Interleukin-6 Gene–Deficient Mice Show Impaired Defense against Pneumococcal Pneumonia

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Induction of pneumonia in C57Bl/6 mice by intranasal inoculation with 10^6 cfu of *Streptococcus pneumoniae* resulted in sustained expression of interleukin (IL)-6 mRNA in lungs and increases in lung and plasma IL-6 concentrations. In IL-6-deficient (IL-6−/−) mice, pneumonia was associated with higher lung levels of the proinflammatory cytokines tumor necrosis factor-α, IL-1β, and interferon-γ and of the antiinflammatory cytokine IL-10 than in wild type (IL-6+/+) mice (all $P < .05$). Also, the plasma concentrations of soluble tumor necrosis factor receptors were higher in IL-6−/− mice ($P < .05$), while the acute-phase protein response was strongly attenuated ($P < .01$). Lungs harvested from IL-6−/− mice 40 h after inoculation contained more *S. pneumoniae* colonies ($P < .05$). IL-6−/− mice died significantly earlier from pneumococcal pneumonia than did IL-6+/+ mice ($P < .05$). During pneumococcal pneumonia, IL-6 down-regulates the activation of the cytokine network in the lung and contributes to host defense.

Bacterial pneumonia remains a common disease, with an estimated incidence in the United States of 4 million cases per year, one-fifth of which require hospitalization [1, 2]. Despite the availability of potent antimicrobial therapy, pneumonia is the sixth leading cause of death and the most frequent cause of death from infectious diseases in developed countries [1, 2]. Among patients with community-acquired pneumonia requiring hospitalization, the mortality rate can be as high as 25% [2–4]. *Streptococcus pneumoniae* is the most frequently isolated microorganism in patients with community-acquired pneumonia [1–4]. Knowledge of the pathogenesis of pneumococcal pneumonia is important, not only because of its relatively high incidence but also because of the increasing occurrence of resistance of *S. pneumoniae* to penicillin and other antimicrobial agents [5].

Interleukin (IL)-6 is a cytokine that is frequently detected in the circulation of patients with bacterial infections [6], including pneumonia [7, 8]. During pneumonia, IL-6 likely is at least in part produced locally in the lung at the site of infection, since in patients with unilateral pneumonia, IL-6 concentrations in bronchoalveolar lavage fluid obtained from the infected lung have been found to be higher than in either lavage fluid from the uninfected lung or in plasma [7]. The role of IL-6 in the pathogenesis of bacterial pneumonia is unknown. Therefore, in the present study, we sought to determine the susceptibility of mice deficient for the IL-6 gene to pneumonia caused by *S. pneumoniae.*

Methods

Animals. The induction of IL-6 during pneumonia was studied in C57Bl/6 mice (weighing 18–20 g). The role of endogenously produced IL-6 in the pathogenesis of pneumonia was determined by comparing IL-6 gene–deficient (IL-6−/−) mice with wild type (C57Bl/6 x 129 Sv; IL-6+/+) mice (both types weighing 20–22 g). The generation of IL-6−/− mice has been described [9].

Induction of pneumonia. Pneumonia was induced as described [10, 11]. Briefly, *S. pneumoniae* serotype 3 was obtained from American Type Culture Collection (ATCC 6303; Rockville, MD). Pneumococci were grown to midlogarithmic phase at 37°C in 5% CO_2 using Todd-Hewitt broth (Difco, Detroit) supplemented with 0.5% yeast extract. After incubation, aliquots were stored at −70°C. For each experiment, 100-μL volumes of thawed suspensions were used to seed 10-mL volumes of fresh Todd-Hewitt broth supplemented with 0.5% yeast extract, which were incubated for 6 h at 37°C in 5% CO_2. Bacteria were pelleted by centrifugation at 450 *g* for 15 min and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of $\sim 2 \times 10^7$ cfu/mL, as determined by plating serial 10-fold dilutions onto sheep blood agar plates. Mice were lightly anesthetized by inhalation of methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL), and 50 μL (10^6 cfu) was inoculated intra-

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nasally. To assess whether this procedure per se induced an inflammatory response in the lungs, some mice were inoculated intranasally with 50 μL of isotonic saline only (i.e., without bacteria).

