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# Effects of IL-10 on Systemic Inflammatory Responses During Sublethal Primate Endotoxemia<sup>1</sup>

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IL-10 protects mice from LPS-induced lethality. To determine the effects of IL-10 on LPS-induced inflammatory responses, six *Papio anubis* baboons were i.v. injected with a sublethal dose of LPS (*Salmonella typhimurium*; 500  $\mu\text{g}/\text{kg}$ ) directly preceded by either human rIL-10 ( $n = 3$ , 500  $\mu\text{g}/\text{kg}$ ) or diluent ( $n = 3$ ). IL-10 strongly inhibited LPS-induced release of TNF, IL-6, IL-8, and IL-12 (all  $p < 0.05$ ). By contrast, IL-10 did neither influence the activation of the coagulation system (plasma levels of thrombin/antithrombin III complexes), nor the activation of the fibrinolytic system (plasma levels of tissue-type plasminogen activator, plasminogen activator inhibitor type I, and plasmin/ $\alpha_2$ -antiplasmin complexes). IL-10 modestly attenuated neutrophilic leukocytosis and neutrophil degranulation (plasma concentrations of elastase/ $\alpha_1$ -antitrypsin complexes) (both  $p < 0.05$ ). Changes in surface TNF receptor expression on circulating granulocytes were not affected by IL-10. These results suggest that during sublethal endotoxemia the predominant anti-inflammatory effect of IL-10 treatment is inhibition of proinflammatory cytokine release. *The Journal of Immunology*, 1997, 158: 1971–1975.

IL-10 is an 18-kDa polypeptide that originally was discovered as a product of murine Th2 cells that inhibited the production of cytokines by Th1 cells (1). It is evident now that IL-10 can be synthesized by various other cell types, including Th1 clones, as well as by B cells, monocytes, and macrophages. IL-10 has multiple biologic effects, of which its potency to inhibit the production of proinflammatory cytokines by stimulated mononuclear cells has received much attention (2–6). This anti-inflammatory property of IL-10 is considered to be responsible, at least in part, for its in vivo protective effect in endotoxemic mice, in which administration of IL-10 results in inhibition of TNF release and a survival advantage (7, 8). The effect of IL-10 on the appearance of cytokines during endotoxemia in primates is unknown.

Sepsis and endotoxemia lead to the activation of a number of host mediator systems, including the hemostatic mechanism and granulocytes, each of which may contribute to the pathologic sequelae of systemic infection (9, 10). It can be argued that IL-10 may attenuate these inflammatory responses in animals in vivo. IL-10 may directly inhibit activation of the coagulation system during endotoxemia by reducing LPS-induced tissue factor expres-

sion by monocytes (11, 12), a process critical for initiation of a coagulant response in experimental sepsis and endotoxemia (13–15). Furthermore, IL-10 may attenuate LPS-induced neutrophil degranulation in an indirect manner, through inhibiting the production of TNF, a cytokine demonstrated to mediate this inflammatory reaction, at least in part, in endotoxemic primates (16, 17). Therefore, in the present study, we sought to determine the effect of recombinant human (rHu)<sup>3</sup> IL-10 on the activation of the cytokine network, coagulation, and fibrinolysis, and on neutrophil degranulation in baboons infused with a sublethal dose of endotoxin.

## Materials and Methods

### Study design

Six *Papio anubis* baboons (10–15 kg), obtained through the National Primate Exchange by Buckshire Laboratories (Chelmsford, PA), were housed and quarantined for 2 to 4 wk at the Research Animal Resource Center of Cornell University Medical College (New York, NY). Baboons were fasted overnight and immobilized with ketamine hydrochloride (10 mg/kg, intramuscular injection) before the experiment. During the study, anesthesia was maintained using i.v. pentobarbital sodium (35 mg/kg, followed by 5 mg/kg/h). Control of the upper airway was obtained using an endotracheal tube. At time zero, all six baboons received a bolus i.v. injection of LPS (*Salmonella typhimurium*, 500  $\mu\text{g}/\text{kg}$ ). In three baboons, the administration of LPS was preceded immediately by a bolus i.v. injection of rHuIL-10 (Schering, Kenilworth, NJ; 500  $\mu\text{g}/\text{kg}$ ) in 10 ml of 0.1% human serum albumin, while the other three baboons received an equivalent volume of 0.1% human serum albumin only at that time. All animals received a continuous infusion of isotonic saline (3 ml/kg/h) for 8 h. Venous blood samples were obtained at 0, 1, 2, 3, 4, 5, 6, 7, and 8 h.

