with Kaplan-Meier. P < 0.05 was considered to represent a statistical significant difference.

Results

Induction of IL-18 in lungs
To determine whether IL-18 is produced within the pulmonary compartment during pneumococcal pneumonia, RT-PCR was performed on lung samples obtained from mice inoculated with saline or at 24 and 48 h after infection with pneumococci. A faint band of IL-18 mRNA was found in lungs of mice receiving saline, indicating that some IL-18 mRNA is constitutively expressed (Fig. 1A). Intranasal infection with S. pneumoniae induced enhanced expression of IL-18 mRNA, as indicated by equal intensity of β actin bands and clear differences in band intensity between control and pneumonia samples for IL-18 RT-PCR products.

To study whether IL-18 protein is produced during pneumococcal pneumonia, IL-18 concentrations were measured in lung homogenates by ELISA. Control mice had high levels of IL-18 concentrations in their lungs (Figure 1B). Infection with S. pneumoniae slightly increased IL-18 concentrations in lung homogenates although this difference was not significant. Recently, it has been demonstrated that pro-IL-18 is expressed constitutively in the liver and spleen of mice (28). Since the ELISA used to detect IL-18 binds both pro-IL-18 and mature IL-18 (data not shown), we performed Western blot analysis to determine whether IL-18 detected in control lungs and after infection with S. pneumoniae consists of pro- or mature IL-18. As shown in Figure 1C, the majority of constitutive IL-18 in the lung consists of pro-IL-18, but also mature IL-18 is present. Infection with S. pneumoniae resulted in increases in the amounts of both pro- and mature IL-18 at 24 h and 48 h after infection.
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**A**

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<thead>
<tr>
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<tr>
<td><em>IL-18</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>saline</td>
<td>24 h</td>
<td>48 h</td>
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**B**

![Graph](image)

**Figure 1.** *IL-18* mRNA and *IL-18* protein expression in lungs of control mice or during pneumococcal pneumonia. Mice (8 per group) were inoculated intranasally with sterile saline (controls) or $1 \times 10^5$ CFU *S. pneumonia*, and sacrificed at the indicated time points.

A. *IL-18* mRNA and *β-actin* mRNA expression in lungs as determined by reverse transcription PCR. Lungs from 3 mice were pooled for each time point.

B. Concentrations of *IL-18* in lung homogenates as measured by ELISA. Data are expressed as mean ± SE of 4-8 mice.

C. Expression of pro- and mature *IL-18* in lung homogenates as assessed by Western blot analysis in individual mice with a specific rat anti-mouse *IL-18* Ab. Each lane shows a representative result of a total of 4-8 mice for each group. Recombinant mouse (rm) pro-*IL-18* and mature *IL-18* (both 2 µg) were used as standards.

**Survival**

To study the contribution of endogenously produced *IL-18* in host defense against pneumococcal pneumonia, survival in *IL-18*−/− and WT mice was studied after intranasal inoculation with *S. pneumoniae*. There was no significant difference in survival in *IL-18*−/− compared to WT mice after inoculation with either $5 \times 10^4$ CFU or $1 \times 10^5$ CFU *S. pneumoniae* (Fig. 2).

**Figure 2.** Survival in *IL-18*−/− and WT mice after intranasal inoculation with $5 \times 10^4$ CFU or $1 \times 10^5$ CFU *S. pneumoniae*. 8-12 mice per group were studied.
IL-18<sup>−/−</sup> mice have increased bacterial outgrowth
To determine the role of IL-18 in early host defense against pneumonia, we compared the bacterial outgrowth in the lungs of WT and IL-18<sup>−/−</sup> mice at 24 and 48 hours after intranasal inoculation with 1 x 10<sup>5</sup> CFU S. pneumoniae. Both at 24 h and 48 h post-infection, IL-18<sup>−/−</sup> mice had significantly more bacteria in their lungs than WT mice (Fig. 3). In addition, the number of IL-18<sup>−/−</sup> mice that developed bacteremia was markedly higher compared to WT mice. At 24 h after infection, 67 % of the IL-18<sup>−/−</sup> mice had positive blood cultures for S. pneumoniae, while none of the WT mice had bacteria in their blood. At 48 h, all IL-18<sup>−/−</sup> mice and only 50 % of WT mice were bacteremic.

Cell influx in BALF
A marked increase in cell numbers in BALF was found at 24 h and 48 h after infection of WT mice with S. pneumoniae as compared to controls, which was mainly the result of granulocyte influx (Fig. 4). The number of recruited granulocytes in the lungs was markedly increased in IL-18<sup>−/−</sup> mice compared to WT mice at 24 h after infection (Fig. 4). At 48 h, the number of granulocytes in BALF did not differ between the two groups.

