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IL-18 Improves the Early Antimicrobial Host Response to Pneumococcal Pneumonia

Fanny N. Lauw,* Judith Branger,*† Sandrine Florquin,‡ Peter Speelman,† Sander J. H. van Deventer,* Shizuo Akira,§ and Tom van der Poll†‡

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. IL-18−/− mice had significantly more bacteria in their lungs and were more susceptible for progressing to systemic infection at 24 and 48 h postinoculation. Similarly, treatment of WT mice with anti-IL-18 was associated with enhanced outgrowth of pneumococci. In contrast, the clearance of pneumococci from lungs of IL-12−/− mice was unaltered when compared with WT mice. Furthermore, anti-IL-12 did not influence bacterial clearance in either IL-18−/− or WT mice. These data suggest that endogenous IL-18, but not IL-12, plays an important role in the early antibacterial host response during pneumococcal pneumonia. The Journal of Immunology, 2002, 168: 372–378.

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 immunocompetent cells and a network of cytokines and chemokines (4). IL-18 is a proinflammatory cytokine which was originally identified in mice during endotoxin shock as a costimulatory factor for the production of IFN-γ (5–8). IL-18 is mainly produced by activated macrophages and is first synthesized as a precursor protein (pro-IL-18, 24 kDa), which requires splicing by IL-1β-converting enzyme to liberate the 18-kDa mature active protein (9, 10). Although IL-18 alone is not a potent stimulator of IFN-γ production, it synergistically enhances IL-12-induced IFN-γ production (11). Besides its IFN-γ-inducing effect, IL-18 has many proinflammatory effects on T and NK cells, enhancing proliferation and cytotoxicity and stimulating the production of cytokines, including TNF, IL-1, IL-2, IL-6, and GM-CSF (11–15). In addition, IL-18 enhances Fas ligand-mediated cytotoxicity of NK and T cells and possesses potent antitumor activity (16–18).

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 and lethality (19, 20). In contrast, IL-18 was protective during infections with Yersinia enterocolitica and intracellular pathogens like Leishmania major and Salmonella typhimurium (21–23). The role of IL-18 in the pathogenesis of bacterial pneumonia is unknown. Therefore, in this 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−/− and wild-type (WT)2 mice. Considering that IL-18 and IL-12 can synergistically activate immunocompetent cells (7, 8), we also determined the role of IL-12 in the innate immune response to pneumococcal pneumonia using IL-12p40 gene-deficient (IL-12−/−) mice. Finally, 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 Ab.

Materials and Methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center of the University of Amsterdam. IL-18−/− mice were generated as described previously (13). IL-18−/− mice were on the C57BL/6 background. IL-12−/− BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Normal C57BL/6 and BALB/c WT mice, used as controls for IL-18−/− and IL-12−/− mice, respectively, were obtained from Harlan Sprague-Dawley (Horst, The Netherlands). Sex- and age-matched (8- to 12-wk old mice) were used in all experiments.

Induction of pneumonia

Pneumonia was induced as described before (24–26). S. pneumoniae serotype 3 was obtained from American Type Culture Collection (6303; Manassas, VA). 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 × g for 15 min and washed twice in sterile 0.9% saline. Bacteria were resuspended in saline at different concentrations (see

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‡ Abbreviations used in this paper: WT, wild-type; rm, recombinant mouse; BALF, bronchoalveolar lavage fluid; MIP-2, macrophage inflammatory protein-2.

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Results, as determined by plating 10-fold dilutions of the suspensions on blood agar plates. Mice were lightly anesthetized by inhalation of isoflu- rane (Uipjohn, Ede, The Netherlands), and 50 μl of bacterial suspension was inoculated intranasally. Control mice received 50 μl of saline.