### Assays

All assays were performed in citrated plasma samples. TNF (18), IL-6 (19), IL-8 (20), and IL-12 p40 (21) were measured by ELISA. Coagulation activation was determined by measuring thrombin/antithrombin III (TAT)

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<sup>3</sup> Abbreviations used in this paper: rHu, recombinant human; cPBS-A, ice-cold phosphate-buffered saline with 0.1% sodium azide; MCF, mean channel fluorescence; PAI-1, plasminogen activator inhibitor type I; PAP, plasmin- $\alpha_2$ -antiplasmin; TAT, thrombin-antithrombin III; tPA, tissue-type plasminogen activator.

complexes (ELISA) (22). Fibrinolytic activation was monitored by measurements of tissue-type plasminogen activator (tPA) (ELISA), plasminogen activator inhibitor type I (PAI-1) (ELISA), and plasmin/ $\alpha_2$ -antiplasmin (PAP) complexes (RIA) (14, 22–25). Levels of PAP complexes are expressed as percentage of the level present in normal baboon plasma in which a maximal amount of PAP complexes was generated by a 1-h incubation with an equal volume of urokinase (50  $\mu\text{g}/\text{ml}$ ) in the presence of 0.4 M of methylamine (22, 25). Neutrophil degranulation was determined by measurement of the plasma concentrations of elastase/ $\alpha_1$ -antitrypsin complexes (RIA) (26).

#### Flow cytometry

Granulocyte counts were determined by flow-cytometric light scatter analysis. Saturation binding of TNF by granulocytes was determined by FACS analysis, as described previously (27–29). Briefly, erythrocytes in 400  $\mu\text{l}$  of blood were lysed with bicarbonate-buffered (pH 7.2), 0.826% ammonium chloride solution. Leukocytes were recovered by centrifugation and washed with ice-cold PBS containing 0.1% sodium azide (cPBS-A). Specific staining was with 1  $\mu\text{g}/\text{ml}$  of biotinylated human TNF, while non-specific staining was with biotinylated TNF plus a 100-fold excess unlabeled human TNF. After incubation on ice for 15 min, cells were washed with cPBS-A and stained with 0.5  $\mu\text{g}/\text{ml}$  of phycoerythrin-conjugated streptavidin for 15 min on ice. Leukocytes were then washed with cPBS-A and resuspended for flow-cytofluorometric analysis. The flow cytometer photomultiplier gain was standardized using phycoerythrin-conjugated beads (Calibrite; Becton Dickinson, San Jose, CA). Mean channel fluorescence (MCF) at  $>570$  nm of forward and side angle scatter-gated monocytes and granulocytes was assessed. Data are presented as the difference (linear units) between MCF intensities of specifically and nonspecifically stained cells.

#### Statistical analysis

All values are expressed as mean  $\pm$  SE. Differences between groups were compared by analysis of variance.  $p < 0.05$  was considered to be significant. It can be argued that the number of baboons studied was small. We chose not to expand the study groups, considering the value of the animals and the fact that responses in the two treatment groups were either already significantly different, or similar to an extent that increasing the number of animals would unlikely influence the analysis (see Results).

