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Endogenous interferon-g impairs bacterial clearance from lungs during Pseudomonas aeruginosa pneumonia.

Abstract

Interferon (IFN-)g is thought to play a role in the resistance to various pathogens. To study the role of IFN-g in the pathogenesis of Pseudomonas pneumonia, IFN-g receptor (R) a-subunit-deficient [IFN-gR(−/−)] mice and wild type mice were intranasally inoculated with Pseudomonas aeruginosa (105 CFU). IFN-gR(−/−) mice demonstrated an enhanced clearance of P. aeruginosa from their lungs when compared to normal wild type mice ($P < 0.05$ at 24 hours after the infection), which was associated with a tendency towards an improved

survival. These findings were not accompanied by a more effective activation of several components of the innate immune system known to contribute to host defense against pneumonia, i.e. the lung concentrations of cytokines and chemokines were similar in IFN-gR(−/−) and wild type mice, while the influx of neutrophils in bronchoalveolar lavage fluid (BALF) was even higher in wild type mice than in IFN-gR(−/−) mice. Remarkably, IFN-gR(−/−) mice had higher nitric oxide levels in the BALF at 24 hours after infection ($P < 0.05$). Endogenous IFN-g impairs rather than augments host defense during pneumonia caused by P. aeruginosa.

Full text

INTRODUCTION

In local infections, such as pneumonia, the initiation, maintenance and resolution of inflammation are considered to be dependent upon the expression of a complex network of pro-inflammatory and anti-inflammatory cytokines, including interferons. Interferon (IFN-)gamma is a glycoprotein that has a variety of immune regulatory functions, including anti-viral activity, and stimulation of macrophages and natural killer (NK) cells [1]. IFN-gamma increases the expression of major histocompatibility complex (MHC) antigens and the inducible synthesis of nitric oxide [1]. The production of IFN-gamma can be induced in several cell types, however, most IFN-gamma secreted during an immune response is produced by activated T and NK-cells [2, 3]. IFN-gamma production is stimulated by macrophage-derived cytokines, especially tumor necrosis factor-alpha (TNF) and interleukin (IL-)12 [4] and is autostimulated by IFN-gamma itself [5].

Through the interaction with a specific cell surface receptor, IFN-gamma initiates its actions on target cells. Disruption of this IFN-gammaR might result in recurrent microbial infections. Indeed, mice carrying a disruption in the murine IFN-gammaR alpha-subunit displayed a phenotype consistent with previous *in vivo* studies of mice treated with neutralizing IFN-gamma-specific antibodies [6, 7]. The ability of these animals to resist infection by a variety of pathogens, including mycobacteria (*M. bovis* and *M. avium*), *Leishmania major* and *Listeria* species was greatly impaired. IFN-gamma may also be important for resistance against Gram-positive respiratory tract infections, since IFN-gamma production is enhanced during murine pneumococcal pneumonia [8, 9], and IFN-gamma

deficient mice showed a markedly increased susceptibility to infection with *Streptococcus pneumoniae* [8]. In contrast, mice lacking the IFN-gamma signal have been demonstrated to have an increased resistance to lipopolysaccharide (LPS) induced toxicity [10-12].

In the present study, we determined the effect of disruption of the IFN-gammaR alpha-subunit on different elements of host defense during pneumonia in mice with the Gram-negative bacterium *Pseudomonas aeruginosa*, the most frequent Gram-negative pathogen causing nosocomial pneumonia [13]. Therefore, we compared bacterial outgrowth, the local production of cytokines and chemokines, cell influx, and nitric oxide (NO) production in lungs, in a mouse model of respiratory tract infection with *P. aeruginosa*.

MATERIALS ET METHODS

Animals. Female *IFN-gammaR*$^{(-/-)}$ with a disruption of the IFN-gammaR alpha-subunit [6] and wild type mice (8-10 weeks), both of a 129SV genetic background, were used in all experiments. Both mice strains were kindly donated by Dr. M. Kopf (Basel Institute for Immunology, Basel, Switzerland). The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Induction of pneumonia. The *P. aeruginosa* strain PA103 (Iglewski Laboratory, Rochester, NY, USA), was grown to mid-logarithmic phase in Luria broth for 6 hours at 37° C, harvested by centrifugation at 1,500 x *g* for 15 min, washed twice in pyrogen-free 0.9% NaCl, and resuspended in 10 ml 0.9% NaCl. The number of bacteria was determined by serial dilution in sterile isotonic saline and culture on blood agar plates for 16 hours. Mice were lightly anesthesized with inhaled isoflurane (Forene® [Abott, Queensborough, Kent, UK]) and an inoculum of 50 mul of the solution was administered intranasally. Some mice were inoculated with 50 mul sterile 0.9% NaCl alone (control mice).

