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

Lauw, F.N.

Citation for published version (APA):
Lauw, F. N. (2000). IL-12, IL-18 and IFN-gamma in the immune response to bacterial infection.
Chapter 7

The role of interferon-γ in murine pneumococcal pneumonia

Anita W. Rijneveld,¹,² Fanny N. Lauw,¹,² Marc J. Schultz,¹,² Sandrine Florquin,³ Anje A. te Velde,¹ Peter Speelman,² Sander J. H. van Deventer,¹ Tom van der Poll ¹,²

Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands: ¹Department of Experimental Internal Medicine, ²Department of Infectious Diseases, Tropical Medicine and AIDS, and ³Department of Pathology.
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Abstract

Interferon-γ (IFN-γ) is considered an important mediator of antibacterial host defense. To determine the role of IFN-γ in host defense against pneumonia, IFN-γ receptor deficient (IFN-γR−−) and 129/Sv wild type (WT) mice were intranasally inoculated with Streptococcus pneumoniae. Infection with S. pneumoniae induced comparable lung concentrations of IFN-γ in IFN-γR−− and WT mice. Mortality did not differ between IFN-γR−− and WT mice inoculated with increasing doses of bacteria. At 48 h after inoculation, IFN-γR−− mice had significantly less pneumococci in their lungs compared to WT mice (P < 0.05). Similarly, IFN-γ−− Balb/c mice had less S. pneumoniae CFU in lungs than WT Balb/c mice at 48 h after infection (P < 0.05). The relatively increased resistance of IFN-γR−− mice to pneumococcal pneumonia was not related to favorable effects on host defense mechanisms known to contribute to antibacterial immunity in lungs, i.e. the influx of neutrophils in bronchoalveolar lavage fluid was reduced, and the levels of protective cytokines and nitric oxide were either similar or lower in IFN-γR−− mice. These data suggest that endogenous IFN-γ, unlike its major protective role in host defense against intracellular pathogens, does not serve a protective role during pneumococcal pneumonia.
Introduction

Streptococcus pneumoniae is a gram-positive bacterium responsible for more than 50% of the cases of community acquired pneumonia. Pneumococcal pneumonia is the fifth leading cause of death worldwide and among the patients with community acquired pneumonia who require hospitalization the mortality rate is as high as 25% (1, 2). Against this background and because of the growing resistance of the pneumococcus to antimicrobial therapy, it is important to gain insight in the pathogenesis of pneumococcal pneumonia (3). Innate defense mechanisms play an important role in the elimination of bacteria from the alveolus. Phagocytic cells, as well as residential natural killer (NK) cells and T-cells, participate in this response via the elaboration of chemotactic and regulatory cytokines (4).

Interferon-γ (IFN-γ) is a potent pro-inflammatory cytokine, mainly produced by antigen activated T and NK cells. IFN-γ exerts several immune regulatory activities, including activation of phagocytes, stimulation of antigen presentation by increasing the expression of major histocompatibility complex (MHC) molecules class I and II on antigen presenting cells (APCs), orchestration of leukocyte-endothelium interactions and stimulation of the respiratory burst (5, 6). IFN-γ is considered to play a pivotal role in host defense against several infectious diseases. Peritoneal and alveolar macrophages can be activated by IFN-γ in vitro to express enhanced antimicrobial activity (7-10). Mice deficient for IFN-γ (IFN-γ<sup>-/-</sup>) or the IFN-γ receptor (IFN-γR<sup>+</sup>) demonstrated impaired pulmonary clearance of Toxoplasma gondii, Listeria monocytogenes, Mycobacterium tuberculosis and Legionella pneumophila (11-15). Furthermore, treatment with IFN-γ improved the outcome in these models (16-19).