Sampling and assays. To assess the production of IL-6 during pneumonia, blood and lungs were collected before and 12, 24, 48, and 72 h after the administration of *S. pneumoniae* (6 mice per time point). It should be noted that C57Bl/6 mice started dying from pneumonia from 48 h after the administration of 10^6 cfu of *S. pneumoniae.* Therefore, data obtained at 72 h after inoculation represent a selection of animals that were still alive. Blood and lungs were also collected at 24 and 48 h after intranasal inoculation with isotonic saline only (5 mice per time point). At the designated time points, mice were anesthetized with inhaled methoxyflurane, blood was collected by cardiac puncture, and whole lungs were harvested for measurement of IL-6. Further, to assess the role of IL-6 in the production of other cytokines, blood and lungs were obtained from IL-6−/− and IL-6+/+ mice at 24 and 40 h after the administration of bacteria (5 or 6 mice/group/time point). The 40-h time point (rather than 48 h) was chosen because preliminary experiments had established that IL-6−/− mice died relatively early after induction of pneumonia. Lungs were processed exactly as described previously [10, 11]. IL-6, IL-10, and interferon (IFN)-γ were measured by ELISA (Pharmingen, San Diego). Tumor necrosis factor (TNF) activity was measured using the WEHI 164 clone 13 fibroblast cytotoxicity assay [12]. IL-1β was measured by ELISA (Genzyme, Cambridge, MA). Plasma concentrations of soluble TNF receptors type I (TNFR-I, p55) and type II (TNFR-II, p75) were measured by ELISA [13]. Cytokine levels are expressed as nanograms per milliliter in plasma and as nanograms pre gram of tissue in lung homogenates. Mouse acute-phase proteins serum amyloid P and C3 were measured by rocket immunoelectrophoresis as described [14]. Myeloperoxidase (MPO) was measured as an index of neutrophil infiltration of the lungs, exactly as described [11, 15].

IL-6 mRNA detection by reverse transcription–polymerase chain reaction. Lungs for RNA isolation were harvested before and at 12, 24, 48, and 72 h after intranasal inoculation with *S. pneumoniae* and at 24 and 48 h after intranasal inoculation with isotonic saline. Lungs from 3 mice per time point were pooled. Total cellular RNA was extracted from snap-frozen lungs using a commercially prepared 5 *M* guanidinium isothiocyanate, acid phenol, and 2-mercaptoethanol solution (RNAzol-B; Bioteck, Friendswood, TX). Total cellular RNA (1 μg) was reverse-transcribed using 2.5 U of murine leukemia virus reverse transcriptase and 0.05 nmol of oligo(dT) (Perkin-Elmer Cetus, Norwalk, CT). cDNA was amplified with 2.5 U of DNA polymerase (AmpliTaq; Perkin-Elmer Cetus) and oligonucleotide primers specific for β-actin [10] or IL-6 (Stratagene, La Jolla, CA) using 35 cycles of DNA amplification on a thermocycler (PTC-100; MJ Research, Watertown, MA). Each cycle included denaturation at 95°C, reannealing of primer and fragment at 55°C, and primer extension at 72°C. The primers used for IL-6 were 456-GACAAAGCCAGAGTCCTTCAGAGAG-480 (sense) and 663-CTAGGTTTGCCGAGTAGATCTC-684 (antisense) [16]. Then 15 μL of the 100-μL reaction mixture was fractionated on a 1% agarose gel. Samples were electrophoresed at 100 V for 1.5 h and stained with ethidium bromide (1 μg/mL).

Determination of plasma and lung bacterial counts. At 40 h after administration of *S. pneumoniae,* blood was obtained aseptically by retroorbital puncture. Then mice were sacrificed by cervical dislocation (5 mice/group), and lungs were removed aseptically and placed in 10 vol of sterile isotonic saline. Lungs were homogenized with a tissue homogenizer that was carefully cleaned and disinfected with 70% alcohol after each homogenization. Serial 10-fold dilutions in sterile isotonic saline were made of blood and lung homogenates, and 20-μL aliquots were plated onto sheep blood agar plates and incubated for 18 h at 37°C and 5% CO_2, after which colonies were counted.