Preparation of blood samples and lung homogenates. At 6 and 24 hours after inoculation, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the *vena cava inferior* in heparin-containing vacutainer tubes. Whole lungs were harvested and homogenized at 4° C in 5 volumes of sterile 0.9% NaCl in a tissue homogenizer that was carefully cleaned and

desinfected with 70% alcohol after each homogenization. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates (and blood), and 50 mul volumes were plated onto sheep-blood agar plates and incubated at 37° C and 5% CO_2. Colony-forming units (CFU) were counted after 24 hours. For cytokine measurements lung homogenates were spun at 1,500 x *g* for 15 min at 4° C, supernatants were filtered through a 35 mum filter (Becton Dickenson, Lincoln Park, NJ, USA) and 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 0.9% NaCl. 0.9-1 ml of lavage fluid was retrieved per mouse, and total cell numbers and differential cell counts were determined from cytospins on each sample. BAL fluid (F) differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, Ill., USA). BALF was then spun at 750 x *g* for 5 min at 4° C, and supernatants were frozen at - 20° C until measurements were performed.

Assays. Cytokine and chemokine levels were measured by ELISA according to the manufacturer's recommendations: TNF (Genzyme, Cambridge, MA, USA), IL-6 (Pharmingen), macrophage inflammatory protein (MIP-) 2, KC, and IFN-gamma (all R&D, Minneapolis, MN, USA). NO_2^-/NO_3^- concentrations were determined in BALF with a commercially available NO assay, according to the manufacturer's recommendations (R&D).

Statistical analysis. Comparisons of survival data were made using the log-rank test. All other data are expressed as means ± standard error (SE). Comparisons between means were conducted using the Wilcoxon test. Significance was set at $P < 0.05$.

RESULTS

Induction of pneumonia and IFN-gamma **production**

Six and 24 hours after inoculation with 10^5 CFU *P. aeruginosa*, lungs appeared swollen and reddish with multiple hemorrhages on the surface. Wet lung weights from mice inoculated with *P. aeruginosa* increased by more than 35%, compared to lungs from control mice inoculated with sterile saline ($P < 0.05$) (Figure 1A).

Mice inoculated with isotonic saline did not have detectable levels of IFN-gamma in lung homogenates after intranasal inoculation. Induction of pneumonia was associated with elevated IFN-gamma levels in lung homogenates (Figure 1B). Remarkably, after 6 hours *IFN-gammaR*$^{(-/-)}$ mice had higher lung IFN-gamma levels than normal wild type mice ($P < 0.05$).

Survival and clearance of P. aeruginosa

Although mortality tended to be lower in *IFN-gammaR*$^{(-/-)}$ mice after inoculation with 10^5 CFU of *P. aeruginosa*, differences with wild type mice did not reach statistical significance. After 10 days, death had occurred in 3/10 of *IFN-gammaR*$^{(-/-)}$ mice and in 6/10 of wild type mice. Mice that survived the first 10 days after infection appeared to be permanent survivors (Figure 2A). We compared outgrowth of *P. aeruginosa* in lungs at 6 and 24 hours post-inoculation. After 6 hours the number of *P. aeruginosa* CFU recovered from lungs was similar in *IFN-gammaR*$^{(-/-)}$ and wild type mice, and none of the animals was bacteremic. At 24 hours after induction of pneumonia, *IFN-gammaR*$^{(-/-)}$ mice had more than 1 log less CFU in their lungs than wild type mice ($P < 0.05$) (Figure 2B). In wild type mice, *P. aeruginosa* CFU in lungs remained virtually unchanged between 6 and 24 hours. After 24 hours, only some of the mice were bacteremic, and the number of CFU recovered from blood of *IFN-gammaR*$^{(-/-)}$ mice and wild type mice was similar (25 ± 25 and 125 ± 100 CFU/ml blood; NS).