The role of IFN-γ in the pathogenesis of bacterial pneumonia is not well defined. In a rat model of chronic Pseudomonas aeruginosa pneumonia, intraperitoneal administration of IFN-γ resulted in improved clearance of bacteria from the lung (20). In addition, in one study, IFN-γ<sup>-/-</sup> mice showed increased mortality during bacteremic pneumococcal pneumonia (21). In the latter investigation, however, neither bacterial outgrowth in lungs, nor associated pulmonary inflammatory responses were reported. Therefore, in the present study we sought to determine the role of IFN-γ in host defense mechanisms during pneumococcal pneumonia using IFN-γR<sup>-/-</sup> and IFN-γ<sup>-/-</sup> mice.
Materials and Methods

Animals
All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center. 10-12 week old male IFN-γR⁻/⁻ and wild type (WT) mice, both on the 129/Sv/Ev x 129Sv/Ev genetic background, were kindly donated by Dr. M. Kopf (Basel Institute for Immunology, Basel, Switzerland) [22]. IFN-γ⁻/⁻ BALB/c mice were purchased from The Jackson Laboratories (Bar Arbor, ME); WT BALB/c mice were from Harland Sprague Dawley Inc., Horst, the Netherlands. In all experiments, sex and age matched mice were used.

Induction of pneumonia
Pneumonia was induced as described previously [23-25]. Briefly, *S. pneumoniae* serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD). Pneumococci were grown for 6 h to midlogarithmic phase at 37°C in 5% CO₂ using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500 x g for 15 minutes, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at different concentrations (see Results), as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abott, Queensborough, Kent, UK), and 50 µl was inoculated intranasally. Some mice were inoculated intranasally with 50 µl of isotonic saline only (control mice).

Preparation of lung homogenates
At 24 and 48 h after inoculation, mice were anesthetized by intraperitoneal injection of Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the vena cava inferior. Whole lungs were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospec Products, Bartlesville, OK) that was carefully cleaned and disinfected with 70% alcohol after each homogenization. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates (and blood), and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37°C and 5% CO₂. CFUs were counted after 16 h. For cytokine measurements lung homogenates were lysed in lysis buffer (300 mM NaCl, 15mM Tris, 2mM MgCl₂, 2mM Triton(X-100), Pepstatin A, Leupeptin, Aprotinine (20 ng/ml), pH 7.4) for 30 min. at 4°C, and centrifuged at 1500 x g at 4°C for 15 minutes. The supernatant was frozen at -20°C until cytokine measurement.
Bronchoalveolar lavage
The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of sterile isotonic saline. 0.9 - 1 ml of lavage fluid (BALF) was retrieved per mouse, and total cell numbers were counted from each sample. BALF differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL.). BALF was then spun at 750 x g for 5 minutes at 4°C and supernatants were frozen at -20°C until measurements were performed.

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

Cell preparation and FACScan analysis
Flow cytometric analysis was performed on cells in BALF and cells isolated from lungs. Lung cells were isolated from freshly derived specimens using an automated desegregation device (Medimachine System; Dako, Glostrup, Denmark) and resuspended in RPMI (Biowhittaker, Verviers, Belgium) with 1% BSA (Sigma, St. Louis, MO). The cell suspension was crushed through a 35μm filter (cell strainer 35μm, Becton &Dickinson labware, New Jersey). Cells were centrifuged at 600 x g for 5 minutes at 4°C, and washed with cold FACS buffer (phosphate buffered saline (PBS) supplemented with 0.01% NaN₃, 0.5% BSA, and 0.3 mM EDTA) and resuspended in FACS buffer. BALF and lung cells from three mice per group were pooled. For staining, 1 x 10⁶ cells/ well (96 well microplate, Greiner B.V. Labor Techniek, Alphen aan de Rijn, the Netherlands) were incubated for 30 minutes at 4°C with rat anti-mouse unconjugated CD11b (clone M1/70) (26). The hybridoma producing anti-mouse CD11b was kindly provided by R. Mebius (Free University, Amsterdam, the Netherlands). After washing with FACS buffer, R-phycoerythrin (R-PE)-conjugated F(ab)2 fragments of goat-anti-rat immunoglobulins (Zymed Inc., Camarillo, CA) were added as a secondary antibody followed by FITC-labeled rat anti-mouse Gr-1 mAb (clone RB6-8C5; PharMingen), which was used for staining of granulocytes. The appropriate isotype controls were included in all experiments. All FACS reagents were used in concentrations recommended by manufacturers. 5,000 Gr-1 positive cells were counted. Results are expressed as the mean cell fluorescence intensity (MFI) after subtraction of control IgG fluorescence.
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**Determination of cytokines, chemokines and nitric oxide concentrations**

Cytokines and chemokines were measured by commercially available ELISAs according to the manufacturers' recommendations: tumor necrosis factor-α (TNF) was obtained from Genzyme (Cambridge, MA), interleukin-6 (IL-6), IL-10, IL-12p40 and IL-12p70 were from Pharmingen, and IFN-γ, macrophage inflammatory protein (MIP-2) and KC were from R&D Systems (Minneapolis, MN). Nitric oxide (NO) production was assessed by measurement of nitrite and nitrate by a colorimetric assay according to the instructions of the manufacturer (Cayman Chemical Company, Ann Arbor, MI). Total NO production was expressed as the sum of both nitrite and nitrate production.

