Pneumonia: an investigation of host defence mechanisms
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The role of interferon-γ in murine pneumococcal pneumonia
To establish the role of interferon-γ (IFN) in pneumonia, IFN-γ Receptor deficient (IFN-γ R<sup>−/−</sup>) and 129/Sv (Wt) mice were intranasally inoculated with *Streptococcus (S.)* pneumoniae. Whereas mortality did not differ between groups, 48h after inoculation, IFN-γ R<sup>−/−</sup> mice had significantly less pneumococci in their lungs compared to Wt mice. Similarly, IFN-γ<sup>−/−</sup> mice had less CFU in lungs than Wt mice. The relatively increased resistance of IFN-γ R<sup>−/−</sup> mice was not related to favourable effects on defense mechanisms known to contribute to antibacterial immunity, i.e. the neutrophilic influx was reduced, and the cytokine and nitric-oxide levels were similar or lower in IFN-γ R<sup>−/−</sup> mice. In contrast, mice treated with anti-IFN-γ did not demonstrate a consistently altered bacterial outgrowth when compared with mice treated with a control antibody. These data suggest that endogenous IFN-γ, unlike its protective role in defense against intracellular pathogens does not serve a protective role during pneumococcal pneumonia.

**Introduction**

*S. 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 patients with community acquired pneumonia who require hospitalization the mortality rate is as high as 25%.<sup>1,2</sup> 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.<sup>3</sup> Innate defense mechanisms play an important role in the elimination of bacteria from the alveolus. Phagocytic cells, as well as resident natural killer (NK) cells and T-cells, participate in this response via the elaboration of chemotactic and regulatory cytokines.<sup>4</sup> 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.<sup>5,6</sup>

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.<sup>7-10</sup> Mice deficient for IFN-γ (IFN-γ<sup>−/−</sup>) or the receptor (IFN-γ R<sup>−/−</sup>) demonstrated impaired pulmonary clearance of *T. gondii, L. monocytogenes, M. tuberculosis* and *L. pneumophila*.<sup>11-15</sup> Furthermore, treatment with IFN-γ improved the outcome in these models.<sup>16-19</sup>

The role of IFN-γ in the pathogenesis of bacterial pneumonia is not well defined. In a rat model of chronic *P. aeruginosa* pneumonia intraperitoneal administration of IFN-γ resulted in improved clearance of bacteria from the lung.<sup>20</sup> In addition, in one study IFN-γ<sup>−/−</sup> mice showed increased mortality during bacteremic pneumococcal pneumonia.<sup>21</sup> 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-γ R−/− and IFN-
γ−/− mice.

Materials and Methods

Animals. Ten to twelve week old male IFN-γ R−/− and wild type (Wt) mice, both on the 129/Sv/Ev genetic background, were kindly donated by Dr. M. Kopf (Basel Institute for Immunology, Basel, Switzerland). 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.

Reagents. The R46A2 rat anti-mouse IFN-γ IgG1 mAb and LO-DNP-2, a control rat IgG1 mAb were produced in ascites and kindly provided by Dr. M. Goldman (Université Libre de Bruxelles, Brussels, Belgium). The endotoxin levels were <15 pg/ml as determined by a Limulus Amoebocyte Lysate essay (LAL-QCL-1000, Whittaker M.A., Bioproducts, Walkersville, MD). 1 mg of anti-IFN-γ mAb or the same amount of control mAb was given to BALB/c mice i.p. 2h before inoculation with S. pneumoniae. Anti-IFN-γ mAb R46A2 given at a dose of 0.5 mg has previously been demonstrated to reduce mortality in mice after administration of Staphylococcal Enterotoxin B24 or endotoxin.25

Induction of pneumonia. Pneumonia was induced as described previously. Briefly, S. pneumoniae serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD). Pneumococci were grown for 6 hours 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 (2 x 10⁵ - 4 x 10⁶ colony forming units (CFU)/ml), as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Abbott, Queensborough, Kent, UK), and 50 µl was inoculated intranasally. Some mice were inoculated intranasally with 50 µl of sterile isotonic saline only (control mice).

Preparation of lung homogenates. At 24 and 48 hours after inoculation mice were anesthetized by intraperitoneal injection of Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam® (Roche, Meidrecht, 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 (Biospect 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. CFU were counted after 16 hours. For cytokine measurements lung homogenates were
lysed in lysis buffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl, 2 mM Triton(X-100), Pepstatin A, Leupeptin, Aprotine (20 ng/ml), pH 7.4) and spun 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 was retrieved per mouse, and total cell numbers were counted from each sample in a hemocytometer. BAL fluid (BALF) differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, Ill.) 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.