Activation of the innate immune system

Local production of cytokines and chemokines within the pulmonary compartment can influence host defense mechanisms during pneumonia [14, 15]. Therefore, we measured the concentrations of TNF, IL-6, MIP-2, and KC in lung homogenates after inoculation with *P. aeruginosa*. TNF, IL-6, MIP-2 and KC levels tended to be lower in lung homogenates from *IFN-gammaR*$^{(-/-)}$ mice compared with wild type mice (Table 1). Neutrophil influx in lungs was not different between wild type mice and *IFN-gammaR*$^{(-/-)}$ mice at 6 hours post-inoculation, while higher neutrophil numbers were found in BALF from wild type mice compared with *IFN-gammaR*$^{(-/-)}$ mice 24 hours post-inoculation (Table 2). Since the production of NO is important for host defense against pneumonia [16], we compared NO levels in BALF after inoculation with *P. aeruginosa*, in *IFN-gammaR*$^{(-/-)}$ mice and wild type mice (Figure 3). NO concentrations increased significantly during pneumonia with *P. aeruginosa* (P < 0.05 *versus* control). NO

levels were higher in *IFN-gammaR*$^{(-/-)}$ mice, compared to wild type mice ($P < 0.05$) at 24 hours, but not at 6 hours.

DISCUSSION

IFN-gamma has been implicated as an important mediator in host defense against several infectious diseases. IFN-gamma enhances the antimicrobial activity of peritoneal and alveolar macrophages. IFN-gamma 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* [14]. The role of IFN-gamma in pneumonia caused by bacteria is not well defined. Recently, IFN-gamma$^{(-/-)}$ mice were reported to have an increased mortality when compared to wild type mice in a model of severe pneumococcal pneumonia [8]. In that study, no data were presented on bacterial outgrowth or induction of innate defense mechanisms. In the present study we evaluated the relevance of the absence of the IFN-gammaR in a model of pneumonia induced by *P. aeruginosa*. *IFN-gammaR*$^{(-/-)}$ mice were found to have an increased resistance against Pseudomonas pneumonia, as reflected by an enhanced clearance of bacteria from the lungs by a mechanism that is not involving local cytokine or chemokine production, or neutrophil influx.

Our investigation should be viewed within the context of previous reports on mouse models of bacterial pneumonia. The majority of these models involved infection with either *Streptococcus pneumoniae, Klebsiella pneumoniae* or *P. aeruginosa* [14, 15]. Cytokines influence host defense during these respiratory tract infections, although their role may vary depending on the pathogen used. Indeed, while endogenous IL-10, a prototypic anti-inflammatory cytokine, hampered bacterial clearance in mouse models of *S. pneumoniae* [17] and *K. pneumoniae* [18], it improved host defense in a model of pneumonia caused by *P. aeruginosa* [19]. Similarly, while endogenous TNF, a prototypic pro-inflammatory cytokine, was important for clearance of *S. pneumoniae* [9] and *K. pneumoniae* [20] from mouse lungs, TNF impaired host defense mechanisms during *Pseudomonas* pneumonia [21]. These data suggest that in more gradually developing pneumonias, such as those caused by *S. pneumoniae* and *K. pneumoniae*, a certain pro-inflammatory cytokine response within the pulmonary compartment is required to combat the invading microorganism, while in more acute forms of pneumonia, such as that caused by *P. aeruginosa*,

an excessive inflammatory response contributes to an adverse outcome. In this light, it is not unexpected that our results contrast with an earlier report of increased mortality of IFN-gamma$^{(-/-)}$ mice during pneumococcal pneumonia [8]. Furthermore, studies in which endotoxin or high dose *Escherichia coli* was administered to mice have documented that endogenous IFN-gamma indeed contributes to an adverse outcome in acute models in which excessive inflammation is accepted as playing a critical role in tissue damage, *i.e.* elimination of IFN-gamma effects converted a strong protection against lethality in these settings [10-12]. It should be noted that *P. aeruginosa* was cleared more effectively from mouse lungs when elevated IFN-gamma levels were already present in the pulmonary compartment at the time of infection. Indeed, pretreatment of mice with a recombinant adenovirus encoding IFN-gamma *via* the intratracheal route, was associated with an enhanced clearance of *Pseudomonas* from the lungs, although CFUs were counted only at 4 hours after the bacterial inoculation [22].