Histopathology
In accordance with the cell count in BALF, the lungs of IL-18<sup>−/−</sup> mice showed significantly more inflammatory infiltrates than WT mice at 24 h after inoculation. As illustrated in Fig. 5a, a massive inflammatory infiltrate was present in IL-18<sup>−/−</sup> mice with vasculitis. Neutrophils were dominant and filled bronchi, bronchioles and adjacent alveolar spaces. In
WT mice, the inflammation clearly was more discrete (Fig. 5b). At 48 h after inoculation, the degree of inflammation was reduced in IL-18−/− mice (Fig. 5c) compared to 24 h but remained higher than in WT animals (Fig. 5d).

![Figure 5. Representative histologic sections of lungs of IL-18−/− mice (a and c) and WT mice (b and d) 24 h (a and b) and 48 h (c and d) respectively after inoculation with 1 x 10⁵ CFU S. pneumoniae. Haematoxylin and eosin staining, original magnification x 25 (24 h) and x 50 (48 h).]

**Cytokine and chemokine response to pneumococcal pneumonia**

Several proinflammatory cytokines and chemokines have been implicated in protective immunity during bacterial pneumonia, including TNF (5, 6, 29), IL-6 (7), IL-12 (30), IFN-γ (8), MIP-2 (31), and KC (32). In contrast, the anti-inflammatory cytokine IL-10 has been found to hamper antibacterial defense in the lung (9, 33). To determine whether alterations in the expression of these mediators contributed to the impaired host defense in IL-18−/− mice, their concentrations were measured in lung homogenates. As shown in Table 1, lung concentrations of IL-6 and the CXC chemokines MIP-2 and KC were higher in IL-18−/− than in WT mice, while IFN-γ levels were lower. Concentrations of TNF, IL-12, and IL-10 were not different in IL-18−/− compared to WT mice.
### Table 1. Cytokine and chemokine concentrations in lung homogenates of IL-18<sup>−/−</sup> and WT mice at 24 and 48 h after intranasal inoculation with 1 x 10<sup>5</sup> CFU <i>S. pneumoniae</i>.

<table>
<thead>
<tr>
<th>(ng/g lung)</th>
<th>24 h</th>
<th>48 h</th>
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<tr>
<td></td>
<td>WT</td>
<td>IL-18&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF</td>
<td>9.2 ± 2.8</td>
<td>14.4 ± 2.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.7 ± 6.5</td>
<td>96.4 ± 29.1&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-12</td>
<td>26.5 ± 4.6</td>
<td>31.8 ± 7.6</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>8.5 ± 0.6</td>
<td>6.0 ± 0.6&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>MIP-2</td>
<td>15.0 ± 5.1</td>
<td>34.7 ± 6.3&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>KC</td>
<td>21.3 ± 2.3</td>
<td>47.5 ± 11.1&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL-10</td>
<td>9.0 ± 0.8</td>
<td>7.8 ± 1.4</td>
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Data are mean ± SE of 8 mice. * P < 0.05 vs. WT mice.

**IL-18 mediated effects are independent of endogenous IL-12**

The combined action of IL-18 and IL-12 can result in synergistic effects on host immune cells, as has been reported on the activation of Th1 cells and the stimulation of IFN-γ production (16, 22). To study whether IL-12 contributes to host defense against <i>S. pneumoniae</i>, and whether IL-18 exerts its protective effect through interaction with IL-12, WT and IL-18<sup>−/−</sup> mice were injected with a neutralizing antibody against IL-12 or control Ab 1 h prior to infection with pneumococci, and mice were sacrificed after 48 h. Injection of anti-IL-12 in WT mice did not significantly influence bacterial outgrowth in the lung compared to control (Fig. 7). Again, IL-18<sup>−/−</sup> mice had more bacterial outgrowth in the lungs compared to WT mice after infection with <i>S. pneumoniae</i>. Administration of anti-IL-12 to IL-18<sup>−/−</sup> mice did not influence bacterial outgrowth in comparison with IL-18<sup>−/−</sup> mice that received a control Ab. These data indicate that IL-18 has IL-12 independent effects in host defense to pneumococcal pneumonia.