*Histological examination.* For histopathological examination only lungs were used. After 24 hours fixation of lungs in 4% paraformaldehyde in PBS and embedding in paraffin, 4 µm thick sections were stained with hematoxylin and eosin. All slides were coded and semi-quantitatively scored by a pathologist without knowledge of the type of mice and treatment.

*Cell preparation and FACScan analysis.* Flow cytometric analysis was performed on cells in BALF and cells isolated from lungs using a FACScan flow cytometer in conjunction with FACScan software (Becton Dickinson, Mountain View, San Jose, CA). Lung cells were isolated from freshly derived specimens using an automated desintegration device (Medimachine System, Dako, Glostrup, Danmark) and resuspended in RPMI (Biowhittaker-Boehringer, Verviers, Belgium) with 1% BSA (Sigma Chemical Co., St. Louis, MO, USA). The cell suspension was crushed through a 35 µm filter (cell strainer 35 µm, 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 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). The hybridoma producing anti-mouse CD11b was kindly provided by R. Mebius, Free University, Amsterdam, the Netherlands. After washing in FACs buffer, R-phycocerythrin (RPE)-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.

* 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) ELISA was obtained from Genzyme (Cambridge, MA), interleukin-6 (IL-6), IL-10, IL-12p40 and IL-12p70 ELISA's were from Pharmingen, and IFN-γ, macrophage inflammatory protein (MIP-2) and KC ELISA's 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 ± SEM, unless indicated otherwise. Comparisons between groups were conducted using the Mann Whitney U test. Survival curves were compared by log-rank test. P-value<0.05 was considered to represent a statistical significant difference.

Results

Induction of IFN-γ. 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 lung homogenates (24h: 13.1 ± 1.0 ng/g; 48h: 16.8 ± 1.9 ng/g; P<0.05 vs. control). IFN-γ R−/− mice had higher lung IFN-γ levels than Wt mice, although the differences were not statistically significant (Figure 1).

Figure 1. IFN-γ protein expression in lungs. IFN-γ protein concentrations in lung homogenates in Wt mice and IFN-γ R−/− 24 and 48h after intranasal inoculation with 5 x 10^5 CFU S. pneumoniae. Data are mean ± SEM; N = 8 for each time point. IFN-γ was undetectable in mice inoculated with sterile saline (controls; data not shown). *P<0.05 vs. control

Survival. Considering that IFN-γ is generally believed to play an important role in antimicrobial host defense,13,22,30,31 we expected IFN-γ R−/− mice to be more susceptible to pneumococcal pneumonia. However, using three different bacterial inocula (10^5, 2 x 10^5, 5 x 10^5 CFU), to induce pneumonia of increasing severity, no differences in mortality between IFN-γ R−/− and Wt mice were observed until ten days after inoculation (Figure 2). Mice that survived for ten days appeared permanent survivors.

Bacterial outgrowth. 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-γ
Figure 2. Survival studies. Survival after intranasal inoculation with $10^4$ (A), $2 \times 10^5$ (B) and $5 \times 10^5$ (C) CFU *S. pneumoniae* in Wt (open circles) and IFN-γ R$^{-/-}$ mice (closed circles). Mortality was assessed twice daily for 10 days. N = 15 per group

R$^{-/-}$ and Wt mice. At 24h after intranasal inoculation of $5 \times 10^5$ CFU *S. pneumoniae*, bacterial counts in the lungs were similar in IFN-γ R$^{-/-}$ and Wt mice (data not shown). At 48h, lungs of IFN-γ R$^{-/-}$ mice contained 1.5 log less *S. pneumoniae* CFU than wild type mice ($P=0.001$), suggesting that endogenous IFN-γ activity hampers antibacterial host defense (Figure 3). 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 R$^{-/-}$ BALB/c and Wt mice. IFN-γ R$^{-/-}$ mice had 1.4 log less CFU *S. pneumoniae* in their lungs than Wt mice 48h after inoculation with $5 \times 10^5$ CFU bacteria ($P=0.01$) (Figure 3).