**Statistical analysis.**

Data are expressed as mean ± SE, unless indicated otherwise. Comparisons between groups were conducted using the Mann Whitney U test. Survival was analyzed with Kaplan-Meier. P < 0.05 was considered to represent a statistical significant difference.

**Results**

**Induction of IFN-γ (Figure 1)**

WT mice did not have detectable levels of IFN-γ in their lung homogenates at baseline or after intranasal inoculation with isotonic saline. Induction of pneumonia was associated with enhanced production of IFN-γ within the pulmonary compartment, as reflected by elevated IFN-γ concentrations in the lung homogenates (24 h: 13.1 ± 1.0 ng/g and 48 h: 16.8 ± 1.9 ng/g lung; P < 0.05 vs. control). IFN-γR−/− mice had higher lung IFN-γ levels than WT mice (24 h: 33.0 ± 12.7 ng/g and 48 h: 34.9 ± 12.8 ng/g lung; P < 0.05 vs. control), although the differences were not statistically significant.

![Figure 1. IFN-γ protein expression in lungs.](image)
Survival (Figure 2)
Considering that IFN-γ is generally believed to play an important role in antimicrobial host defense (13, 22, 27, 28), we expected IFN-γR−/− mice to be more susceptible to pneumococcal pneumonia. However, using three different bacterial inocula (1 x 10⁴, 2 x 10⁵ and 5 x 10⁵ CFU S. pneumoniae), inducing pneumonia of increasing severity, no differences in mortality between IFN-γR−/− and WT mice were observed until ten days post inoculation. Mice that survived for ten days appeared permanent survivors.

Bacterial outgrowth (Figure 3)
To obtain further insight in the role of IFN-γ in host defense against pneumococcal pneumonia, we evaluated the outgrowth of pneumococci in the lungs of IFN-γR−/− and WT mice. At 24 h after intranasal inoculation of 5 x 10⁵ CFU S. pneumoniae, bacterial counts in the lungs were similar in IFN-γR−/− and WT mice (data not shown). At 48 h, lungs of IFN-γR−/− mice contained 1.5 log less bacteria than WT mice (P = 0.001), suggesting that endogenous IFN-γ activity hampers antibacterial host defense. To confirm this finding and to demonstrate that it is not unique for the 129 Sv/Ev background, we repeated this experiment in IFN-γ−/− Balb/c and WT mice. IFN-γ−/− mice had 1.4 log less bacteria in their lungs than WT mice at 48 h after infection (P = 0.01).
Histological changes (Figure 4)

Histologically, at 24h after inoculation the IFN-γR−/− mice showed more pronounced inflammatory infiltrates in the lung when compared to WT mice without differences in the architecture of the inflammation. At 48 h after inoculation with *S. pneumoniae* lungs of IFN-γR−/− mice showed clearly more apoptotic bodies and histiocytes (macrophages) corresponding to the clearance phase when compared to WT mice, in which the inflammatory infiltrates were more pronounced.

**Figure 3.** Bacterial outgrowth in lungs. A. Bacterial outgrowth in lungs of IFN-γR−/− and WT mice at 48 h after intranasal inoculation with $5 \times 10^5$ CFU *S. pneumoniae*. B. Bacterial counts in lungs of IFN-γ−/− and WT and mice at 48 h after inoculation of $5 \times 10^5$ CFU *S. pneumoniae*. Horizontal lines represent the medians within the groups.