Statistical analysis. All values are expressed as mean ± SE. Two sample comparisons were made by the Wilcoxon test for unpaired samples. Survival curves were compared with the log-rank test. $P < .05$ was considered significant.

Results

Development of pneumonia. As reported previously [10, 11], C57Bl/6 mice did not show any sign of illness for the first 16–24 h after inoculation with *S. pneumoniae.* Thereafter, they developed signs of systemic toxicity, including lethargy and piloerection. From 36 to 48 h, mice developed progressive respiratory distress. Administration of 10^6 cfu of *S. pneumoniae* resulted in 100% lethality by postinoculation day 5. Histologically, lungs at 24 h after inoculation showed areas of acute inflammation, consisting mainly of neutrophils, associated with the terminal airways. Pulmonary vessels were congested and contained large numbers of neutrophils, often marginating. At 48 h after inoculation, lung lesions were much more severe, with alveolar cell necrosis, alveolar hemorrhage, fibrinous exudate, and perivascular edema.

Induction of IL-6 (figures 1, 2). Neither normal mice nor mice 24 or 48 h after intranasal inoculation with sterile saline had detectable IL-6 (>0.10 ng/mL) in their circulation. Similar amounts of IL-6 were detectable in lung homogenates obtained

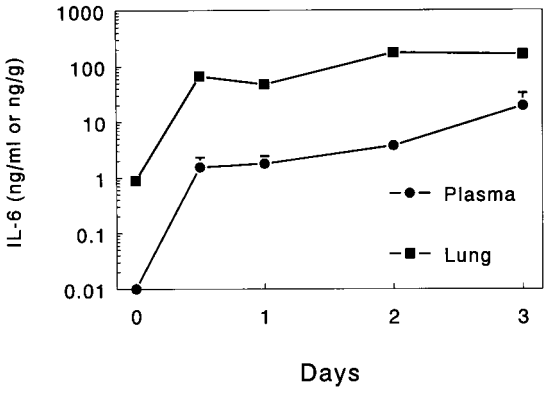

Figure 1. Induction of IL-6 during pneumococcal pneumonia: mean (± SE) plasma and lung concentrations of IL-6. At time 0, mice were inoculated with 50 μL (10^6 cfu) of *Streptococcus pneumoniae.* At each designated time point, 6 mice were sacrificed, and their plasma and lungs were harvested for measurement of IL-6.

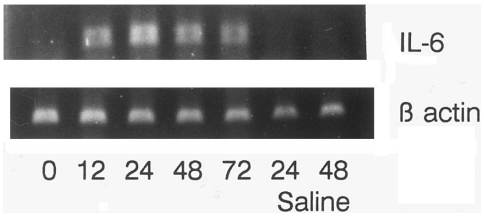

Figure 2. IL-6 mRNA and β-actin mRNA expression in lungs as determined by reverse transcription–polymerase chain reaction at indicated time points (bottom, h) before and after intranasal inoculation with 10^6 cfu of *Streptococcus pneumoniae.* Saline = 24 or 48 h after inoculation with sterile isotonic saline only (i.e., without bacteria). Lungs from 3 mice were pooled at each time point.

from normal mice (0.89 ± 0.29 ng/g) and in lungs obtained after inoculation with saline (24 h: 0.84 ± 0.25 ng/g; 48 h: 0.97 ± 0.46 ng/g; nonsignificant). Administration of *S. pneumoniae* was associated with a marked increase in IL-6 concentrations in both plasma and lungs. Lung IL-6 levels reached a plateau between 48 and 72 h (48 h: 175.35 ± 19.76 ng/g) and plasma IL-6 concentrations plateaued at 72 h (19.38 ± 13.22 ng/mL). Intranasal administration of *S. pneumoniae* resulted in the induction of IL-6 mRNA in the lung within 12 h. The expression of IL-6 mRNA was sustained for up to 72 h. No detectable IL-6 mRNA was noted in the lungs of normal mice or mice given saline.

