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Z Xing, ... , X F Lei, M K Achong

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### **Research Article**

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# IL-6 Is an Antiinflammatory Cytokine Required for Controlling Local or Systemic Acute Inflammatory Responses

Zhou Xing,\* Jack Gauldie,\* Gerard Cox,\* Heinz Baumann,† Manel Jordana,\* Xue-Feng Lei,\* and Michelle K. Achong\*

\*Immunology and Infection Program, Department of Pathology, McMaster University, Hamilton, Ontario, L8N 3Z5 Canada; and

†Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263

## Abstract

IL-6 is induced often together with the proinflammatory cytokines TNF $\alpha$  and IL-1 in many alarm conditions, and circulating IL-6 plays an important role in the induction of acute phase reactions. However, whether this endogenous IL-6 plays any additional pro- or antiinflammatory roles in local or systemic responses remains unclear. In this study, the role of IL-6 in acute inflammatory responses was investigated in animal models of endotoxic lung or endotoxemia by using IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice. Aerosol exposure of endotoxin induced increased IL-6 and proinflammatory cytokines TNF $\alpha$  and MIP-2 and a neutrophilic response in the lung of IL-6 $^{+/+}$  mice. However, the levels of TNF $\alpha$  and MIP-2 and neutrophilia were significantly higher in the lung of IL-6 $^{-/-}$  mice. The rate of neutrophil apoptosis in these mice was similar to that in IL-6 $^{+/+}$  mice. A low constitutive level of antiinflammatory cytokine IL-10 was not enhanced by endotoxin and remained similar in the lung in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice. Systemically, intraperitoneal delivery of endotoxin resulted in much more pronounced circulating levels of TNF $\alpha$ , MIP-2, GM-CSF, and IFN $\gamma$  in IL-6 $^{-/-}$  mice than in IL-6 $^{+/+}$  mice, and administration of recombinant IL-6 to IL-6 $^{-/-}$  mice abolished these differences. In contrast, circulating IL-10 levels were induced to a similar degree in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice. Thus, our studies reveal that endogenous IL-6 plays a crucial antiinflammatory role in both local and systemic acute inflammatory responses by controlling the level of proinflammatory, but not antiinflammatory, cytokines, and that these antiinflammatory activities by IL-6 cannot be compensated for by IL-10 or other IL-6 family members. (*J. Clin. Invest.* 1998. 101:311–320.) Key words: cytokines • endotoxin • inflammation • lung • neutrophils

## Introduction

IL-6 is a pleiotropic cytokine that is commonly produced at local tissue sites and released into circulation in almost all situa-

tions of homeostatic perturbation typically including endotoxemia, endotoxic lung, trauma, and acute infections (1, 2). In addition to its critical participation in the generation of immunity against chronic intracellular infections, circulating IL-6, together with other alarm cytokines TNF $\alpha$  and IL-1, is known to be required for the induction of acute phase reactions composed of fever, corticosterone release, and hepatic production of acute phase proteins many of which are protease inhibitors (3, 4). Overall, the induction by IL-6 of these acute phase reactions has been regarded as part of an attempt to maintain homeostasis. However, it still remains unclarified whether during local or systemic acute inflammatory responses, IL-6 is also directly involved in the modulation of other aspects of inflammation, particularly cytokine responses and tissue inflammatory infiltration. In this regard, clinically, serum IL-6 levels appear to correlate with mortality of patients with endotoxic syndrome or trauma, yet the assumption that IL-6 may play a detrimental role in these conditions has been restricted by the difficulty in separating the biologic effect of IL-6 from the result of tissue injury (2). Experimentally, the use of antibodies in abrogating endogenous IL-6 in endotoxic models has unfortunately yielded very conflicting information (5–7). Apparently, the clarification of functional roles of IL-6 in these conditions is of importance in enhancing our understanding of molecular mechanisms of inflammation and in designing appropriate therapeutic strategies. In this study, we have elected to use IL-6 gene knock-out mice to address the regulatory role of IL-6 in local or systemic acute inflammatory responses elicited by either local lung or systemic exposure to endotoxin.

## Methods

**Mice.** The generation of mice deficient in IL-6 gene (C57Bl/6  $\times$  129Sv) and normal control littermates was described elsewhere (8). These mice were bred and maintained under a 12-h light–dark cycle in our Level B pathogen-free facility. Mice were used at 10–16 wk of age. All experimentation described in this study was approved by the Animal Research Ethics Board of McMaster University.

**Models of endotoxin-induced acute lung inflammation and endotoxemia.** To set up a model of endotoxin-induced acute lung inflammation, both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice at groups of eight were placed in a Plexiglas chamber and exposed to an aerosol of endotoxin (LPS) (055:B5 *Escherichia coli* derived; Sigma Chemical Co., St. Louis, MO) nebulized at a rate of 10 liters per minute for 30 min (30  $\mu$ g LPS/ml saline). To elicit endotoxemia, both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice were injected intraperitoneally with LPS at a sublethal dose of 4  $\mu$ g/g body weight after a standard procedure previously described (9). In separate experiments, recombinant rat IL-6 was injected subcutaneously and intraperitoneally to IL-6 $^{-/-}$  mice at a total dose of 0.4  $\mu$ g/g body weight 30 min before intraperitoneal endotoxin challenge. The concentration of this recombinant rat IL-6 was determined based upon its bioactivity in a B9 hybridoma growth factor bioassay. In the mortality experiment, both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice were injected intraperitoneally with a lethal dose of LPS (20  $\mu$ g/g body weight), and mortality was recorded in the following 4–5 d.

Address correspondence to Zhou Xing, M.D., Ph.D., Room 4H13, Health Sciences Center, Department of Pathology, McMaster University, Hamilton Ontario, L8N 3Z5, Canada. Phone: 905-525-9140 Ext. 22473; FAX: 905-522-6750; E-mail: xingz@fhs.csu.mcmaster.ca