Another component of host defense against invading pathogens in lungs is the influx of neutrophils into the pulmonary compartment. Earlier experiments in a murine *K. pneumoniae* pneumonia model, in which the potent chemotaxin MIP-2 was inactivated with anti-MIP-2-serum have demonstrated that a 60% decrease in influx of neutrophils into lungs, was associated with decreased early mortality and an attenuated clearance of bacteria from the lungs [23]. Furthermore, in a murine endotoxemia model, in which pretreatment with anti-MIP-1alpha caused a 40% reduction in LPS-induced increases in neutrophil influx, the reduced neutrophil influx was associated with a reduction of lung permeability and a modest decrease in survival [24]. We demonstrated that there was not differece in neutrophil influx in lungs between wild type mice and *IFN-gammaR*$^{(-/-)}$ mice at 6 hours post-inoculation, while at 24 hours higher neutrophils numbers were found in BALF of wild type mice than that of *IFN-gammaR*$^{(-/-)}$ mice. Taking the studies cited above into account [23, 24], the difference in neutrophil counts in BALF of *IFN-gammaR*$^{(-/-)}$ and that of wild type mice at 24 hours may have been of pathophysiological relevance. It is conceivable that higher neutrophil numbers in BALF are directly proportional to the number of bacteria present at that moment in our experiments. Hence, the lower bacterial load in lungs of *IFN-gammaR*$^{(-/-)}$ mice (thus providing less pro-inflammatory stimuli) could have been responsible for the attenuated neutrophil recruitment at later time points. It should be noted that in mice with thermal wounds, treatment with IFN-gamma led to a reduced

neutrophil influx into skin, suggesting that at least in the skin, IFN-gamma has the ability to inhibit, rather than to augment, neutrophil recruitment [25].

The IFN-gamma receptor (R) consists of two subunits. The ligand-binding component of the IFN-gammaR is referred to as the IFN-gammaR alpha-chain or alpha-subunit, the second subunit has been designated the IFN-gammaR beta-subunit [2]. One function of the beta-subunit is to stabilize the complex formed between the ligand and the alpha-subunit. Evidence exists that expression of the two IFN-gammaR subunits differs significantly. The alpha-subunit chain is expressed at moderate levels on the surface of nearly all cells, and is not regulated by external stimuli. The beta-subunit is constitutively expressed at extremely low levels, but expression can be regulated in certain cell types by external stimuli. Regulation of the beta-subunit gene is a critical factor in determining the responsiveness to IFN-gamma in certain cells, and can be regulated either positively or negatively in a stimulus-specific manner. One can speculate that mice that are deficient for the alpha-subunit of the IFN-gammaR have developed mechanisms to compensate for the absence of this receptor. However, previous findings with these mice display a phenotype consistent with studies of mice treated with neutralizing IFN-gamma monoclonal antibodies, with a greatly impaired ability to resist infections by various pathogens. Thus we consider it likely that our present results truely reflect the role of endogenous IFN-gamma in *Pseudomonas* pneumonia.

Previously, *Klebsiella* pneumonia was found to be associated with elevated nitrite concentrations in lungs of mice [16]. Treatment with N-omega-nitro-L-arginine methylester (L-NAME), a competitive inhibitor of NO synthase, impaired the clearance of *Klebsiella* from the pulmonary compartment and resulted in an increased mortality [16]. It is therefore possible that the elevated NO levels observed in the present study in *IFN-gammaR*$^{(-/-)}$ mice may have contributed to the improved clearance of *Pseudomonas* in these animals. Reactive nitrogen intermediates, particularly NO, are generated in murine macrophages as a result of IFN-gamma-dependent transcription of the gene encoding the inducable form of nitric oxide synthase (iNOS), which catalyzes the formation of large amounts of NO. The physiological relevance of IFN-gamma as an inducer of iNOS is evident in the difficulty with which iNOS is expressed in macropahges of mice deficient for its receptor [6]. However, numerous cytokines and microbial products, often acting synergistically, stimulate iNOS [26]. Further studies are

warranted to establish the interaction between endogenous IFN-gamma and NO during pneumonia.

CONCLUSION

In conclusion, we found an increased bacterial clearance in mice with a disrupted IFN-gammaR gene, during pneumonia caused by *P. aeruginosa*. These data demonstrate the complex role of IFN-gamma in innate immunity during pulmonary infection and may have important implications for the development and use of cytokine/anti-cytokine therapies in the future.