Inflammatory responses in IL-6−/− and IL-6+/+ mice (figure 3 and table 1). As expected, IL-6 could not be detected in plasma or lungs of IL-6−/− mice during pneumonia. In addition, no IL-6 mRNA was found in the lungs of these mice after administration of *S. pneumoniae* (data not shown). In IL-6+/+ mice, plasma IL-6 levels were 0.59 ± 0.10 ng/mL after 24 h and 3.76 ± 1.50 ng/mL after 40 h; lung IL-6 concentrations were 50.74 ± 4.91 and 228.37 ± 41.57 ng/g, respectively. Lung TNF levels were 3- to 4-fold higher in IL-6−/− mice than in IL-6+/+ mice ($P < .05$). In plasma, TNF remained undetectable (<0.03 ng/mL) in all but 1 IL-6+/+ mouse. By contrast, all IL-6−/− mice had detectable plasma TNF levels at 40 h after inoculation ($P < .01$ vs. IL-6+/+ mice). Lung IL-1β and IFN-γ levels were higher in IL-6−/− mice at 24 h after inoculation ($P < .05$). In plasma, IL-1β remained undetectable (<0.12 ng/mL) in all but 2 mice. Plasma IFN-γ was higher in IL-6−/− mice at 24 h after induction of pneumonia ($P < .05$). Lung levels of the antiinflammatory cytokine IL-10 were higher in IL-6−/− mice at 40 h after inoculation ($P < .05$), while IL-10 remained undetectable (<0.12 ng/mL) in plasma in all mice. Also, plasma concentrations of soluble TNFR-I and TNFR-II were higher in IL-6−/− mice than in IL-6+/+ mice ($P < .05$).

The acute-phase protein response was strongly attenuated in IL-6−/− mice ($P < .01$ vs. IL-6+/+ mice). Lung MPO activity was higher in IL-6−/− mice than in IL-6+/+ mice ($P = .01$).

Bacterial clearance. IL-6−/− mice had ~6-fold more *S. pneumoniae* colonies isolated from lung homogenates at 40 h after inoculation than did IL-6+/+ mice (608 ± 346 × 10^7 and 112 ± 59 × 10^7 cfu, respectively; $P < .05$). From all mice, *S. pneumoniae* could be cultured from blood. Although