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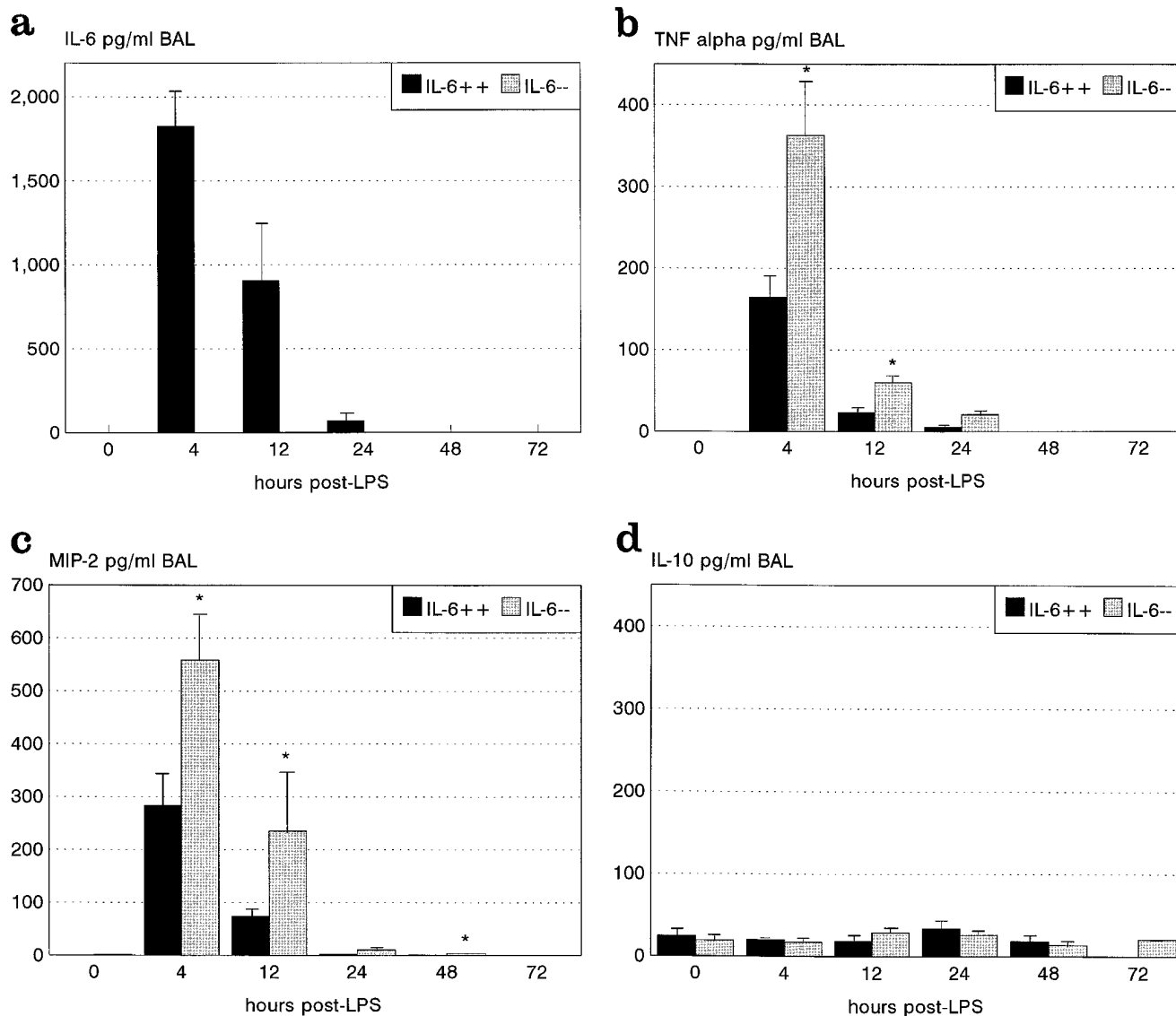


Figure 1. Cytokine responses in LPS-induced acute lung inflammation in IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice. BAL fluids were collected at various times post-LPS aerosol exposure and measured by a bioassay for IL-6 (a) or by ELISA for TNF $\alpha$  (b), MIP-2 (c), and IL-10 (d). Results are expressed as mean  $\pm$  SEM from four mice per time point. \* $P \leq 0.05$ .

*Preparation and analysis of bronchoalveolar lavage, lung tissue, and peripheral blood samples.* At various times after LPS delivery to the lung, mice were subjected to retro-orbital bleeding and then killed. The lungs were removed from the thoracic cavity and bronchoalveolar lavage (BAL)<sup>1</sup> was performed twice in a total volume of 0.45 ml of PBS through an intratracheal polyethylene tube attached to a 1-ml syringe. Routinely, about 0.4 ml of BAL was retrieved from each mouse. Cell pellets were resuspended in PBS for total and differential cell counting and supernatants stored at  $-70^{\circ}\text{C}$  for cytokine assays. Differential counting was performed on Diff-Quik stained cytopspins and routinely 400–500 cells were counted on each cytopspin. For assessment of the extent of inflammation clearance in the lung, cytopspins from BAL samples were also used to determine the percentages of apoptotic neutrophils according to our previously de-

scribed criteria (10). For histology, lungs were fixed by perfusion with 10% buffered formaline for 24 h, processed, and stained with H&E. Total peripheral blood leukocytes were determined after red blood cell lysis with a lysis buffer and differential counts determined on Diff-Quik stained blood smears made from the whole blood (11). At various times after intraperitoneal endotoxin delivery, mice were subjected to retro-orbital bleeding and sera prepared as above for cytokine measurement.

*Cytokine assays and measurement of acute phase proteins.* The concentration of TNF $\alpha$ , IL-10, GM-CSF, IFN $\gamma$ , or macrophage inflammatory protein-2 (MIP-2) in BAL and serum was measured by specific ELISA (R & D Systems, Minneapolis, MN). The sensitivity of these ELISA kits was all equal to or smaller than 5 pg/ml. TGF $\beta$ 1 content in BAL was measured by using an ELISA kit for human TGF $\beta$ 1 (R & D Systems; sensitivity, 7 pg/ml). This kit also detects TGF $\beta$ 1 in other species with the same sensitivity because of the almost identical homology of this cytokine across species. IL-6 content was measured by a modified IL-6-dependent B9 hybridoma growth assay as previously described (12). The sensitivity of this assay was 0.5 pg/ml. For

1. Abbreviations used in this paper: BAL, bronchoalveolar lavage; MIP-2, macrophage inflammatory protein-2.

detection of acute phase proteins  $\alpha$ 1-acid glycoprotein and serum amyloid P in serum, rocket electrophoresis was performed by using specific rabbit anti-mouse antibodies as previously described (12).

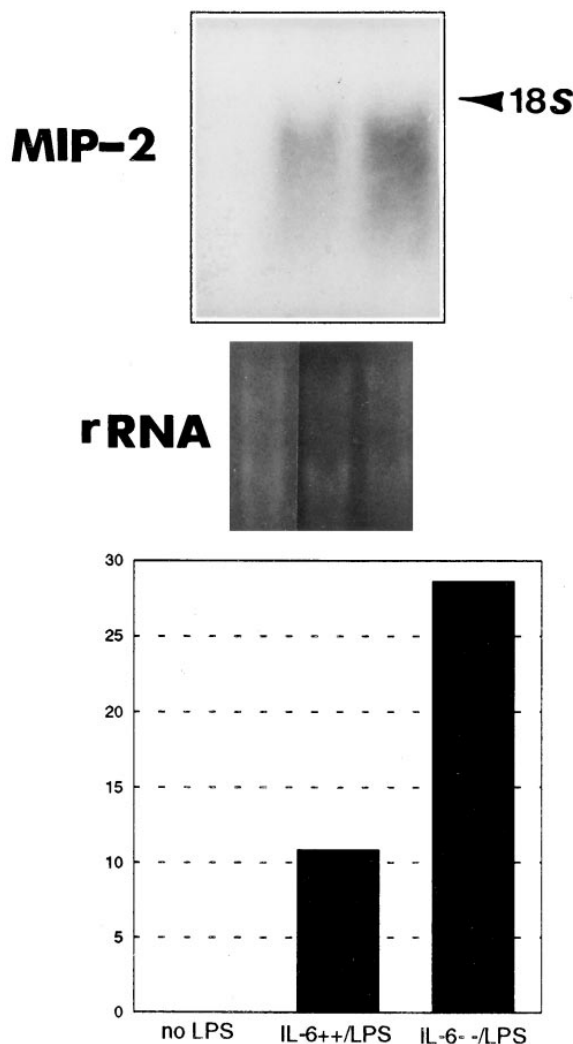
**Total lung tissue RNA extraction and Northern hybridization.** In separate experiments, lungs were homogenized and total tissue RNA was extracted. Total RNA (30  $\mu$ g per lane) was separated in a 1% agarose formaldehyde gel and transferred onto a Biotrans nylon membrane (Pall Corporation, East Hills, NY). The blot was then hybridized in a buffer containing  $\alpha$ - $^{32}$ P-CTP-labeled cDNA probe for MIP-2 and autoradiographed according to a previously described protocol (13).