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Figures

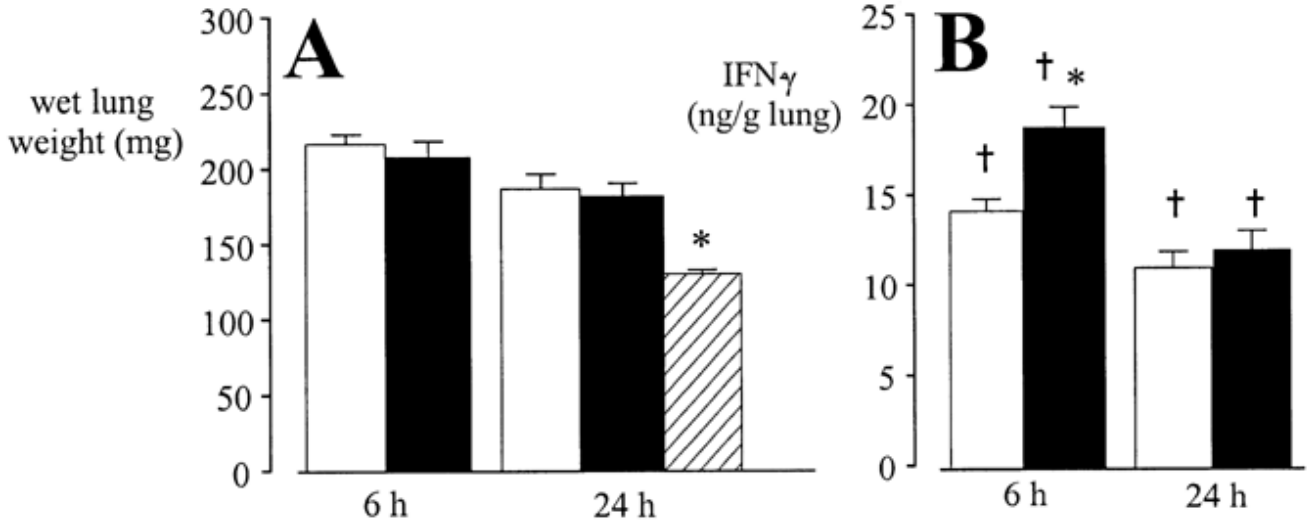


Figure 1

A: wet lung weights from *IFN-gammaR*$^{(-/-)}$ (solid bars), and wild type mice (open bars) inoculated with *P. aeruginosa*, and from mice inoculated with sterile saline (*III*-hatched bars).

Data are means (± SE); N = 8 mice per group at each time point. * $P < 0.05$ *versus* mice inoculated with *P. aeruginosa*.

B: IFN-g protein expression in lungs. IFN-g concentrations in lung homogenates in IFN-*g*R$^{(-/-)}$ (solid bars) and wild type mice (open bars) 6 and 24 hours after intranasal inoculation with 10^5 CFU Pseudomonas aeruginosa.

Data are means (± SE); N = 8 mice per group at each time point. IFN-gamma was undetectable in control mice inoculated with sterile saline (data not shown). Ý $P < 0.05$ *versus* control mice; * $P < 0.05$ *versus* wild type mice.