**Figure 6.** Bacterial outgrowth in lungs of IL-18<sup>−/−</sup> and WT mice at 48 h after intranasal inoculation with 1 x 10<sup>5</sup> CFU <i>S. pneumoniae</i> in combination with anti-IL-12 Ab or control IgG injected i.p. 1 h prior to infection with <i>S. pneumoniae</i>. Data are mean ± SE of 8 mice. * P < 0.05 vs. WT mice receiving control IgG.
Discussion

IL-18 was originally described as an important cofactor for IFN-γ production from T and NK cells in the presence of costimulatory signals, especially together with IL-12 (10, 11, 16). Recent studies have demonstrated that IL-18 has many other biologic activities, including stimulation of proliferation and cytotoxicity of T and NK cells, induction of Fas ligand expression, potentiation of IL-12-induced activation of Th1 cells, and the induction of cytokine production (12, 15-20). IL-18 is produced during clinical infection and in various animal models of infection (10, 11, 23, 34). Importantly, endogenous IL-18 has a protective role in mice during infection with *Y. enterolitica*, and the intracellular pathogens *L. major* and *S. typhimurium* (23-25).

In the present study we demonstrate the important role of IL-18 during gram-positive bacterial infection in the lung. IL-18 mRNA and IL-18 protein, mainly consisting of pro-IL-18, were found to be constitutively expressed within the lung. This is in agreement with earlier studies, which showed that IL-18 mRNA is expressed in lungs and other organs of normal mice (23, 28, 35-37). Alveolar macrophages, the resident phagocytes within the airways, are likely important producers of IL-18 within the lung, since IL-18 is known to be mainly produced by activated macrophages (22). Cameron et al. reported that the majority of IL-18 mRNA within lung tissue of mice was localized to airway epithelium cells, although inflammatory cells, mostly lymphocytes, within the airway wall and parenchyma also expressed IL-18 mRNA (35). In addition, IL-18 mRNA expression was also found in granulocytes present in the lungs of LPS-treated mice. As demonstrated by Western blot analysis, constitutively expressed IL-18 mainly consisted of biologically inactive pro-IL-18, although also low concentrations of mature IL-18 were present. Intranasal infection with *S. pneumoniae* induced the upregulation of IL-18 mRNA expression, and a modest increase in the concentrations of both pro- and mature IL-18 protein in the lung. Importantly, bacterial outgrowth in both lungs and blood was significantly increased in the early phase of infection in IL-18−/− mice compared to WT mice, although this did not result in differences in survival. These data suggest that, although the release of IL-18 locally within the lung is not strongly upregulated during pneumococcal pneumonia, IL-18 plays an important regulatory role in the early localized antimicrobial host defense against *S. pneumoniae*.

Previous studies have demonstrated that locally produced cytokines play an important role in the regulation of host defense against bacterial pneumonia (4). In a mouse model of pneumococcal pneumonia, the absence of TNF or IL-6 activity was associated with enhanced bacterial outgrowth in the lung and increased mortality (5-7). In contrast, the anti-inflammatory cytokine IL-10 impaired bacterial clearance from the lung by attenuating the proinflammatory response (9). In our study, IL-18−/− mice did not have an impaired ability to produce TNF and IL-6, and no overproduction of IL-10 was found, making it unlikely
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that an altered production of these cytokines contributed to their decreased host response to pneumococcal pneumonia.

Pneumonia is characterized by the recruitment of phagocytic cells, mainly granulocytes, to the site of infection (38, 39). Granulocyte influx in the lung was markedly increased in IL-18−/− mice at 24 h after *S. pneumoniae* inoculation. Accordingly, a severe bronchopneumonia with signs of vasculitis was observed in the lungs of IL-18−/− mice at this time point. The inflammatory infiltrate was much more discrete in the lungs of WT mice. At 48 h, the number of granulocytes in BALF and the degree of inflammation was reduced in IL-18−/− mice compared to 24 h but remained higher than in WT animals. Chemokines are a family of small proteins, which play an essential role in leukocyte trafficking during inflammation (40, 41). Based on their structure, chemokines are divided in several families. CXC chemokines have stimulatory and chemotactic activities on neutrophils, while CC chemokines mainly target mononuclear cells (41). Previously, the CXC chemokines MIP-2 and KC were found to serve a protective role during *Klebsiella* pneumonia in mice by the increased recruitment of neutrophils to the lungs associated with improved bacterial clearance (31, 32). In the present study, lung concentrations of MIP-2 and, most strikingly, of KC were increased in IL-18−/− mice compared to WT mice. Previously, IL-18 has been reported to increase the in vitro production of IL-8, the human prototypic CXC chemokine, which was mediated through enhanced release of TNF from non-CD14+ mononuclear cells (18). Also, TNF has been found to induce the secretion of MIP-2 and KC in mice (42). In this respect, it is surprising that higher concentrations of MIP-2 and KC were found in lungs of IL-18−/− mice than in WT mice, in the presence of comparable TNF concentrations. Nonetheless, it is conceivable that the increased KC and MIP-2 concentrations in lungs of IL-18−/− mice contributed to the concurrently enhanced granulocyte influx in BALF. Alternatively, the increased lung inflammation observed in IL-18−/− mice could merely be a reflection of an increased proinflammatory stimulus provided by the higher bacterial load.