**Data analysis.** Wherever applicable, results and differences were statistically analyzed by using a Minitab statistical software package (MINITAB, State College, PA). The difference was considered statistically significant when  $P \leq 0.05$ .

## Results

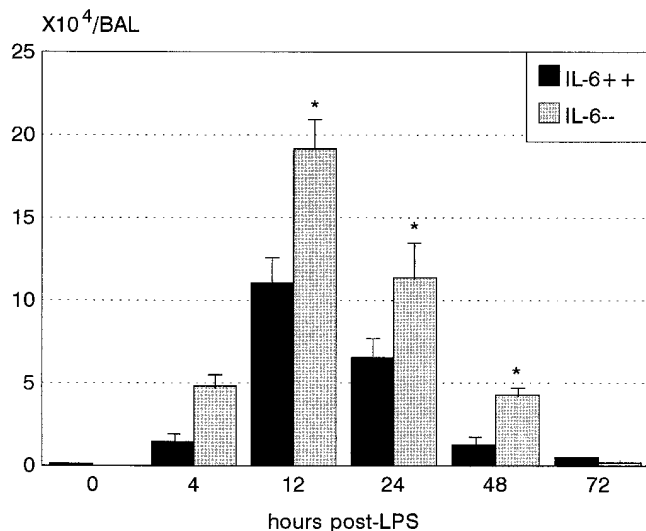
**Cytokine profiles in endotoxin-induced acute lung inflammation.** To investigate the role of IL-6 in acute lung inflammation, we first examined the content of cytokines including IL-6 and proinflammatory TNF $\alpha$  and MIP-2 in BAL in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice during acute lung inflammation elicited by local lung exposure to LPS aerosol. Previously, we have shown that TNF $\alpha$  and MIP-2 expression is closely correlated with the onset of lung neutrophilia after LPS exposure (14). LPS exposure induced a marked release of IL-6 in the lung of IL-6 $^{+/+}$  but not IL-6 $^{-/-}$  mice, which peaked at 4 h and significantly declined at 24 h (Fig. 1 a). While LPS induced a TNF $\alpha$  response in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice, which reached a maximum level in BAL at 4 h and markedly declined by 24 h, the magnitude of TNF $\alpha$  response was more than 100% higher at various time points in IL-6 $^{-/-}$  mice than in IL-6 $^{+/+}$  mice (Fig. 1 b). Similarly, the level of MIP-2 markedly increased in the lung of IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice was 100–400% higher in the lung of IL-6 $^{-/-}$  mice between 4 to 48 h (Fig. 1 c). Interestingly, there were similarly small constitutive levels of antiinflammatory cytokine IL-10 detected in BAL from both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice that were not enhanced by LPS exposure (Fig. 1 d). Since TGF $\beta$  is also capable of a number of antiinflammatory activities (15) and co-administration of this cytokine has been shown to inhibit endotoxin-induced neutrophilia in the lung (16), we also measured the content of total TGF $\beta$ 1 protein in BAL. Similar to IL-10, only small amounts of TGF $\beta$ 1 were detected in BAL and there was no statistically significant difference found between IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice ( $60.1 \pm 15.5$  versus  $44.7 \pm 3.5$  at 4 h and  $36.8 \pm 7.1$  versus  $49.0 \pm 4.5$  pg/ml at 12 h after LPS-aerosol, IL-6 $^{+/+}$  versus IL-6 $^{-/-}$  mice, respectively). To examine whether the difference in cytokine content also occurred at the mRNA level, we chose to examine mRNA expression in lung tissue of MIP-2, a potent neutrophil chemokine in rodents, at 4 h after LPS challenge. As shown in Fig. 2, MIP-2 mRNA expression was significantly induced by LPS in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice but was at least 150% stronger in the lung of IL-6 $^{-/-}$  mice (Fig. 2), suggesting that the greater amount of cytokine proteins in the lung of IL-6 $^{-/-}$  mice was a result of greater mRNA expression.

**Neutrophilia and neutrophil apoptosis in BAL and lung tissue in endotoxin-induced acute lung inflammation.** We and others have previously shown that local endotoxin challenge in the lung primarily elicits an acute neutrophilic inflammation (14, 17–19). To investigate whether enhanced proinflamma-



**Figure 2.** Lung MIP-2 mRNA expression in LPS-induced acute lung inflammation in IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice. Total lung tissue RNA was extracted from mice 4 h after LPS aerosol exposure and subjected to Northern hybridization. The bottom panel shows quantitative measurement of relative density of MIP-2 message by using a PhosphorImager/ImageQuant system (Molecular Dynamics, Sunnyvale, CA). The results shown are representative of two separate hybridizations using total RNA preparations from two mice per condition.

tory cytokine responses to endotoxin exposure in the lung of IL-6 $^{-/-}$  mice were also accompanied by a more pronounced neutrophilic response, neutrophil accumulation in BAL was examined. Consistent with our previous findings, normal lung contained few neutrophils, but LPS exposure induced a significant neutrophil accumulation in BAL which was apparent as early as 4 h, peaked at about 12 h and dropped markedly close to basal levels at 72 h in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice (Fig. 3). However, this neutrophilic response was found to be significantly more pronounced in BAL of IL-6 $^{-/-}$  mice throughout the entire course of inflammation as compared to that in IL-6 $^{+/+}$  mice (Figs. 3 and 4), 62 versus 18% at 4 h, 78 versus 52% at 12 h, 57 versus 32%, and 35 versus 4% at 48 h. Of note, there were much more red blood cells found in BAL from the



**Figure 3.** Neutrophilic responses in LPS-induced acute lung inflammation in IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice. BAL fluids were collected at various times post-LPS aerosol exposure. Total BAL and neutrophil cell numbers were determined. Results are expressed as mean  $\pm$  SEM from four mice per time point. \* $P \leq 0.05$ .

lung of IL-6<sup>-/-</sup> mice, compared to IL-6<sup>+/+</sup> mice, between 4 and 12 h after LPS challenge (Fig. 4), suggesting a greater degree of pulmonary vascular leakage or damage. Since we have demonstrated previously that neutrophil apoptosis contributes to the resolution of acute pulmonary neutrophilic inflamma-