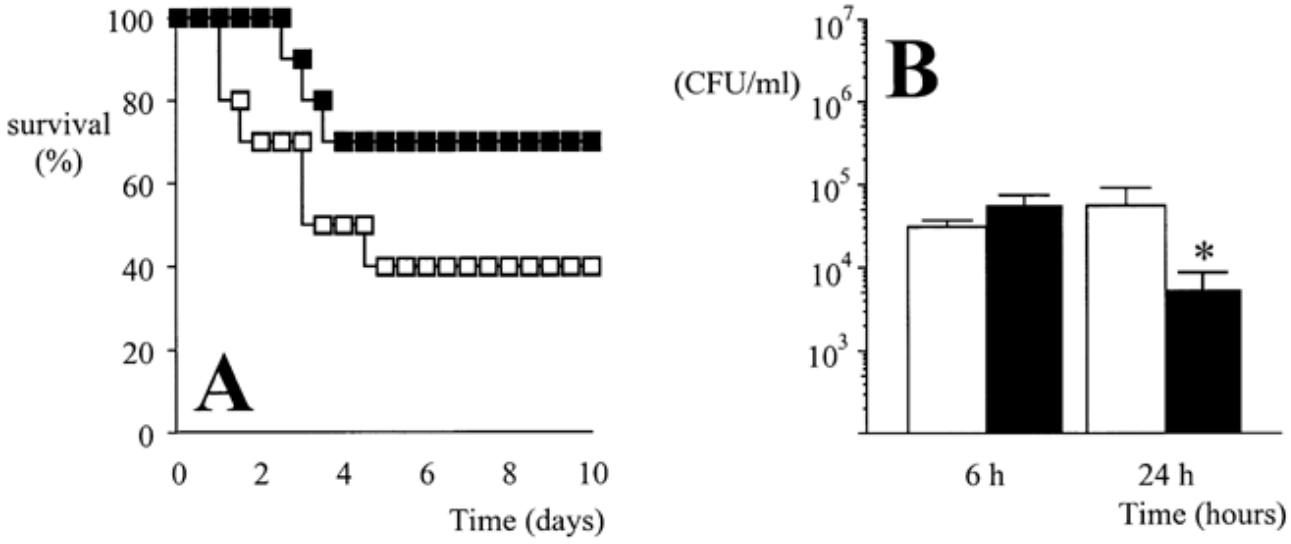


Figure 2

A: survival of *IFN-gammaR*$^{(-/-)}$ mice (solid bars-solid bars, N = 10) and wild type mice (open bars-open bars, N = 10) infected with *Pseudomonas aeruginosa*.

Mice were challenged with 10^5 CFU of *P. aeruginosa*. Differences were not statistically significant. **B: Clearance of bacteria is enhanced in IFN-*g*R$^{(-/-)}$ mice.**

Mean (± SE) *P. aeruginosa* CFU in lungs 6 and 24 hours after intranasal inoculation with 10^5 CFU in wild type mice (open bars) and *IFN-gammaR*$^{(-/-)}$ mice (solid bars). N = 8 per strain at each time point. * $P < 0.05$ *versus* wild type mice.

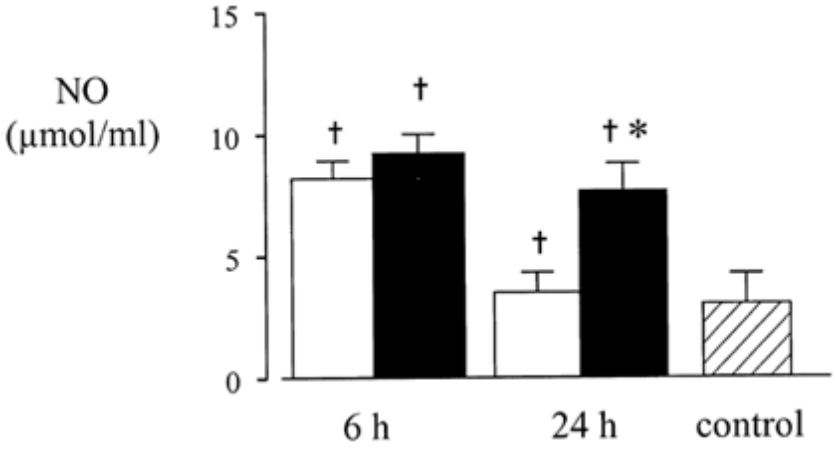


Figure 3

Mean (± SE) NO concentrations in BALF at 6 and 24 hours after induction of pneumonia with *P. aeruginosa* in *IFN-gammaR*$^{(-/-)}$ mice (solid bars) and wild type mice (open bars).

Control mice [///-hatched bars] were intranasally inoculated with isotonic saline and sacrificed at 24 hours. N = 8 per strain at each time point. Ý $P < 0.05$ *versus* control mice; * $P < 0.05$ *versus* wild type mice.

Table 1
Cytokine and chemokine concentrations in lung homogenates at 6 and 24 hours after induction of pneumonia

	T = 6 hours		T = 24 hours		Control	
	IFN-gammaR$^{-/-}$	Wild type	*IFN-gammaR*$^{-/-}$	Wild type	*IFN-gammaR*$^{-/-}$	Wild type
Cytokine						
TNF (ng/ml)	3.3 ± 0.5†	4.2 ± 0.6†	1.1 ± 0.2†	1.2 ± 0.4†	ND	ND
IL-6 (ng/ml)	169.9 ± 23.2†	181.3 ± 13.7†	32.1 ± 10.2†	50.2 ± 21.8†	1.3 ± 0.2	1.4 ± 0.1
KC (ng/ml)	39.2 ± 11.4†	55.0 ± 14.1†	4.8 ± 1.1†	10.7 ± 5.9†	0.1 ± 0.1	ND
MIP-2 (ng/ml)	202.6 ± 35.9†	158.0 ± 18.7†	21.2 ± 6.3†	31.3 ± 10.8†	4.3 ± 0.4	4.1 ± 0.4

Mice were intranasally inoculated with *Pseudomonas aeruginosa* (10^5 CFU) at T = 0 hour. Control mice received sterile saline intranasally and were sacrificed after 24 hours. Data represent means (± SE) of eight mice per group at each time point. ND = not detectable. † $P < 0.05$, compared to control mice.