RT-PCR

Lungs were harvested at 24 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 three mice per time point were pooled and homogenized in 1 ml of TRIzol Reagent (Life Technologies, Grand Island, NY). Total RNA was isolated using chlo-roform extraction and isopropanol precipitation. The RNA pellet was dis-solved in 100 μl of diethylpyrocarbonate-treated water and quantified by spectrophotometry. Reverse transcription was performed by mixing 2 μg of total cellular RNA with 0.5 μg of oligo(dT) (Life Technologies) in a total volume of 12 μl. The mixture was incubated at 72°C for 10 min. Afterward, 8 μl of a solution containing 4 μl of 5’s First Strand buffer (Life Technologies), 10 mM DTT (Life Technologies), 1.25 mM dNTPs (Amersharm Pharmacia Biotech, Little Chalfont, U.K.), and 100 U of Su-perscript Reverse Transcriptase (Life Technologies) was added and the mixture was incubated at 42°C for 1 h. Finally, the tubes were heated to 72°C for 10 min after which 180 μl of 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 1× PCR buffer (67 mM Tris-HCl (pH 8.8), 6.7 mM MgCl2, 10 mM 2-ME, 0.67 μg of BSA, 16.6 mM (NH4)2 SO4, 2% DMSO (Merck, München, Germany), 1.25 μg of BSA (New England Biolabs, Beverly, MA), 0.5 U of AmpliTaq DNA polymerase (PerkinElmer, Branchburg, NJ), and 75 ng of sense- and antisense oligonucleotide primers specific for IL-18 and β-actin (internal standard). The PCR were performed in a thermocycler (Gene Amp. PCR System 9700, PerkinElmer) 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, vari-able 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, 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, and IL-18 was found to be linear at 29 amplification cycles. The primers used for IL-18 (433 bp) were 5′-ACTGTACACCGCAGTATACCGG-3′ (sense) and 5′-AGTGGAC ATTCAGAATTTACCC-3′ (antisense), and for β-actin (617 bp) 5′-GT CAGAAGAAGACTCTATGGT-3′ (sense) and 5′-GCTGGTGCCAAATAG TGATG-3′ (antisense). PCR products were visualized by agarose gel electrophoresis.

Determination of bacterial outgrowth

At 24 and 48 h after infection, mice were anesthetized by 7.0 ml/kg FFM (fentanyl citrate 0.079 mg/ml, midazolam 1.25 mg/ml, and 5 μg/ml detomidine). The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalve-olar lavage was performed by instilling 0.5-ml aliquots of sterile saline. Approximately 1 ml of bronchoalveolar 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 Diagnostics, McGraw Park, IL.).

Histologic examination

Lungs for histologic examination were harvested at 24 and 48 h after in-fection, fixed in 10% formaline, and embedded in paraffin. Four-microme-ter sections were stained with H&E and analyzed by a pathologist who was blinded for groups.

Reagents

Rabbit anti-murine IL-18 antisem, kindly donated by Dr. C. Dinarello, was used at a dose of 200 μl. The anti-IL-18 antisem (200 μl) was given i.p. 1 h before and 24 h after intranasal admin-istration of bacteria. This dose significantly reduced endotoxin-induced IFN-γ release and lethality in mice (20). Rabbit serum (Sigma-Aldrich, St. Louis, MO) was used as control. Polyclonal sheep anti-murine IL-12 was adminis-trated at a dose of 200 μg i.p. 1 h before infection with S. pneu-moniae. Anti-IL-12 was prepared as described previously (30) and was kindly supplied by the Bioanalytical Sciences Department of Genetics In-stitute (Cambridge, MA). Sheep IgG (Sigma-Aldrich) was used as a control.

Assays

IL-18, TNF, IL-12, INF-γ, macrophage inflammatory protein-2 (MIP-2), and KC were measured by ELISAs according to the instructions of the manufacturer (R&D Systems).

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. Values of p < 0.05 were considered to represent a statistically 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 pneumococcus. 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 pneumococ-cial pneumonia, IL-18 concentrations were measured in lung homogen-ates by ELISA. Control mice had high levels of IL-18 concentrations in their lungs (Fig. 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). Because the ELISA was used to detect IL-18 and 48 h after infection. 67% of the IL-18−/− 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−/− mice and only 50% of WT mice were bacteremic. Despite these differences in early antibacterial defense, survival did not consistently differ among IL-18−/− and WT mice. Infection of 10^5 CFU of pneumococci resulted in lethality between days 2 and 5 in both strains and an overall survival of 1 of 14 IL-18−/− mice and 3 of 14 WT mice (nonsignificant). In addition, in another experiment with a lower bacterial inoculum (5 × 10^4 CFU), mortality also tended to be higher in IL-18−/− mice than in WT mice (survival: two of eight IL-18−/− mice and three of eight WT mice; nonsignificant). 

To further confirm the early protective role of endogenous IL-18 in pneumonia, we treated WT mice with a neutralizing anti-IL-18 polyclonal Ab before and 24 h after intranasal inoculation with 10^5 CFU of S. pneumoniae. In accordance with the results obtained in IL-18−/− mice, anti-IL-18-treated WT mice demonstrated an enhanced outgrowth of pneumococci in lungs when compared with WT mice treated with a control Ab (Fig. 2B).

**Cell influx in BALF**

A marked increase in cell numbers in BALF was found at 24 and 48 h after infection of WT mice with S. pneumoniae as compared with controls, which was mainly the result of granulocyte influx (Fig. 3). The number of recruited granulocytes in the lungs was markedly increased in IL-18−/− mice compared with WT mice at 24 h after infection (Fig. 3). At 48 h, the number of granulocytes in BALF did not differ between the two groups.
FIGURE 3. Numbers of granulocytes in BALF of IL-18−/− and WT mice after intranasal administration of 1 × 10^5 CFU of S. pneumoniae. Data are mean ± SE of four mice per group at each time point. *p < 0.05 vs WT mice.