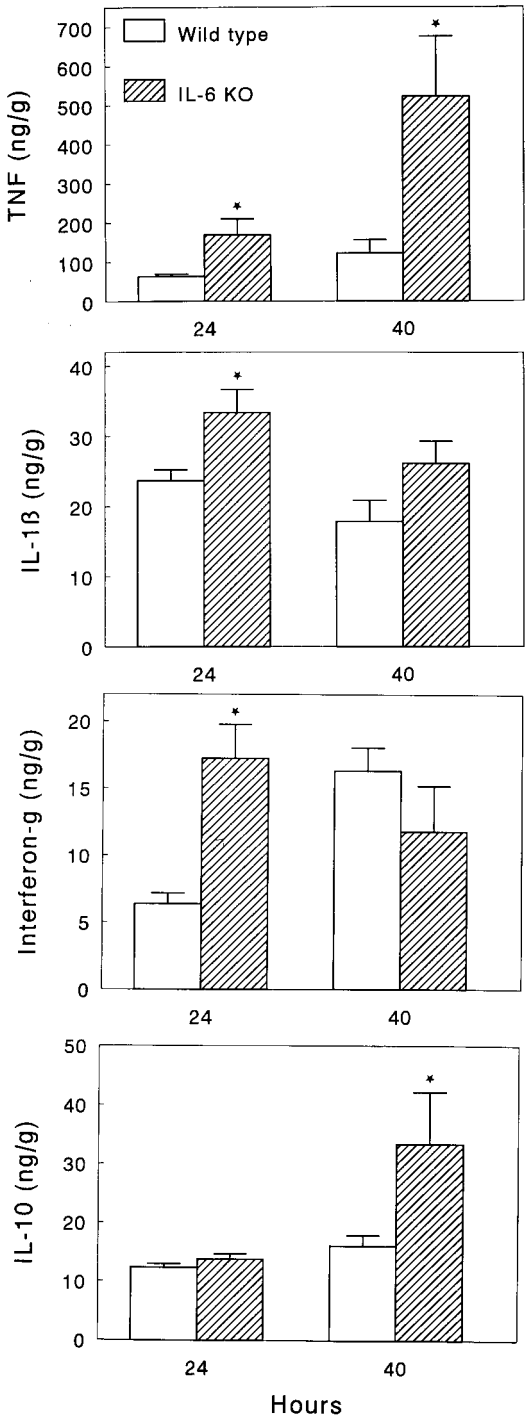

Figure 3. Mean (± SE) lung concentrations of tumor necrosis factor (TNF)-α, IL-1β, interferon (IFN)-γ, and IL-10 at 24 and 40 h after intranasal inoculation with 10^6 cfu of *Streptococcus pneumoniae* in IL-6−/− (IL-6 KO) and IL-6+/+ (wild type) mice. There were 5 or 6 mice/group/time point. * $P < .05$, Wilcoxon test, vs. IL-6+/+ mice.

Table 1. Inflammatory responses during pneumococcal pneumonia in IL-6−/− and IL-6+/+ mice.

	24 h		40 h	
	IL-6+/+	IL-6−/−	IL-6+/+	IL-6−/−
Plasma				
TNF (ng/mL)	<0.03	0.17 ± 0.17	0.01 ± 0.01	0.56 ± 0.35*
IFN-γ (ng/mL)	<0.12	2.09 ± 1.58*	0.38 ± 0.22	0.29 ± 0.08
sTNFR-I (ng/mL)	0.28 ± 0.02	0.42 ± 0.05*	0.31 ± 0.02	0.62 ± 0.04*
sTNFR-II (ng/mL)	12.95 ± 3.96	42.02 ± 5.15*	32.48 ± 6.59	71.16 ± 9.93*
SAP (μg/mL)	ND	ND	36.5 ± 0.5	7.7 ± 0.7*
C3 (μg/mL)	ND	ND	129.5 ± 14.5	25.7 ± 7.6*
Lung MPO (U/g)	ND	ND	137.3 ± 2.5	171.7 ± 14.2*

NOTE. Data are mean ± SE. At time 0, mice were inoculated with 50 μL (10^6 cfu) of *S. pneumoniae;* 5 or 6 mice/group/time point vs. IL-1β was undetectable in plasma in all but 2 mice. IL-10 could not be detected in plasma of any mouse. ND, not determined; TNF, tumor necrosis factor; IFN, interferon; sTNFR, soluble TNF receptor; SAP, serum amyloid P; MPO, myeloperoxidase.

* $P < .05$ vs. IL-6+/+ mice by Wilcoxon test.

the mean number of colonies was much higher in IL-6−/− mice, the difference with IL-6+/+ mice was not significant due to large interindividual variation (182 ± 109 × 10^4 and 13 ± 3 × 10^4/mL, respectively; nonsignificant).

Survival (figure 4). Survival studies were performed in 16 IL-6−/− and 16 IL-6+/+ mice. Mortality was assessed every 12 h. IL-6−/− mice succumbed significantly earlier from pneumococcal pneumonia than did IL-6+/+ mice ($P < .05$). Eight IL-6−/− mice were dead at 48 h after inoculation versus 2 IL-6+/+ mice; all IL-6−/− mice had died by 60 h, at which time 6 IL-6+/+ mice were still alive.