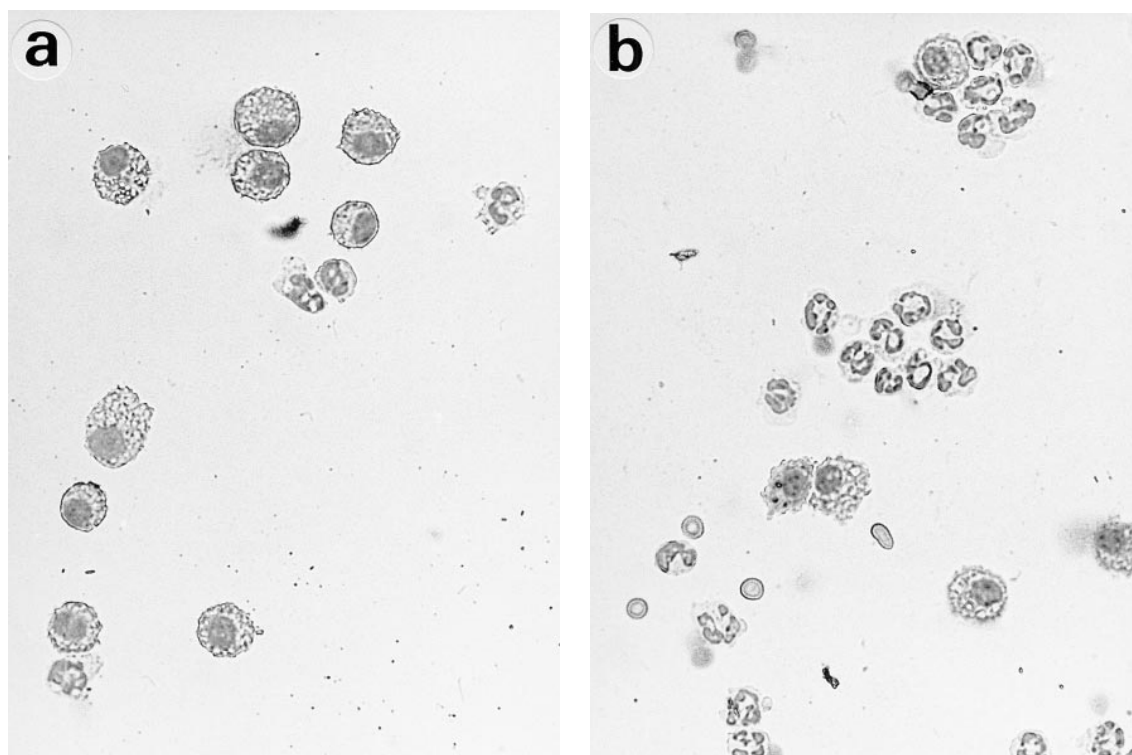
**Table I.** Percentage of Apoptotic Neutrophils in BAL from IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> Mice

	4 h	24 h	72 h
IL-6 <sup>+/+</sup>	4 $\pm$ 0.8	4 $\pm$ 1.1	8.7 $\pm$ 4.7
IL-6 <sup>-/-</sup>	1.3 $\pm$ 0.6*	2.6 $\pm$ 0.8	12 $\pm$ 2.3

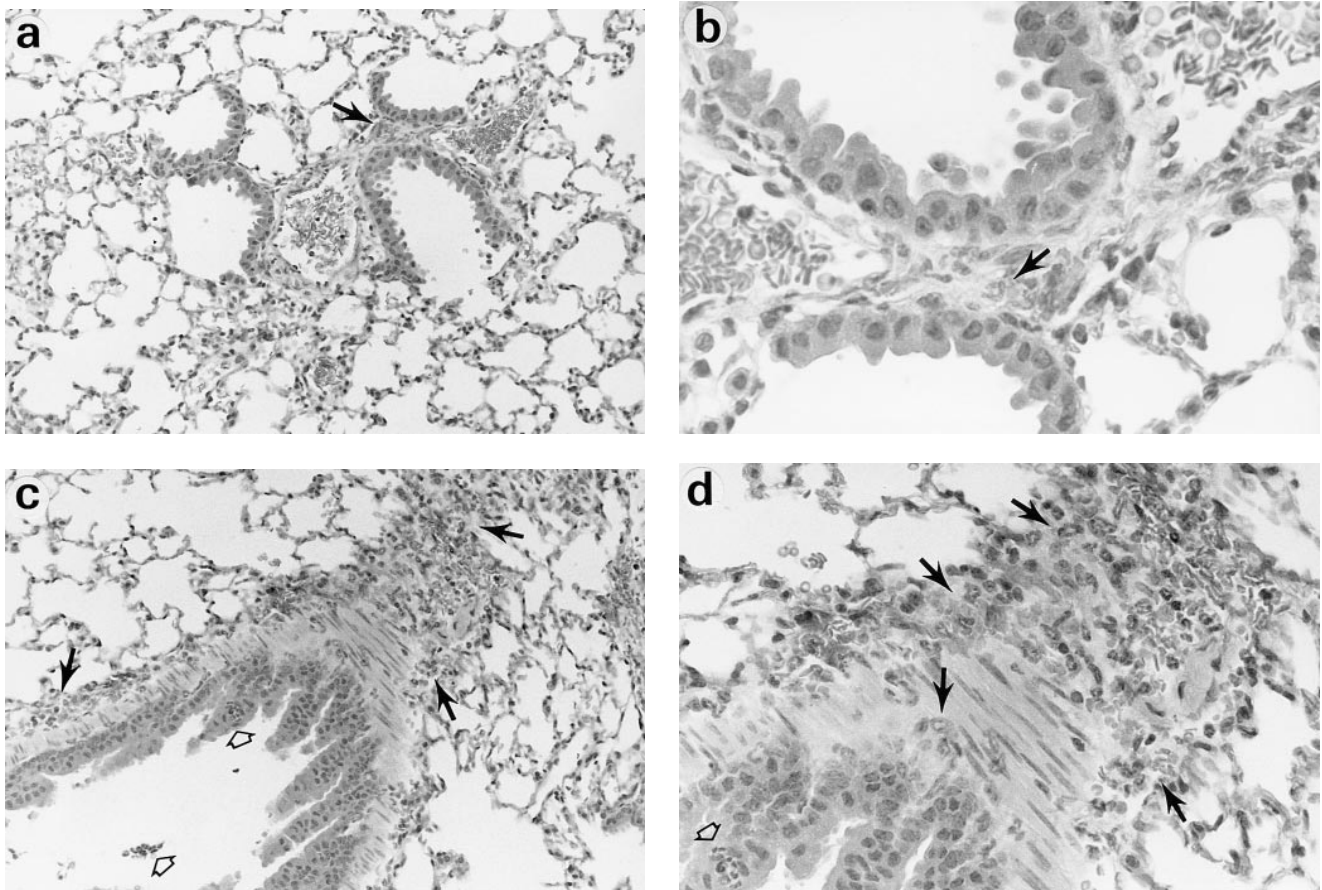
Apoptosis was assessed on Diff-Quik stained cytopins according to the criteria we previously described (10). Results are expressed as mean percentage  $\pm$  SEM from four mice. No significant difference was found between IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice at 24 or 72 h after endotoxin exposure in the lung. \*The difference at 4 h was marginally statistically significant ( $P = 0.044$ ).

tion (10) and in vitro studies have suggested that IL-6 is able to enhance neutrophil apoptosis (20), we examined whether this IL-6-mediated mechanism was operating in vivo and thus contributed to a greater degree of neutrophil accumulation in the lung of IL-6<sup>-/-</sup> mice. Although the percentage of apoptotic neutrophils in BAL from IL-6<sup>-/-</sup> mice appeared slightly smaller than in IL-6<sup>+/+</sup> mice at 4 h, there was no significant difference observed between IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice at other time points (Table I). Thus, the absence of IL-6 did not seem to markedly affect the clearance of neutrophilic response in the lung and this was consistent with a similar resolving process of neutrophilic inflammation observed in IL-6<sup>-/-</sup> mice compared to that in IL-6<sup>+/+</sup> mice (3 d in both instances; Fig. 3).