**Figure 4.** Histopathology of lungs

Histological sections of lungs of WT (A + C) and IFN-γR−/− (B + D) 24h (A + B) and 48 h (C + D) respectively after inoculation with $5 \times 10^5$ CFU *S. pneumoniae*. Haematoxylin and eosin staining, original magnification x 50. Sections are representative for the groups at the different time point.
Neutrophilic cell influx and activation markers (Figure 5 and 6)

In a first attempt to determine the mechanism by which endogenous IFN-γ could facilitate the outgrowth of bacteria during pneumococcal pneumonia, we determined the influx and the activation state of the granulocytes in BALF at 48 h after intranasal inoculation with S. pneumoniae. Neutrophil counts in BALF of IFN-γR−/− mice were lower than in BALF of WT mice ($P < 0.05$). Neutrophils isolated from lung tissue and BALF during pneumococcal pneumonia displayed signs of activation, as reflected by enhanced expression of CD11b, when compared to neutrophils obtained from control mice. However, these neutrophil activation markers did not differ between IFN-γR−/− and WT mice.

![Figure 5. Granulocyte influx in BALF.](image)

**Figure 5.** Granulocyte influx in BALF. Mean (± SE) of granulocyte numbers in BALF obtained from IFN-γR−/− and WT mice at 48 h after intranasal inoculation of $5 \times 10^5$ CFU S. pneumoniae. n=6 mice per group.

![Figure 6. CD11b expression on granulocytes](image)

**Figure 6.** CD11b expression on granulocytes CD11b expression on Gr-1 positive cells in BALF and lung cells from IFN-γR−/− and WT mice at 48 h after inoculation of saline (control mice) or of $5 \times 10^5$ CFU S. pneumoniae. Data are mean ± SE of 6 mice per group. * $P < 0.05$ vs. controls.

Lung concentrations of cytokines, chemokines and NO (Table 1)

A second mechanism by which IFN-γ could facilitate the outgrowth of bacteria during pneumococcal pneumonia is by modulation of cytokine or chemokine production. In particular, the cytokines TNF, IL-6 and IL-12, and the chemokines KC and MIP-2 have been found to contribute to the host defense in murine models of pneumonia (23, 25, 29-32). Of these mediators, lung concentrations of TNF and MIP-2 were similar in IFN-γR−/− and wild type mice. By contrast, IL-6, IL-12(p70) and KC levels were lower in IFN-γR−/− mice. Hence, alterations in local production of "protective" cytokines and chemokines cannot explain the relatively decreased susceptibility of IFN-γR−/− mice during pneumonia.

IFN-γ is able to enhance NO production (5, 33, 34). To assess whether this mechanism was affected in the IFN-γR−/− mice, we measured NO levels in BALF of both groups at 48h after induction of pneumonia. IFN-γR−/− mice had lower NO levels when compared to WT mice ($9.5 \pm 1.1$ μM and $13.6 \pm 1.6$ μM respectively), although this difference was not significant.
Table 1. Cytokine and chemokine concentrations in lung homogenates after intranasal inoculation with 5 x 10⁵ CFU S. pneumoniae.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>24 h</th>
<th>48 h</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>IFN-γR&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF</td>
<td>1215 ± 207</td>
<td>1238 ± 106</td>
</tr>
<tr>
<td>IL-6</td>
<td>342 ± 103</td>
<td>202 ± 51</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>30 ± 75</td>
<td>58 ± 19</td>
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<tr>
<td>IL-12p70</td>
<td>9 ± 1</td>
<td>10 ± 1</td>
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<tr>
<td>KC</td>
<td>656 ± 151</td>
<td>62 ± 12&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>MIP-2</td>
<td>223 ± 44</td>
<td>458 ± 210</td>
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</table>

Data are mean ± SE of 8 mice per group at each time point. * P < 0.05 vs. WT mice.

Discussion

IFN-γ is a proinflammatory cytokine that has protective effects in a variety of infectious diseases. The role of IFN-γ in bacterial pneumonia is not well defined. Recently, Rubins and Pomeroy reported that IFN-γ<sup>−/−</sup> mice demonstrated an increased mortality when compared to WT mice in a model of severe pneumococcal pneumonia (21). In that study, however, no data were presented on bacterial outgrowth or induction of innate defense mechanisms. We here report that IFN-γR<sup>−/−</sup> mice do not have an increased mortality after induction of pneumococcal pneumonia induced by three different doses of bacteria. After the first 48h of the disease, both IFN-γR<sup>−/−</sup> and IFN-γ<sup>−/−</sup> mice had a reduced outgrowth of pneumococci in lungs when compared to their respective WT strains. Our results suggest that endogenous IFN-γ may hamper early antibacterial defense in the lung compartment, but that the lack of functional IFN-γ eventually does not influence survival.