Discussion

During overwhelming immune activation, especially in the absence of a localized infectious source such as after bolus injection of endotoxin, inhibition of proinflammatory cytokines may be beneficial [17, 18]. In such models, high levels of proinflammatory cytokines appear in the circulation, and inhibition of their systemic effects confers protection against tissue injury and lethality. However, these models of systemic challenges do not provide insight into the potential beneficial effects of proinflammatory cytokines at the site of an infection. We recently found that local production of TNF within the lung is important for host defense in this murine model of pneumococcal pneumonia [11], while IL-10 produced in lungs hampers host defense [10]. In accord, neutralization of IL-10 in mice with pneumonia caused by *Klebsiella pneumoniae* was associated with a decrease in *Klebsiella* colonies in lungs and an increase in survival [19].

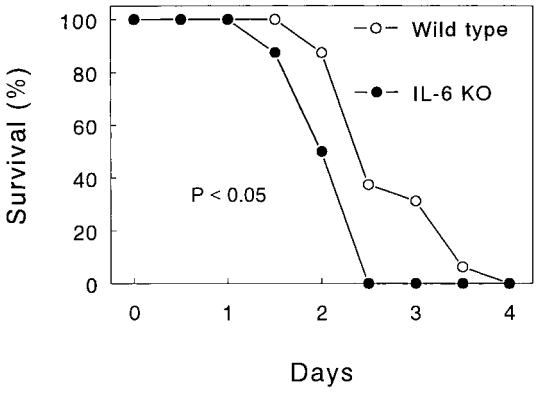

Figure 4. IL-6−/− mice show impaired defense against pneumococcal pneumonia. Survival after intranasal inoculation with 10^6 cfu of *Streptococcus pneumoniae* of IL-6−/− (IL-6 KO) and IL-6+/+ (wild type) mice. There were 16 mice/group. IL-6−/− mice died significantly earlier than IL-6+/+ mice ($P < .05$, log-rank test).

In models of systemic inflammation induced by bolus administration of endotoxin, TNF is a mediator of toxicity and death [17], while IL-10 is protective [18] (i.e., completely the opposite of their respective roles in models of gram-negative [19, 20] and gram-positive [10, 11] pneumonia, even though, in such models, blood cultures are positive). It should be noted, however, that in endotoxin-challenge models, lipopolysaccharide is injected directly into the circulation, while during pneumonia, bacteria likely are shed gradually or intermittently from the source of the infection, which apparently does not result in detectable cytokine levels in the circulation [10, 11] (present data). Hence, animal studies of pneumonia and systemic endotoxin effects represent two completely different entities, the former being more clinically relevant.

Although IL-6 is not an important mediator of endotoxin-induced inflammatory responses [9, 21–23], recent studies indicate that this cytokine may have a significant role in host defense against bacterial infection. IL-6 appears to be essential for defense against *Listeria monocytogenes,* since IL-6 gene–deficient mice succumb early from this gram-positive infection [9, 24]. Further, IL-6–deficient mice demonstrated enhanced

mortality after intraperitoneal administration of live *Escherichia coli,* which is associated with increased bacterial numbers in organs during the course of this infection [23]. The objective of the present study was to determine the role of endogenous IL-6 in the pathogenesis of pneumococcal pneumonia, the most frequent manifestation of community-acquired pneumonia.

The absence of an IL-6 response in IL-6−/− mice was associated with enhanced induction of both agonist and antagonist members of the cytokine network. Some of these findings were anticipated from previous studies. Indeed, IL-6 has been considered an antiinflammatory cytokine by virtue of its ability to inhibit endotoxin-induced TNF and IL-1 production by mononuclear cells in vitro [25, 26] and to reduce TNF release in endotoxemic mice in vivo [25]. In accord, endotoxin-treated IL-6−/− mice produce 3 times more TNF than wild type controls [22]. However, administration of IL-6 to cancer patients results in an increase in the plasma levels of soluble TNFR-I [27]. Therefore, the enhanced release of soluble TNFR-I and TNFR-II in IL-6−/− mice was unexpected. Interestingly enough, IL-6−/− mice also had increased pulmonary levels of IL-10, a pleiotropic cytokine known to inhibit the production of proinflammatory cytokines under various in vitro and in vivo conditions [18]. Hence, it should be noted that IL-6−/− mice had elevated proinflammatory cytokine levels despite concurrently elevated IL-10 concentrations.