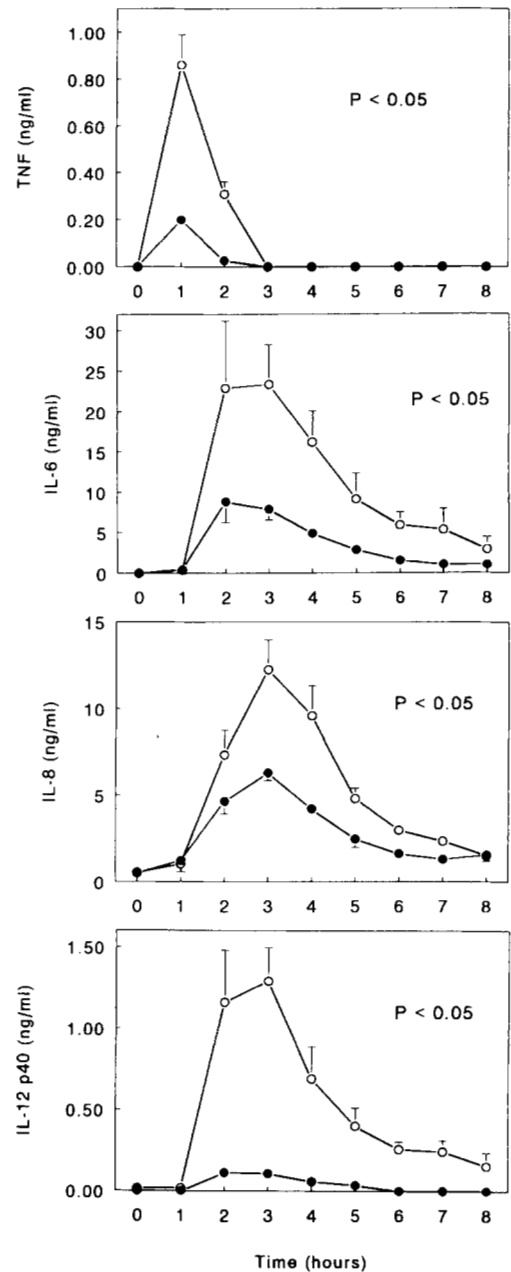
## Results

### Cytokines

Intravenous injection of LPS elicited rises in the plasma concentrations of TNF, IL-6, IL-8, and IL-12 p40 (all  $p < 0.05$  vs baseline; Fig. 1). Treatment with rHuIL-10 caused significant reductions in these LPS-induced cytokine responses (all  $p < 0.05$  vs LPS only). Peak plasma concentrations, after LPS alone and after LPS with rHuIL-10, respectively, were: TNF,  $0.86 \pm 0.13$  and  $0.20 \pm 0.01$  ng/ml; IL-6,  $23.33 \pm 4.91$  and  $8.78 \pm 2.57$  ng/ml; IL-8,  $12.26 \pm 1.72$  and  $6.29 \pm 0.44$  ng/ml; and IL-12 p40,  $1.29 \pm 0.20$  and  $0.11 \pm 0.03$  ng/ml.

### Coagulation and fibrinolysis

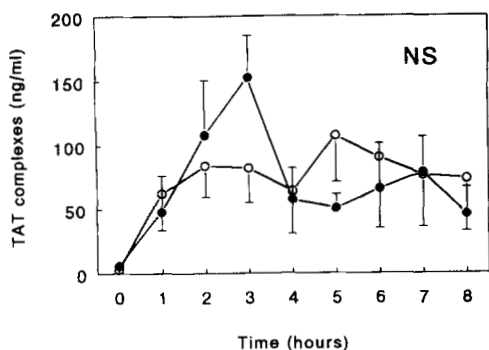
Administration of LPS was associated with activation of both the coagulation system and the fibrinolytic system. Treatment with rHuIL-10 did not influence the coagulant response to LPS (Fig. 2). Peak plasma concentrations of TAT complexes, indicative of the formation of thrombin, were  $107.9 \pm 36.5$  ng/ml after LPS only, and  $152.7 \pm 32.3$  ng/ml after LPS with rHuIL-10 ( $p = 0.70$  for the difference between groups). rHuIL-10 also did not affect LPS-induced plasmin formation, as reflected by similar increases in the plasma levels of PAP complexes in both treatment groups (peak levels  $0.82 \pm 0.41\%$  after LPS only, and  $0.92 \pm 0.44\%$  after LPS with rHuIL-10;  $p = 0.64$  for the difference between groups; Fig. 3). In accordance, the rises in tPA and PAI-1 levels were similar in both groups. Peak tPA concentrations were  $5.1 \pm 1.5$  ng/ml after LPS only and  $5 \pm 1.3$  ng/ml after LPS with rHuIL-10 ( $p = 0.89$ ); peak concentrations of PAI-1 were  $971 \pm 233.5$  and  $664.2 \pm 153.4$  ng/ml, respectively ( $p = 0.55$ ) (Fig. 3).