Histopathology

In accordance with the cell count in BALF, the lungs of IL-18−/− mice showed significantly more inflammatory infiltrates than WT mice at 24 h after inoculation. As illustrated in Fig. 4A, a massive inflammatory infiltrate was present in IL-18−/− mice with vasculitis. Neutrophils were dominant and filled bronchi, bronchioles, and adjacent alveolar spaces. In WT mice, the inflammation clearly was more discrete (Fig. 4B). At 48 h after inoculation, the degree of inflammation was reduced in IL-18−/− mice (Fig. 4C) compared with 24 h but remained higher than in WT animals (Fig. 4D).

Cytokine and chemokine response to pneumococcal pneumonia

To determine whether alterations in the expression of cytokines and/or chemokines contributed to the impaired host defense in IL-18−/− mice, their concentrations were measured in lung homogenates. As expected, the lung concentrations of IFN-γ were lower in IL-18−/− mice than in WT mice (Table I). In contrast, CXC chemokines MIP-2 and KC were higher in IL-18−/− than in WT mice. Concentrations of TNF and IL-12 were similar in both groups.

The effect of anti-IL-18 on cytokine levels in lungs was also measured; remarkably, anti-IL-18 did not reduce IFN-γ concentrations (3.3 ± 0.2 ng/g lung vs 3.0 ± 0.2 ng/g in mice treated with preimmune serum). Anti-IL-18 did reduce IL-12 levels (18.9 ± 1.6 vs 68.7 ± 14.6 ng/g, p < 0.05).

| Cytokine and chemokine concentrations in lung homogenates of IL-18−/− and WT mice at 24 and 48 h after intranasal inoculation with 1 × 10^5 CFU S. pneumoniae* |
|---------------------------------|-----------------|-----------------|-----------------|
|                                | WT              | IL-18−/−        | WT              | IL-18−/−        |
| 24 h                           |                 |                 | 48 h            |                 |
| IFN-γ (μg/g lung)              | 8.5 ± 0.6       | 6.0 ± 0.6*      | 5.2 ± 0.4       | 3.4 ± 0.4*      |
| MIP-2 (ng/g lung)              | 15.0 ± 5.1      | 34.7 ± 6.3*     | 48.5 ± 23.9     | 100.9 ± 21.7    |
| KC (ng/g lung)                 | 21.3 ± 2.3      | 47.5 ± 11.1*    | 26.9 ± 3.5      | 45.6 ± 10.2*    |
| IL-12 (ng/g lung)              | 26.5 ± 4.6      | 31.8 ± 7.6      | 88.8 ± 14.7     | 44.1 ± 5.4      |
| TNF (ng/g lung)                | 9.2 ± 2.8       | 14.4 ± 2.7      | 11.5 ± 2.2      | 14.4 ± 2.3      |

* Data are mean ± SE of eight mice and are measured in nanograms per gram lung. *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 (7, 8). To study whether IL-12 contributes to host defense against S. pneumoniae, we induced pneumonia in IL-12−/− and WT mice. Bacterial outgrowth in lungs (Fig. 5) and blood (data not shown) appeared indistinguishable between both mouse strains. Furthermore, survival did not differ between IL-12−/− mice (5 of 16) and WT mice (2 of 16; nonsignificant). To determine whether IL-12 exerts its protective effect through interaction with IL-12, WT and IL-18−/− mice were injected with a neutralizing Ab against IL-12 or control Ab 1 h before 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 with control, confirming the results obtained with IL-12−/− mice (Fig. 6). Again, IL-18−/− mice had more bacterial outgrowth in the lungs compared with WT mice after infection with S. pneumoniae. Administration of anti-IL-12 to IL-18−/− mice did not influence bacterial outgrowth in comparison with IL-18−/− mice that received a control Ab. These data indicate that IL-18 has IL-12-independent effects in host defense to pneumococcal pneumonia. IL-12 did interact to enhance IFN-γ production in lungs, i.e., IL-12−/− mice had lower IFN-γ concentrations in their lungs than did WT mice.
after induction of pneumonia, and anti-IL-12 treatment reduced IFN-γ levels in lungs of both WT and IL-18−/− mice (Table II).

**Discussion**

IL-18 was originally described as an important co-factor for IFN-γ production from T and NK cells in the presence of costimulatory signals, especially together with IL-12 (12). 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 (7, 8, 11–17). IL-18 is produced during clinical infection and in various animal models of infection (5, 6, 20, 21, 23, 31). Importantly, endogenous IL-18 has a protective role in mice during infection with Y. enterolitica and the intracellular pathogens L. major and S. typhi (21-23).