Figure 3. Bacterial outgrowth in lungs. (A) CFU *S. pneumoniae* in lungs of Wt mice and IFN-γ R$^{-/-}$ mice 48h after inoculation with $5 \times 10^5$ CFU *S. pneumoniae*. (B) CFU in lungs of Wt mice and IFN-γ R$^{-/-}$ 48h after inoculation of $5 \times 10^5$ CFU *S. pneumoniae*. Horizontal lines represent the medians within the groups

Histological changes. At 24h after inoculation IFN-γ R$^{-/-}$ mice showed more pronounced inflammatory infiltrates in the lung when compared to Wt mice without differences in the composition of the inflammation. At 48 hours after inoculation with *S. pneumoniae* lungs of IFN-γ R$^{-/-}$ mice showed clearly more apoptotic bodies and macrophages corresponding to the clearance phase (Figure 4). At this time point Wt mice still displayed a pronounced and more active inflammation in the lungs.
Figure 4. Histopathology of the lungs. Histological sections of lungs of Wt mice (A + C) and IFN-γ R−/− mice (B + D) 24h (A + B) and 48h (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 each time point. N = 5 per group

Neutrophilic cell influx and activation markers. 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 of neutrophils and the activation state of the granulocytes in BALF at 48h after intranasal inoculation with S. pneumoniae. Neutrophil counts in BALF of IFN-γ R−/− mice were lower than in BALF of Wt mice (P<0.05; Figure 5).

Figure 5. Granulocytic influx in BALF. Mean ± SEM of granulocytic influx in BALF obtained from Wt mice and IFN-γ R−/− mice at 48h after intranasal inoculation of $5 \times 10^5$ CFU S. pneumoniae. N = 6 per group

Neutrophils isolated from lung tissue and BALF displayed signs of activation, as reflected by enhanced expression of CD11b, when compared to neutrophils obtained from control mice. However, this neutrophil activation marker did not differ between IFN-γ R−/− and Wt mice.
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 chemokine or cytokine 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.\textsuperscript{27,28,32-35} Of these mediators, lung concentrations of TNF and MIP-2 were similar in IFN-γ R\textsuperscript{−/−} and Wt mice. By contrast, IL-6, IL-12(p70) and KC levels were lower in IFN-γ R\textsuperscript{−/−} mice. Hence, alterations in local production of “protective” cytokines and chemokines can not explain the relatively decreased susceptibility of IFN-γ R\textsuperscript{−/−} mice during pneumonia. IFN-γ is able to enhance NO production.\textsuperscript{5,36,37} To assess whether this mechanism was affected in the IFN-γ R\textsuperscript{−/−} mice we measured nitrate levels in BALF of both groups at 48h after induction of pneumonia. IFN-γ R\textsuperscript{−/−} mice had lower nitrate levels when compared to Wt mice (9.5 ± 1.1 μM and 13.6 ± 1.6 μM, respectively), although this difference was not significant.

| Table 1. Cytokine and chemokine levels in lung homogenates |
|------------------|-----------------|------------------|------------------|
|                  | 24h after inoculation | 48h after inoculation |
|                  | Wild type | IFN-γ R\textsuperscript{−/−} | Wild type | IFN-γ R\textsuperscript{−/−} |
| TNFα             | 1215 ± 207 | 1238 ± 106 | 1623 ± 230 | 1631 ± 190 |
| IL-6             | 342 ± 103  | 202 ± 51   | 254 ± 62   | 68 ± 20*   |
| IL-12p40         | 30 ± 75    | 58 ± 19    | 203 ± 29   | 136 ± 38   |
| IL-12p70         | 9 ± 0.4    | 10 ± 0.6   | 11 ± 0.6   | 8 ± 0.5*   |
| KC               | 656 ± 151  | 62 ± 12 *  | 697 ± 221  | 25 ± 0.5*  |
| MIP-2            | 223 ± 44   | 458 ± 210  | 465 ± 267  | 211 ± 5.9  |

Data are mean ± SEM of 8 mice per group per timepoint. Mice were inoculated intranasally with 10\textsuperscript{5} CFU S. pneumoniae at t = 0. *P<0.05 vs. Wt

Anti-IFN-γ treatment during pneumococcal pneumonia in Wt mice. To exclude the possibility that the decreased susceptibility in these gene deficient mice were due to compensatory changes, we performed additional experiments, in which we pretreated normal
BALB/c mice intraperitoneally with anti-mouse IFN-γ mAb or control mAb 2h before inoculation with 1 or 5 x 10^5 CFU *S. pneumoniae*, and determined bacterial outgrowth 48h postinfection. These studies yielded inconsistent results, i.e. in the first experiment anti-IFN-γ treated mice tended to have an enhanced outgrowth (19.3 ± 4.2 x 10^6 vs. 6.5 ± 4.5 x 10^6/ml lung homogenate in anti-IFN-γ and control mice respectively), whereas in the second experiment bacterial outgrowth was indistinguishable in both groups (2.5 ± 1.1 x 10^6 vs. 2.4 ± 2.2 x 10^6 CFU/ml lung homogenate in control mice).