The mechanism by which the absence of an IL-6 response resulted in enhanced release of soluble TNF receptors and IL-10 remains to be elucidated. Possibly, the elevated TNF levels in IL-6−/− mice contributed to these exaggerated antiinflammatory responses, since TNF can induce release of its receptors and of IL-10 in humans, and anti-TNF treatment of endotoxemic primates abrogates the appearances of soluble TNF receptors and IL-10 [28, 29].

TNF was not detectable in plasma of wild type mice during pneumonia. Although we cannot exclude the possibility that TNF was detectable in plasma at early time points after inoculation, we consider this unlikely, since during pneumonia TNF is produced within lungs, and the highest lung TNF levels are found after ⩾24 h [11]. Rather, these data suggest that TNF production is compartmentalized within lungs and that plasma TNF levels are a weak reflection of lung TNF concentrations.

The acute-phase protein response was markedly reduced in IL-6−/− mice with pneumococcal pneumonia. These data extend previous studies with IL-6−/− mice, demonstrating that IL-6 is not required to generate an acute-phase protein response to endotoxin but is an important mediator of acute-phase protein release during sterile inflammation induced by subcutaneous injection of turpentine and during infection with *L. monocytogenes* [9, 22].

Significantly more pneumococci were recovered from lungs of IL-6−/− mice than from lungs of IL-6+/+ mice 40 h after inoculation. Although a large interindividual variation existed, we consider this difference clinically relevant, since the increased number of bacterial colonies in IL-6−/− mice was associated with reduced survival time in these animals. It has been postulated that the absence of an adequate neutrophilic response in IL-6–deficient mice contributes to the increased susceptibility to *L. monocytogenes* and *E. coli* infection [23, 24]. In our study, IL-6−/− mice had enhanced pulmonary MPO activity, as determined 40 h after inoculation with *S. pneumoniae.* Although neutrophil numbers were not actually counted in our study, it has previously been shown that MPO activity corresponds well with the number of neutrophils in tissue sections of inflamed lungs [30]. Conceivably, the elevated lung TNF levels in IL-6−/− mice contributed to increased neutrophil influx, since TNF is considered to play a significant role in this characteristic inflammatory response [20].

In comparison with other cytokines, IL-6 has been reported most consistently in the circulation of septic patients [31]. Until recently, IL-6 was merely considered a marker for the severity of the bacterial challenge, rather than a significant player in the pathogenesis of bacterial infection. This and other studies [9, 23, 24] indicate that the role of IL-6 in host defense against bacteria is more important than previously recognized. Our study does not elucidate the mechanisms by which IL-6 contributes to antibacterial mechanisms or activation of inflammatory responses. Considering the limited (direct) effects of IL-6 in various in vitro systems, it is conceivable that IL-6 is involved indirectly and influences the activity of mediators acting more directly on inflammatory processes. Nonetheless, these results indicate that neutralization of IL-6 activity in humans, as has been propagated for some diseases [32, 33], may hamper a specific resistance against bacterial infections.

Pneumococcal pneumonia is the most common community-acquired pneumonia. In our study, IL-6 was produced locally within the lung during pneumonia caused by *S. pneumoniae.* Endogenous IL-6 played a major role in the induction of the cytokine network within the lung, controlling the activation of both agonist and antagonist mediators during pneumonia. The net effect of IL-6 was protective, reducing the growth of pneumococci and prolonging survival. Thus, IL-6 was an important mediator of inflammation within lungs during pneumococcal pneumonia. According to our findings and those of earlier studies addressing the role of cytokines in the pathogenesis of bacterial pneumonia [10, 11, 19, 20], it becomes increasingly clear that local inflammation, facilitated directly or indirectly by proinflammatory cytokines, is essential for local defense against bacteria. Neutralization of proinflammatory cytokines in patients with bacterial infections may therefore be potentially hazardous and may be one reason, among others, why clinical trials with strategies directed against TNF, based primarily on animal models in which bacteria or their products were administered as a bolus, have not been successful [17, 34].

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