Together with alveolar macrophages, the resident phagocytic cells in the lung, infiltrating granulocytes are the first line of defense against infectious agents (38, 43). They eliminate invading microorganisms by phagocytosis and subsequent intracellularly killing them (38, 39). In addition, these cells play a critical role in orchestrating the inflammatory response by the secretion of bioactive lipids, cytokines and chemokines. Little is known about the effect of IL-18 on the function of phagocytic cells, since most studies focussed on the effects of IL-18 on T and NK cells. Additional studies are required to determine whether IL-18, either directly or through soluble mediators, affects the phagocytosis and/or intracellular killing capacity of AM and granulocytes, which may provide an explanation for the initial impaired clearance of *S. pneumoniae* from the lungs.

IL-18, originally named IFN-γ-inducing factor (IGIF), has traditionally been viewed upon as an important stimulator, together with IL-12, of IFN-γ production (22).
accordance, IL-18−/− mice had lower IFN-γ concentrations in their lungs than WT mice during pneumonia. Previous studies have suggested that IL-12 and IFN-γ are involved in protective immunity during pneumonia (8, 30). However, data from the present and previous studies, indicate that the protective role of endogenous IL-18 is mediated via IL-12 and IFN-γ independent mechanisms. Indeed, injection of anti-IL-12 did not influence pneumococcal outgrowth in either WT or IL-18−/− mice. We confirmed these results in IL-12p40−/− and IL-12p35−/− mice, which did not differ in bacterial outgrowth in their lungs and lethality as compared to WT mice during pneumococcal pneumonia (44 and data not shown). These findings contrast with an earlier report in which anti-IL-12 treatment was found to hamper host defense in Klebsiella pneumonia (30). Possibly, the role of IL-12 is more prominent during gram-negative (K. pneumoniae) pneumonia than in gram-positive (S. pneumoniae) pneumonia. Also, we found that IFN-γR−/− mice and IFN-γ−/− mice are not more susceptible to, or even slightly protected, to pneumococcal pneumonia (45). These results in mice lacking IFN-γ activity, which were repeatedly confirmed in many experiments, contrast with a previous study by Rubins et al. (8). These authors used much larger inocula of S. pneumoniae (up to 10⁸ CFU), associated with rapid mortality, which may have less relevance for clinical pneumonia. Together, these data strongly favor a role for IL-18 during pneumococcal pneumonia that is unrelated to effects of IL-12 or IFN-γ.

Despite the availability of potent anti-microbial agents, pneumonia remains an important cause of illness and mortality worldwide. The gram-positive bacterium S. pneumoniae is the most frequently isolated pathogen in patients with community-acquired pneumonia (1, 2). Therefore, insight into the immune response against S. pneumoniae may contribute to potential adjuvant immunomodulatory therapies. We here demonstrate that endogenous IL-18 has a protective role in the early immune response during murine pneumococcal pneumonia by promoting bacterial clearance from the lung and delaying the progression to systemic infection. This is in line with previous studies which demonstrated that local inflammation, in which pro-inflammatory cytokines play a pivotal role, is essential for local host defense against respiratory pathogens (5-7). We here show that IL-18 possesses important immunoregulatory activities independent of IL-12 and IFN-γ in vivo, and should therefore not merely be considered as an IFN-γ-inducing cytokine. The mechanisms by which IL-18 exerts its protective effects remain to be investigated in further experiments.

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References

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34. Lauw FN, Simpson AJH, Prins JM, Smith MD, Kurimoto M, van Deventer SJH, Speelman P, Chaowagul W, White NJ, van der Poll T. Elevated plasma concentrations of interferon-γ (IFN-γ) and the IFN-γ-
Role of IL-18 in pneumococcal pneumonia


44. Kortlang C, Magram JS, Specelman P, Zaat SAJ, van Deventer SJH, van der Poll T. Interleukin-12 deficient mice have intact defense mechanisms against pneumococcal pneumonia in spite of defective interferon-γ production. 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada; September 28-October 1, 1997; (abstract G-12).