To verify the differential neutrophilic response in the lung, histopathology of lung tissues taken at 4 h after LPS exposure



**Figure 4.** Cytologic representation of differential neutrophilic responses in LPS-induced acute lung inflammation in IL-6<sup>+/+</sup> (a) and IL-6<sup>-/-</sup> (b) mice. BAL fluids were collected 4 h after LPS aerosol exposure and cytopins stained with Diff-Quik.



**Figure 5.** Histologic representation of differential neutrophilic responses in LPS-induced acute lung inflammation in IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup>. Lungs from IL-6<sup>+/+</sup> (a and b) and IL-6<sup>-/-</sup> (c and d) mice were fixed by perfusion with 10% formalin, processed and H&E stained 4 h after LPS aerosol exposure. *Solid arrows*, peribronchial and perivascular neutrophilia; *open arrow heads*, intrabronchoepithelial and intraluminal neutrophil infiltration. a and c:  $\times 200$ ; b and d:  $\times 760$ .

was examined. In consistent with neutrophilic response detected in BAL, there was a mild degree of neutrophilia found primarily in the perivascular and peribronchial areas with little neutrophilic infiltrate in the epithelium in the lung of IL-6<sup>+/+</sup> mice (Fig. 5, a and b). In contrast, in the lung of IL-6<sup>-/-</sup> mice, not only was there a more diffused and intensified neutrophilic accumulation in the perivascular and peribronchial areas, but neutrophils were often seen infiltrating the bronchial epithelium and lumen (Fig. 5, c and d). In some areas, neutrophils were also seen in alveolar spaces. This more pronounced BAL

or lung tissue neutrophilic response to lung endotoxin challenge observed in IL-6<sup>-/-</sup> mice was unlikely a result of a greater number of peripheral blood neutrophils because the number of peripheral blood neutrophils in IL-6<sup>-/-</sup> mice, before or after endotoxin exposure, was found similar to or even slightly lower, although not statistically significant, than that in their normal counterparts (Table II). Smaller numbers of peripheral blood neutrophils in IL-6<sup>-/-</sup> mice before and after treatment were also observed in other experimental models (21).

*Cytokine responses during endotoxemia.* To investigate the

**Table II.** Peripheral Blood Total Leukocytes and Neutrophils in IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> Mice ( $\times 10^4/ml$ )

	no LPS		LPS aerosol			
	WBC	PMN	24 h		72 h	
			WBC	PMN	WBC	PMN
IL-6 <sup>+/+</sup>	698.0 $\pm$ 114.0	65.0 $\pm$ 26.3	551.0 $\pm$ 174.0	96.8 $\pm$ 32.1	541.0 $\pm$ 126	58.9 $\pm$ 15.7
IL-6 <sup>-/-</sup>	351.6 $\pm$ 66.9	25.2 $\pm$ 5.3	410.1 $\pm$ 52.2	41.5 $\pm$ 12.5	412.9 $\pm$ 18.7	24.9 $\pm$ 3.7

Total leukocyte counts (WBC) and neutrophil numbers in peripheral blood were determined using peripheral blood samples collected from both IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice before and after endotoxin aerosol challenge. Results are expressed as means $\pm$ SEM from four mice. The differences between IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice are not statistically significant.