The essential role of endogenous IFN-γ in host defense against infection has in particular been demonstrated for intracellularly growing micro organisms (11-14). In models of acute systemic infection with extracellular bacteria, IFN-γ has been found to play a detrimental role. Indeed, treatment with anti-IFN-γ antibodies reduced mortality after intravenous or intraperitoneal injection of high doses of *Escherichia coli*, (35, 36). The detrimental role of IFN-γ in these acute infection models is in line with findings that treatment with anti-IFN-γ antibodies profoundly reduced mortality in mice exposed to high doses of endotoxin (37, 38) In a subacute model of *Staphylococcus aureus* sepsis, resulting in a 100% lethality in normal WT mice over a 10-day period, IFN-γR<sup>−/−</sup> mice were relatively protected against lethality, which was associated with a reduced number of *S. aureus* CFUs in blood when compared to WT mice (39). This study taken together with our results,
suggest that IFN-\(\gamma\) may facilitate bacterial outgrowth in conditions in which the experimental animal is not overwhelmed by a high dose of bacteria (or bacterial products).

Our study does not provide a clear explanation for the reduced outgrowth of pneumococci in lungs of mice lacking functional IFN-\(\gamma\). A protective mechanism could neither be demonstrated for the diminished outgrowth of staphylococci during subacute \(S.\) \textit{aureus} sepsis (39). We evaluated several innate responses known to contribute to antibacterial defense in the lung compartment. None of these responses were altered in IFN-\(\gamma\)R\(^{-/-}\) mice in a way that would enhance bacterial clearance. Indeed, the induction of some "protective" responses was even attenuated in IFN-\(\gamma\)R\(^{-/-}\) mice, including the recruitment of neutrophils to the lung. Further, although we found an upregulation of CD11b on granulocytes in BALF of mice with pneumonia, indicative for an enhanced activation state (40, 41), CD11b expression did not differ between IFN-\(\gamma\)R\(^{-/-}\) and WT mice. Earlier research has suggested that IFN-\(\gamma\) has the ability to inhibit, rather than to augment, neutrophil recruitment in vivo, i.e. neutrophil influx in skin was diminished in mice with thermal wounds after treatment with IFN-\(\gamma\) (42), and intraperitoneally administered IFN-\(\gamma\) decreased the inflammatory response in rats with chronic \textit{Pseudomonas aeruginosa} pneumonia by reducing neutrophilic influx (20). Conceivably, the reduced KC concentrations played a role in the attenuated neutrophil influx in BALF of IFN-\(\gamma\)R\(^{-/-}\) mice, considering that inhibition of this CXC chemokine diminished neutrophil accumulation in lungs after intratracheal administration of endotoxin (43) and transgenic overexpression of KC in mouse lungs resulted in enhanced neutrophil migration within the lung compartment (44). In addition, the lower bacterial load in lungs of IFN-\(\gamma\)R\(^{-/-}\) mice (thus providing less proinflammatory stimuli) could have been responsible for the attenuated neutrophil recruitment. This may also explain the lower concentrations of IL-6 and IL-12, two other "protective" cytokines during pneumonia (23, 31), in IFN-\(\gamma\)R\(^{-/-}\) mice.

IFN-\(\gamma\) has been implicated as a pivotal mediator in host defense against a variety of respiratory pathogens. IFN-\(\gamma\) was found to be important for cell-mediated immunity against fungi and intracellular microorganisms that can cause chronic pneumonias, including \textit{Mycobacteria, Mycoplasma, Chlamydia} and \textit{Histoplasma} (reviewed in (45)). We here show that endogenous IFN-\(\gamma\) is not required for, and may even impair, an effective pulmonary defense in pneumonia due to \textit{S. pneumoniae}, the most frequently isolated organism in patients with community acquired pneumonia. These data exemplify the complex role of IFN-\(\gamma\) in innate immunity during pulmonary infection.
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References


IFN-γ during pneumococcal pneumonia