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 (21, 28, 32–34). Alveolar macrophages, the resident phagocytes within the airways, are likely important producers of IL-18 within the lung, because IL-18 is known to be mainly produced by activated macrophages (19). Cameron et al. (32) 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. 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 up-regulation of IL-18 mRNA expression, and a modest increase in the concentrations of both pro-IL-18 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 with WT mice. Furthermore, the role of endogenous IL-18 was confirmed by the finding that anti-IL-18 treatment was associated with an enhanced outgrowth of pneumococci in lungs of WT mice when compared with WT mice treated with a control Ab. These data suggest that, although the release of IL-18 locally within the lung is not strongly up-regulated during pneumococcal pneumonia, IL-18 plays an important regulatory role in the early localized antimicrobial host defense against S. pneumoniae.

Pneumonia is characterized by the recruitment of phagocytic cells, mainly granulocytes, to the site of infection (35). 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 were reduced in IL-18−/− mice compared with 24 h but remained higher than in WT animals. The increased recruitment of granulocytes to the alveolar compartment of IL-18−/− mice may at least in part have been mediated by the elevated lung concentrations of the CXC chemokines MIP-2 and KC, which are known to contribute to granulocyte attraction to sites of bacterial infection in the lung (36, 37). Normally, granulocytes serve a protective role in the defense against pneumonia, as indicated by studies in which Abs against either MIP-2 or the type 2 CXCR diminished granulocyte recruitment and bacterial clearance during Klebsiella and Pseudomonas pneumonia respectively (36, 36), and conversely by an investigation in which transgenic overexpression of KC in the lungs resulted in an enhanced granulocyte recruitment to lungs and an improved bacterial clearance (37). However, we consider it likely that the increased lung inflammation observed in IL-18−/− mice is a reflection of an increased proinflammatory stimulus provided by the higher bacterial load. Indeed, in systemic sterile inflammation, induced by i.p. injection of Escherichia coli endotoxin in mice, anti-IL-18 reduced neutrophil accumulation in various tissues, including the lungs, which was associated with reduced lung concentrations of MIP-2 (20). Furthermore, IL-18 has been
reported to increase rather than to inhibit the production of IL-8, the human prototype CXC chemokine, in vitro (14).

Previous studies have demonstrated that locally produced cytokines play an important role in the regulation of host defense against bacterial pneumonia (4). In murine pneumonia caused by *S. pneumoniae* or *Klebsiella pneumoniae*, the absence of the proinflammatory cytokine TNF was associated with enhanced bacterial outgrowth in the lung and increased mortality (25, 39, 40). However, neutralization of the anti-inflammatory cytokine IL-10 impaired bacterial clearance from the lung in these models (24, 41). Hence, a local proinflammatory milieu seems required for an adequate antibacterial defense at the site of the infection. Our present findings are in line with the documented proinflammatory properties of IL-18, revealing that this cytokine, like TNF, plays a protective role in host defense against bacterial respiratory tract infection.

IL-18, originally named IFN-γ-inducing factor, has traditionally been viewed as an important stimulator, together with IL-12, of IFN-γ production (19). In accordance, IL-18−/− mice had lower IFN-γ concentrations in their lungs than did WT mice during pneumonia. Previous studies have suggested that IL-12 and IFN-γ are involved in protective immunity during pneumonia (42, 43). 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, IL-12−/− mice demonstrated a normal defense to pneumococcal pneumonia, which is in line with earlier investigations with IL-12p35−/− mice in this model (44). Furthermore, injection of anti-IL-12 did not influence pneumococcal outgrowth in either WT or IL-18−/− mice. These findings contrast with an earlier report in which anti-IL-12 treatment was found to hamper host defense in *Klebsiella pneumoniae* pneumonia (45). Indeed, both the role of endogenous IL-18 is unrelated to IL-12 and IFN-γ and anti-IL-18 antiserum. previous studies by Rubins et al. (43) reporting an increased mortality of IL-12−/− mice. These authors, who did not evaluate the effect of IFN-γ deficiency on bacterial outgrowth, used much larger inocula of *S. pneumoniae* (up to 10^8 CFU), associated with rapid mortality, which may have less relevance for clinical pneumonia. It should be noted that in the current studies anti-IL-18 treatment of WT mice was not associated with reduced IFN-γ concentrations, contrasting with results obtained in IL-18−/− mice. Although a clear explanation for this is not available, all in all 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 antimicrobial 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. In this study we 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 proinflammatory cytokines play a pivotal role, is essential for local host defense against respiratory pathogens (4).

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