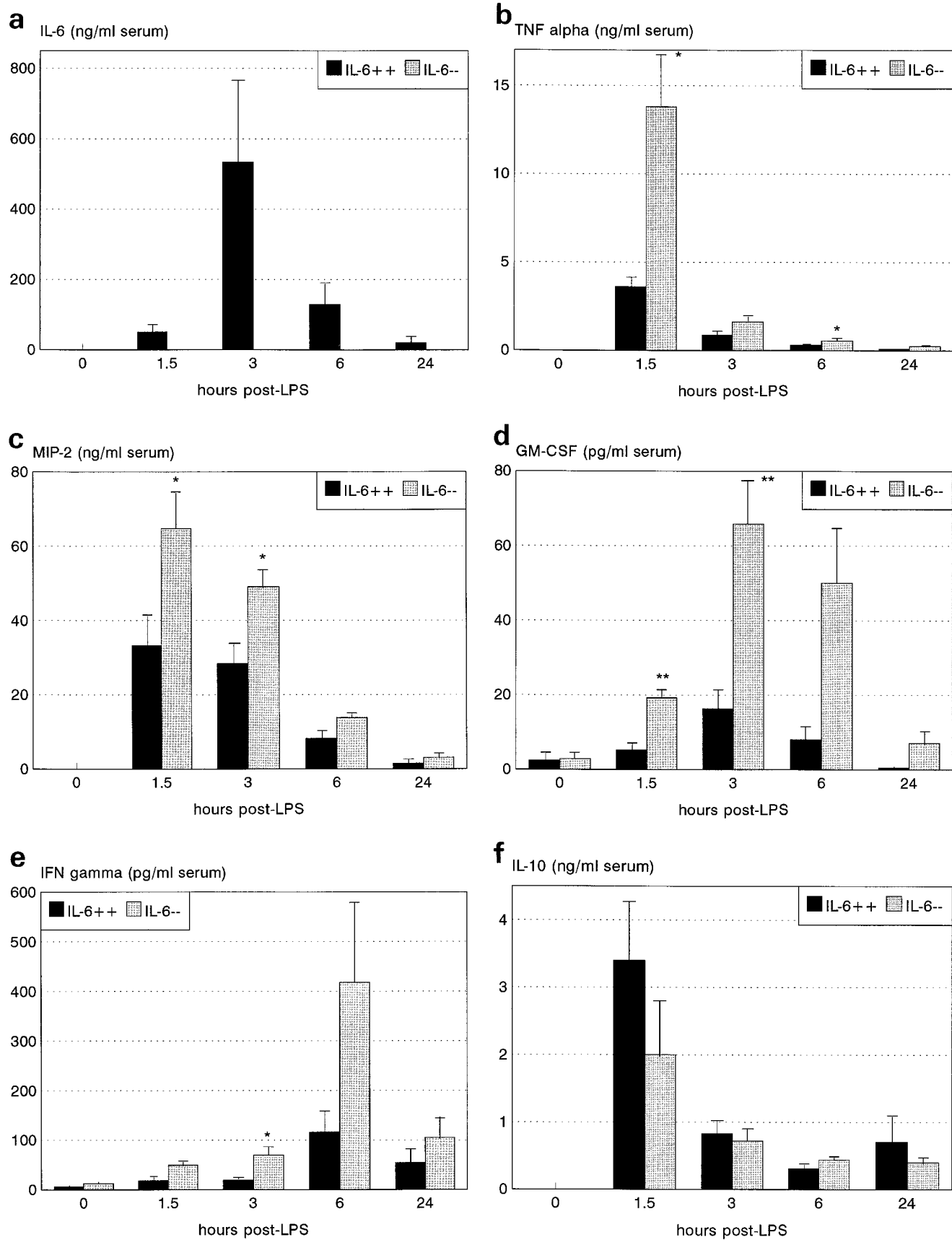


Figure 6. Cytokine responses in endotoxemia in IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice. Sera were collected at various time points after intraperitoneal LPS delivery and measured by a bioassay for IL-6 (a) or by ELISA for TNF $\alpha$  (b), MIP-2 (c), GM-CSF (d), INF $\gamma$  (e), and IL-10 (f). Results are expressed as mean  $\pm$  SEM from seven mice per group. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ .

role of IL-6 in systemic acute inflammatory responses, we examined the concentration in circulation of cytokines, both proinflammatory TNF $\alpha$ , GM-CSF, MIP-2, and IFN $\gamma$  and anti-inflammatory IL-10, in IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice during endotoxemia elicited by intraperitoneal administration of LPS. Systemic delivery of LPS triggered the release of IL-6 into the circulation only in IL-6 $^{+/+}$  but not IL-6 $^{-/-}$  mice, which markedly rose at 1.5 h, peaked at 3 h, and significantly dropped by 24 h after LPS delivery (Fig. 6a). This systemic delivery of LPS induced a quick rise in circulating levels of TNF $\alpha$  which peaked at 1.5 h and markedly declined by 6 h. The levels of this cytokine in IL-6 $^{-/-}$  mice were about 300% higher than in their normal counterparts at the peak time and remained higher up to 24 h afterwards (Fig. 6b). Similarly, the levels of neutrophil chemokine MIP-2 peaked at 1.5 h and markedly declined by 24 h and were  $\sim$ 100% higher in IL-6 $^{-/-}$  mice (Fig. 6c). The levels of GM-CSF peaked at 3 h and remained still high by 6 h and were 300–500% higher in IL-6 $^{-/-}$  mice than in IL-6 $^{+/+}$  mice (Fig. 6d). The levels of IFN $\gamma$  did not reach a maximum until 6 h and were constantly higher in IL-6 $^{-/-}$  mice (Fig. 6e). We next examined the circulating level of anti-inflammatory cytokine IL-10. While mice had little measurable IL-10 in the peripheral blood, systemic endotoxin challenge resulted in a quick increase in circulating IL-10 in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice which similar to TNF $\alpha$  and MIP-2, peaked at 1.5 h and markedly declined at 24 h (Fig. 6f). This was different from local lung exposure to endotoxin that induced little IL-10 release in the lung. Of most prominence was that, in stark contrast to proinflammatory cytokines, the levels of IL-10 in IL-6 $^{-/-}$  mice were similar to those in IL-6 $^{+/+}$  mice. IL-10 level at 1.5 h in IL-6 $^{-/-}$  mice was even slightly lower than in IL-6 $^{+/+}$  mice but this difference was not statistically significant.

To further demonstrate that enhanced proinflammatory cytokine responses during endotoxemia were a result of the lack of IL-6, rIL-6 protein was injected subcutaneously and intraperitoneally into IL-6 $^{-/-}$  mice before endotoxin challenge and cytokine responses were compared. As shown in Table III, pretreatment with rIL-6 in IL-6 $^{-/-}$  mice almost completely abolished the overreactivity of cytokine responses and brought the level of proinflammatory cytokines in these mice back close to that in normal counterparts, suggesting an IL-6-specific regulatory effect on the level of proinflammatory cytokines during endotoxin-induced acute inflammatory responses.

To examine whether overreactive proinflammatory cytokine responses during endotoxemia could lead to more pronounced

Table III. Enhanced Cytokine Responses in IL-6 $^{-/-}$  Mice

	Without rIL-6		With rIL-6	
	1.5 h	3 h	1.5 h	3 h
TNF $\alpha$	2.7	1.0	0.04	-0.6
GM-CSF	2.8	2.9	0.05	0.04

IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice were challenged intraperitoneally with 4  $\mu$ g LPS/per gram body weight and sera were collected at 1.5 and 3 h after LPS for TNF $\alpha$  or GM-CSF content by ELISA. Some of IL-6 $^{-/-}$  mice ( $n = 4$ ) were injected intraperitoneally and subcutaneously with 0.2  $\mu$ g/g body weight of recombinant rat IL-6 30 min before intraperitoneal LPS challenge. Results are expressed as fold increase of each cytokine measured over IL-6 $^{+/+}$  controls.

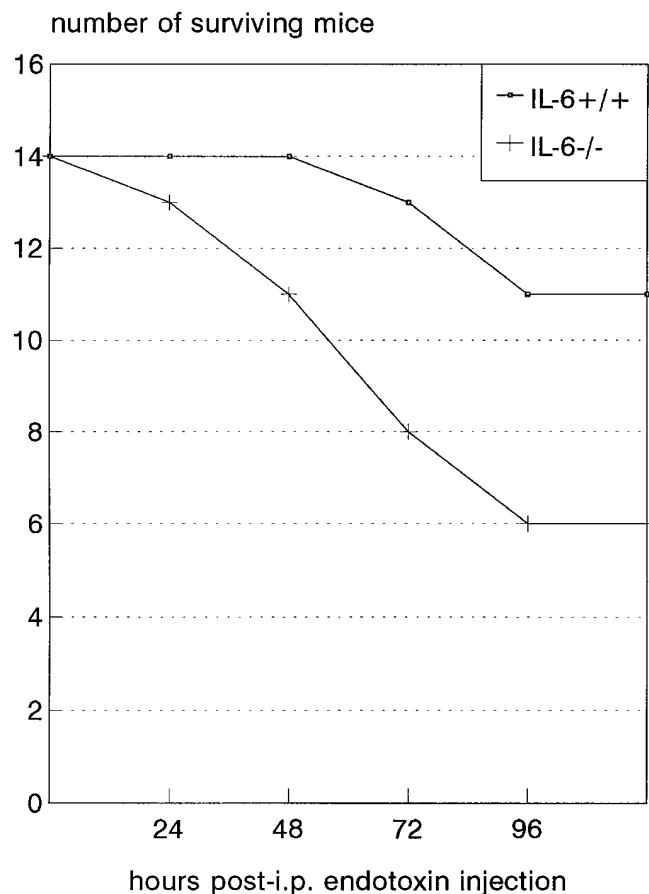


Figure 7. Mortality of IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice during endotoxemia. 14 of each mice were injected intraperitoneally with 20  $\mu$ g/g body weight of endotoxin and mortality was recorded in the next 5 d.

tissue injury in IL-6 $^{-/-}$  mice, a lethal dose of endotoxin was given intraperitoneally to IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice and mortality was observed. The survival rate of IL-6 $^{-/-}$  mice was about 50% lower than IL-6 $^{+/+}$  mice (78 versus 42% by day 4, Fig. 7).