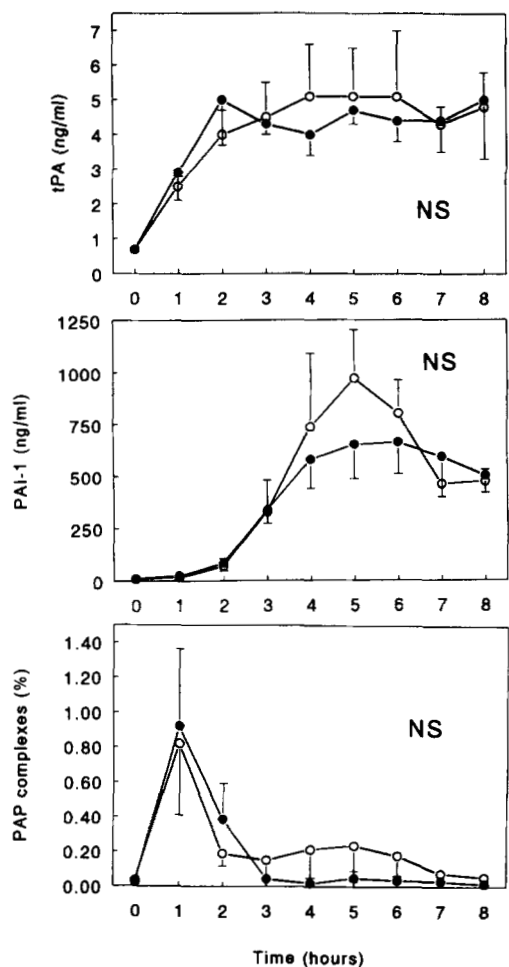
**FIGURE 1.** Mean ( $\pm$  SE) plasma concentrations of TNF, IL-6, IL-8, and IL-12 p40 after i.v. injection of *S. typhimurium* LPS (500  $\mu\text{g}/\text{kg}$ ) immediately preceded by a bolus i.v. injection of rHuIL-10 (500  $\mu\text{g}/\text{kg}$ ; ●) or placebo (○).  $p$  values indicate the difference between groups by analysis of variance.

### Granulocyte responses

Granulocyte responses during endotoxemia are given in Figure 4. Injection of LPS elicited a granulocytosis at 5 h and onward, which was significantly attenuated by rHuIL-10. Peak granulocyte numbers were  $13.6 \pm 4 \times 10^9/\text{L}$  after LPS only, and  $8.3 \pm 0.3 \times 10^9/\text{L}$  after LPS with rHuIL-10 ( $p < 0.05$  for the difference between groups). Infusion of rHuIL-10 also inhibited neutrophil degranulation, as indicated by an abrogated rise in the plasma concentrations of elastase/ $\alpha_1$ -antitrypsin complexes. Peak levels of elastase/ $\alpha_1$ -antitrypsin complexes were  $184 \pm 67$  ng/ml after LPS only, and  $119 \pm 14$  ng/ml after LPS with rHuIL-10 ( $p < 0.05$  for the