**Discussion**

IFN-γ is a pro-inflammatory cytokine that has protective effects in a variety of infectious diseases. The essential role of endogenous IFN-γ in host defense against infection has in particular been demonstrated for intracellularly growing micro-organisms.11-14 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 associated with bacteremia.21 In that study, however, no data were presented on bacterial outgrowth or induction of innate host defense mechanisms. We here report that IFN-γ R<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-γ R<sup>-/-</sup> and IFN-γ<sup>-/-</sup> mice had a reduced outgrowth of pneumococci in lungs when compared to their respective Wt strains, whereas studies with a neutralizing anti-IFN-γ mAb in Wt mice yielded inconsistent results. Our results suggest that endogenous IFN-γ does not improve antibacterial defense in the lung compartment.

Accordingly, in a subacute model of *S. aureus* sepsis, resulting in a 100% lethality in normal Wt mice over a 10-day period, IFN-γ R<sup>-/-</sup> mice were relatively protected against lethality, which was associated with a reduced number of *S. aureus* CFU’s in blood when compared to Wt mice.38 This study taken together with our results in knockout mice, suggest that IFN-γ may facilitate bacterial outgrowth in conditions in which the experimental animal is not overwhelmed by a high dosis of bacteria. On the opposite 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*.39,40 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.25,41

Our study does not provide a clear explanation for the reduced outgrowth of pneumococci in lungs of mice lacking functional IFN-γ. A protective mechanism could neither be demonstrated for the diminished outgrowth of staphylococci during subacute *S. aureus* sepsis.38 We evaluated several innate responses known to contribute to antibacterial defense in the lung compartment. None of these responses was altered in IFN-γ R<sup>-/-</sup> mice in a way that
INTERFERON-γ would enhance bacterial clearance. Indeed, the induction of some "protective" responses was even attenuated in IFN-γ 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, CD11b expression did not differ between IFN-γ R⁻/⁻ and Wt mice. Earlier research has suggested that IFN-γ 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-γ, and intraperitoneally administered IFN-γ decreased the inflammatory response in rats with chronic Pseudomonas aeruginosa pneumonia by reducing neutrophilic influx. Conceivably, the reduced KC concentrations played a role in the attenuated neutrophil influx in BALF of IFN-γ R⁻/⁻ mice, considering that inhibition of this CXC chemokine diminished neutrophil accumulation in lungs after intratracheal administration of endotoxin and transgenic overexpression of KC in mouse lungs resulted in enhanced neutrophil migration within the lung compartment. In addition, the lower bacterial load in lungs of IFN-γ 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, in IFN-γ R⁻/⁻ mice.

Our results were obtained using mice that were genetically deficient for IFN-γ or IFN-γ R, and should therefore be interpreted with caution. In fact, we could not confirm the diminished pneumococcal outgrowth measured in IFN-γ R⁻/⁻ and IFN-γ⁻/⁻ mice in experiments in which we treated Wt mice with a neutralizing anti-IFN-γ mAb. Several factors could possibly explain this discrepancy. First, the dose of anti-IFN-γ mAb could have been insufficient to neutralize all IFN-γ. Although this possibility seems less likely considering that we gave twice the amount of this particular mAb (1 mg) needed to reduce staphylococcal enterotoxin B or endotoxin-induced lethality (0.5 mg), it is not excluded that the mAb did not penetrate well enough in the pulmonary compartment. This could not be checked directly due to the lack of a reliable and specific IFN-γ bioassay. Second, knockout mice may differ from Wt mice not only with respect to the product of the deleted gene; hereditary deficiency of a protein may result in compensatory changes. However, it should be noted in this context that the role of endogenous IFN-γ in inflammation and infection has been studied in many investigations using knockout mice only, and these results have provided valuable information on the role of IFN-γ in these models.

IFN-γ has been implicated as a pivotal mediator in host defense against a variety of respiratory pathogens. IFN-γ was found to be important for cell-mediated immunity against fungi and intracellular microorganisms that can cause chronic pneumonias, including Mycobacteria, Mycoplasma, Chlamydia and Histoplasma (reviewed in reference 4). We here show that endogenous IFN-γ is not required for an effective pulmonary defense in pneumonia due to S. pneumoniae, the most frequently isolated organism in patients with community acquired pneumonia. These data exemplify the complex role of IFN-γ in innate immunity.
during pulmonary infection.

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