**Acute phase protein release during endotoxemia.** Since previous studies have indicated that the optimal time to measure circulating acute phase proteins is 24 h after intraperitoneal LPS challenge (8, 22), the circulating levels of acute phase proteins  $\alpha$ 1 acid glycoprotein and serum amyloid P were examined with serum samples saved at 24 h after intraperitoneal LPS delivery by rocket electrophoresis. The levels of these acute phase proteins were similar in both naive unchallenged IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice. While there was an increase in the level of these acute phase proteins in both IL-6 $^{+/+}$  and IL-6 $^{-/-}$  mice after endotoxin challenge, the levels of  $\alpha$ 1 acid glycoprotein and particularly, serum amyloid P appeared lower in IL-6 $^{-/-}$  mice although the differences were not statistically significant (Table IV). These findings are in line with the previous observations from others (8, 22).

## Discussion

Cytokines are soluble signals of paramount importance mediating cell-cell communication during inflammatory and im-



Table IV. Acute Phase Protein Responses in IL-6+/+ and IL-6-/-

	$\alpha$ 1-acid glycoprotein	Serum amyloid P
IL-6+/+	175 $\pm$ 31	306 $\pm$ 105
IL-6-/-	142 $\pm$ 29	170 $\pm$ 32

Sera were collected before or 24 h after intraperitoneal endotoxin challenge and measured for  $\alpha$ 1-acid glycoprotein and serum amyloid P by an immunoassay using rocket electrophoresis. Results are expressed as percentage increase over control levels measured in samples obtained before challenge (mean $\pm$ SEM from four mice). There is no statistically significant difference between IL-6+/+ and IL-6-/- mice.

immune responses. Among the cytokines of first discovery is IL-6, which is still a subject of intensive investigations today because of its ubiquity and functional diversity (1-3). Indeed, IL-6 was found to be produced by cells at tissue sites and released into circulation in such acute situations of homeostatic perturbation as endotoxemia, endotoxic lung or trauma. In these processes, circulating IL-6 has been known to play an important role in the induction of acute phase reactions by acting upon the liver and the hypothalamic-pituitary-adrenal axis (22, 23). However, IL-6 is induced together with other alarm cytokines TNF $\alpha$  and IL-1 that are also involved in the elicitation of acute phase reactions (3). While there is little doubt about the proinflammatory nature of TNF $\alpha$  and IL-1, it has remained unclear whether IL-6 is merely an acute phase reaction-inducing cytokine or has any additional proinflammatory or antiinflammatory activities in these processes. Indeed, the lack of understanding of other functional aspects of this cytokine in both local and systemic acute inflammatory responses has led to a confounding description about the nature of endogenous IL-6 in literature which has been described as either proinflammatory or antiinflammatory.

In this study, we have revealed an antiinflammatory nature of IL-6 in both local and systemic acute inflammatory responses elicited by local lung or systemic exposure to endotoxin by using IL-6 gene knock-out mice. We demonstrate that IL-6 is critically required to control the extent of local or systemic acute inflammatory responses, particularly the level of proinflammatory cytokines in the local and systemic compartments, respectively. Locally in endotoxic lung, the absence of IL-6 not only resulted in a more pronounced response of proinflammatory cytokines but a greater extent of tissue neutrophilia. The latter likely resulted from higher levels of MIP-2, a well-known neutrophil chemoattractant involved in the elicitation of pulmonary neutrophilia (14, 19, 24), and TNF $\alpha$ , a cytokine capable of enhancing expression of neutrophil adhesion molecules on the endothelium and amplifying many other inflammatory processes (25). Previously, we have shown that TNF $\alpha$  and MIP-2 are two cytokines of earliest induction upon endotoxin challenge in the lung (14). Our current evidence that IL-6-/- mice did not demonstrate a severely impaired ability to resolve, by apoptosis, the acute pulmonary neutrophilic response, further supports a direct contribution of a greater proinflammatory cytokine response to enhanced neutrophilia. These findings for the first time reveal a regulatory role of endogenous IL-6 in local acute inflammatory responses.

We have also demonstrated that systemically, the absence

of IL-6 resulted in a remarkably higher circulating level of proinflammatory cytokines during endotoxemia including TNF $\alpha$ , MIP-2, GM-CSF, and IFN $\gamma$ . Each of these cytokines has been previously shown to be critically involved in the elicitation of toxic syndromes in endotoxemia (26-28). These findings highlight an irreplaceable role of IL-6 by other IL-6 family members such as LIF (29) in controlling the level of acute inflammatory responses during endotoxemia. Since we have shown previously that elevated circulating levels of proinflammatory cytokines during endotoxemia are attributed at least in part to enhanced cytokine gene expression at various solid tissue sites (9), local and systemic IL-6 likely modulates the level of proinflammatory cytokine responses at multiple tissue sites. The antiinflammatory nature of IL-6 is further supported by our observation that IL-6-/- mice suffered an increased mortality rate upon challenge with a lethal dose of endotoxin. In contrast to overreactive proinflammatory cytokine responses in IL-6-/- mice, the response of acute phase proteins was only moderately or marginally impaired in endotoxemia. These findings confirm the observations from Kopf et al. (8) and Fattori et al. (22), suggesting that the role of IL-6 in the induction of acute phase proteins in such settings may be partially compensated for by other cytokines. Previous studies have shown that corticosterone release is not affected during endotoxemia in IL-6-/- mice (22).

Further, we have demonstrated that the absence of IL-6 has little effect on the level of antiinflammatory cytokine IL-10 either locally in the lung during acute lung inflammation or systemically in circulation during endotoxemia, and nor did the lack of IL-6 have effect on the level of TGF $\beta$  in the lung. These suggest that in a normal host, one function of inducible IL-6 during acute responses is to suppress the level of proinflammatory cytokines without compromising the level of antiinflammatory cytokines. While IL-10 has been shown to play an antiinflammatory role during endotoxemia or acute lung inflammation (27, 30, 31), our findings suggest that IL-6 is also an antiinflammatory molecule whose function cannot be compensated for by other antiinflammatory molecules such as IL-10. Although the pharmacologic effect of a given cytokine may differ from its biologic effect, the antiinflammatory nature of IL-6 has also been suggested by studies where pharmacologic use of recombinant IL-6 was found to induce the release of antiinflammatory molecules IL-1 receptor antagonist and soluble TNF $\alpha$  receptor in normal subjects (32) and to inhibit TNF $\alpha$  release in experimental endotoxemia or endotoxic lung (16, 33, 34). Thus, these findings allow us to compare the biologic function of IL-6 with IL-10. Both of them inhibit proinflammatory cytokine responses and tissue neutrophilia and enhance the release of antiinflammatory molecules such as anti-protease inhibitors. On the other hand, IL-6 has been shown to be required in or enhance the generation of specific immune responses against intracellular infections and tumor (8, 21, 35, 36), and recent evidence has also suggested a proimmune nature of IL-10 in certain circumstances such as autoimmune diseases and tumorigenesis (37, 38).