Table 2
Leukocyte counts and differentials in bronchoalveolar lavage fluid at 6 and 24 hours after induction of pneumonia

	T = 6 hours		T = 24 hours		Control	
	IFN-gammaR$^{-/-}$	Wild type	*IFN-gammaR*$^{-/-}$	Wild type	*IFN-gammaR*$^{-/-}$	Wild type
(x 10^5 cells/ml)						
Leukocyte count	5.2 ± 0.4†	5.1 ± 1.1†	5.7 ± 1.0†	9.6 ± 0.8*†	0.9 ± 0.1	0.8 ± 0.1
Polymorphonuclear cells	4.8 ± 0.2†	4.6 ± 0.5†	5.3 ± 0.5†	9.2 ± 0.7*†	0.0 ± 0.0	0.0 ± 0.0
Lymphocytes	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Macrophages	0.3 ± 0.1†	0.4 ± 0.0†	0.3 ± 0.1†	0.4 ± 0.1†	0.8 ± 0.0	0.8 ± 0.0

Mice were intranasally inoculated with *Pseudomonas aeruginosa* (10^5 CFU) at T = 0 hour. Control mice received sterile saline intranasally and were sacrificed after 24 hours. Data represent means (± SE) of six mice per group at each time point.* $P < 0.05$ *versus* wild type mice, † $P < 0.05$, compared to control mice.

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In this issue

Regulation of interferon-g receptor (INF-gR) chains: a peculiar way to rule the life and death of human lymphocytes.

P. Bernabei, A. Allione, L. Rigamonti, M. Bosticardo, G. Losana, I. Borghi, G. Forni, F. Novelli
human, IFN-g, IFN-g receptor, signal transduction, cellular proliferation, apoptosis.

The interleukin-6 cytokine system in embryonic development, embryo-maternal interactions and cardiogenesis.

P. Seiler, G. Plenz, M.C. Deng
interleukin-6, embryo, blastocyst, endometrium, heart, gp130.

Role of cytokines in the modulation of nitric oxide production by cyclic AMP.

Z. Zidek
cyclic AMP, prostaglandins, nitric oxide, interleukin-10, tumor necrosis factor-a, interferon-g.

The 3' untranslated region of tumor necrosis factor-a is highly conserved in idiopathic pulmonary fibrosis (IPF).

R.W. Freeburn, H. Kendall, L. Dobson, J. Egan, N.J. Simler, A.B. Millar
TNF-a, idiopathic pulmonary fibrosis, polymorphism.

Endogenous interferon-g impairs bacterial clearance from lungs during Pseudomonas aeruginosa pneumonia.

M.J. Schultz, A.W. Rijneveld, P. Speelman, S.J.H. van Deventer, T. van der Poll
interferon-g, cytokines, host defense, Pseudomonas aeruginosa, pneumonia.

Development of allergic contact dermatitis requires activation of both tumor necrosis factor-receptors.

F.M. Becke, T. Hehlgans, G. Brockhoff, D.N. Männel
allergic contact dermatitis, p75TNF receptor, p55TNF receptor, Langerhans cells, dendritic cells.

Transforming growth factor beta-1 and interferon-alpha in the aids dementia complex: possible relationship with cerebral viral load?

O. Perrella, P.B. Carreiri, A. Perrella, C. Sbreglia, F. Gorga, D. Guarnaccia, G. Tarantino
transforming growth factor-beta1, HIV-RNA, AIDS-dementia complex, interferon-alpha.

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interferon-beta, natural autoantibodies, multiple sclerosis.

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interleukin-6, cord blood, gene polymorphism, inflammatory mediators.

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neuroblastoma, immunotherapy, gene therapy, cytokine, IL-1b, TNF-a.

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TNF, cytokine, LPS.

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apoptosis, caspases, Hsp27, Hsp70, T lymphocytes.

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MHC class II antigens, murine macrophages, c-Fos.

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MRC5, fibroblasts, TNF receptor, RT-PCR, binding, flow cytometry.

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IL-10, recombinant, rat, cytokines.

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