Collectively, our observations have significantly clarified the conflicting results regarding the role of endogenous IL-6 during endotoxemia obtained in models by using monoclonal antibodies against IL-6. IL-6 has been considered, in two previously published reports, to play a detrimental role during endotoxemia (5, 6). However, in the study by Starnes et al., the circulating level of IL-6 was not measured post-anti-IL-6 ad-

ministration, hence a lack of indication regarding the efficacy of this antibody (5). It is likely that instead of abrogating IL-6, such antibodies may have enhanced IL-6 release or prolonged the half-life of IL-6, as suggested in studies by Heremans et al. (6) where while the use of anti-IL-6 antibodies decreased the mortality of mice, it unexpectedly increased the level of circulating IL-6 bioactivity during endotoxemia. Moreover, the role of contaminating LPS in the anti-IL-6 preparation used in the former study (5) in inducing endotoxin tolerance cannot be entirely ruled out, since in an independent study, the same antibody but a different batch free of LPS was found to enhance the mortality of mice during endotoxemia (7). Thus, there is more evidence supporting a protective role of IL-6 in endotoxemia from these studies. Nonetheless, the inconsistency in results from these studies indicates the complexity and difficulty in using anti-IL-6 antibodies to examine the function of endogenous IL-6. In comparison, the use of IL-6<sup>-/-</sup> mice may circumvent a number of potential problems associated with the use of anti-IL-6 antibodies and provide a unique opportunity to study the function of IL-6 in the authentic absence of endogenous IL-6. The IL-6<sup>-/-</sup> mice used in this study previously have been shown to have a normal development of organ systems and normal expression of functional cell surface markers on immune cell types with only slightly smaller numbers of thymocytes and peripheral blood T cells (8). We found a slightly smaller, but not statistically different, number of peripheral neutrophils in these mice before or after LPS exposure. Apart from these, we found that the number of alveolar macrophages in the lung was similar between untreated IL-6<sup>+/+</sup> and IL-6<sup>-/-</sup> mice, and others found an unimpaired release of nitric oxide by LPS/IFN $\gamma$ -stimulated peritoneal macrophages from IL-6<sup>-/-</sup> mice in vitro (8). Of importance, while we have demonstrated an enhanced response of proin-

flammatory cytokines in IL-6<sup>-/-</sup> mice during endotoxemia, the release of antiinflammatory cytokine IL-10 in response to systemic endotoxin exposure is not altered in these mice, indicating a selective effect of IL-6. Furthermore, the release of acute phase proteins was normal and exaggerated cytokine responses in IL-6<sup>-/-</sup> mice were abolished by pretreatment with recombinant IL-6 protein. All of these suggest that the more pronounced inflammatory responses observed in IL-6<sup>-/-</sup> mice were a consequence of the lack of direct suppressive mechanisms by IL-6, rather than from aberrant or indirect compensatory functions of the host.

Thus, based upon all of our current and previous findings and those from others, a model of the role of IL-6 in both acute and chronic inflammatory diseases can be proposed (Fig. 8). Since the release of acute phase proteins and corticosterone (a global inflammation inhibitor) is a major feature of acute phase reactions and many of acute phase proteins are antiprotease inhibitors in nature, a main function of acute phase reactions can be regarded as part of antiinflammatory mechanisms mediated by IL-6. In acute inflammatory responses typically elicited by nonimmune stressors such as endotoxin, trauma and acute infections, with induction of acute phase reactions (8, 22, 23), inhibition of proinflammatory cytokines, and perhaps induction of antiinflammatory molecules and extrahepatic protease inhibitors (32, 39, 40), IL-6 operates to control the extent of tissue inflammatory responses. In chronic diseases typically exemplified by immune stressors including chronic intracellular infections and tumor, IL-6 serves not only as an inducer of acute phase reactions but an important player in the elicitation of cellular immune responses against affected cells and of mucosal humoral responses against reinfection (8, 12, 21, 23, 35, 36). All of these activities by IL-6 may be viewed as an attempt to bring the host back to homeostasis.

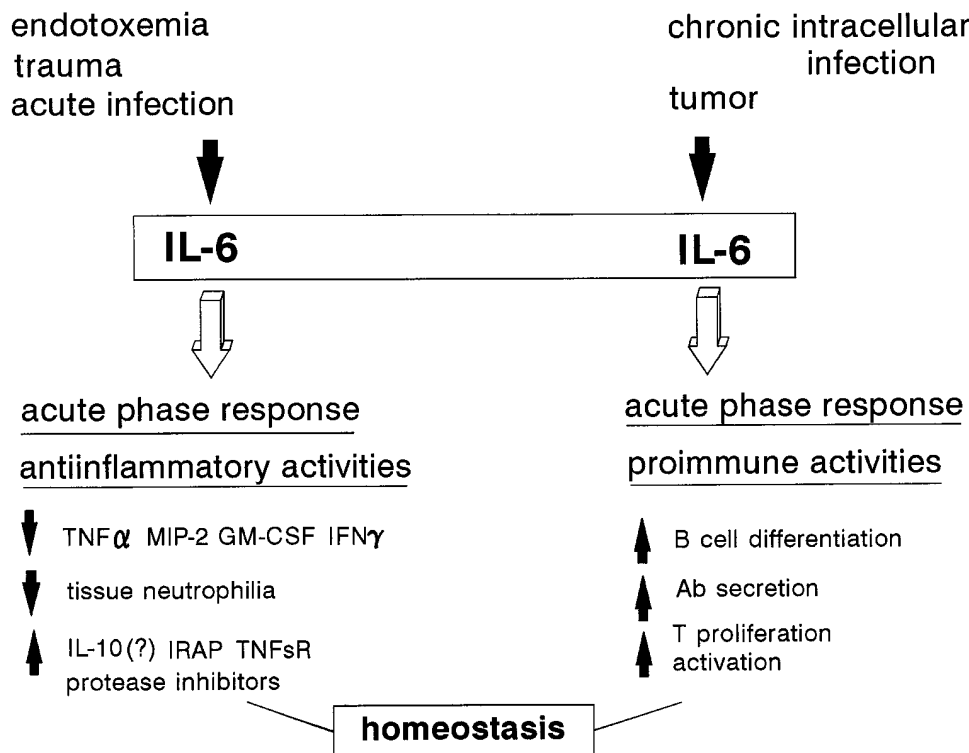


Figure 8. A model of biologic functions of IL-6 in acute and chronic diseases. During local or systemic acute inflammatory responses, IL-6 is involved in the induction of acute phase reactions and controlling the level of acute inflammatory responses by downregulating the expression of proinflammatory cytokines and upregulating antiinflammatory molecules including IL-1 receptor antagonist protein (*IRAP*), TNF soluble receptor (*TNFsR*), and extrahepatic protease inhibitors. During chronic diseases, particularly intracellular viral or bacterial infections, IL-6 is involved not only in the elicitation of acute phase reactions but the development of specific cellular and humoral immune responses including end-stage B cell differentiation, immunoglobulin (*Ab*) secretion and T cell activation.

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