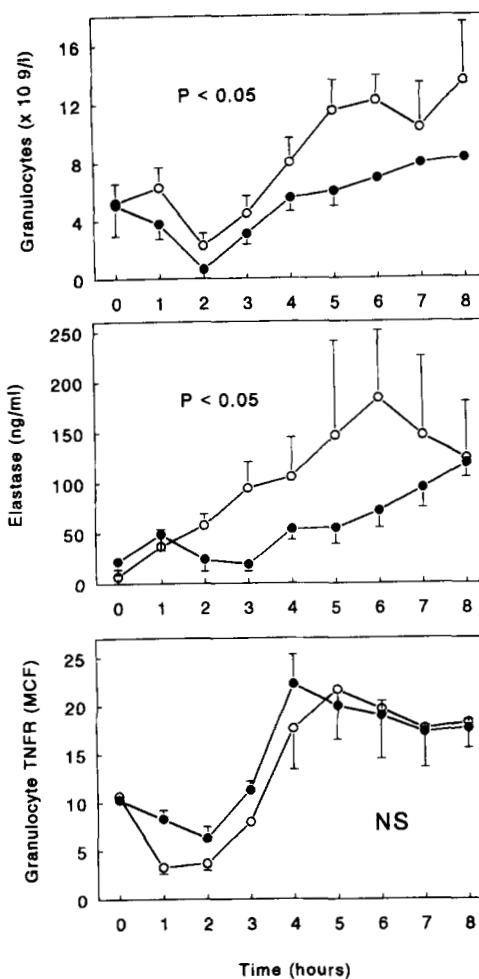


**FIGURE 2.** Mean ( $\pm$  SE) plasma concentrations of TAT complexes after i.v. injection of *S. typhimurium* LPS (500  $\mu$ g/kg) immediately preceded by a bolus i.v. injection of rHuIL-10 (500  $\mu$ g/kg; ●) or placebo (○). NS = nonsignificant for the difference between groups by analysis of variance.



**FIGURE 3.** Mean ( $\pm$  SE) plasma concentrations of tPA, PAI-1, and PAP complexes after i.v. injection of *S. typhimurium* LPS (500  $\mu$ g/kg) immediately preceded by a bolus i.v. injection of rHuIL-10 (500  $\mu$ g/kg; ●) or placebo (○). NS = nonsignificant for the difference between groups by analysis of variance.

difference between groups). By contrast, rHuIL-10 did not influence the reduced expression of TNF receptors on circulating granulocytes after administration of LPS. After injection of LPS only, TNF binding by granulocytes decreased to  $30.9 \pm 5.5\%$  of granulocyte TNF binding at baseline, compared with  $62.1 \pm 13.2\%$



**FIGURE 4.** Mean ( $\pm$  SE) granulocyte counts, plasma concentrations of elastase/ $\alpha_1$ -antitrypsin complexes, and granulocyte TNF receptor expression after i.v. injection of *S. typhimurium* LPS (500  $\mu$ g/kg) immediately preceded by a bolus i.v. injection of rHuIL-10 (500  $\mu$ g/kg; ●) or placebo (○). Saturation binding of biotinylated TNF by granulocytes (lower panel) was determined by FACS analysis, as described in *Materials and Methods*. Results are expressed as the difference between specific MCF and nonspecific MCF (mean  $\pm$  SE). *p* values indicate the difference between groups by analysis of variance.

after injection of LPS with rHuIL-10 ( $p = 0.50$  for the difference between groups).

### Discussion

Systemic administration of LPS leads to release of proinflammatory cytokines and the activation of a number of host counter-regulatory mechanisms, including the release of anti-inflammatory cytokines (e.g., IL-10), cytokine inhibitors (e.g., IL-1 receptor antagonist), and soluble cytokine receptors (e.g., soluble TNF receptors). Inhibition of proinflammatory cytokine activity during models of severe Gram-negative sepsis is associated with a significant increase in survival rates, as demonstrated by protection of baboons with *Escherichia coli* bacteremia by treatment with anti-TNF or IL-1 receptor antagonist (30). Similarly, down-regulation of the proinflammatory cytokine response by administration of rIL-10 has been found to protect mice against lethality associated with injection of LPS (7, 8). In accordance with these mouse studies, we report in this work that infusion of rHuIL-10 strongly inhibits the release of TNF during sublethal endotoxemia in baboons.

Furthermore, we established that rHuIL-10 also attenuated the endotoxin-induced appearances of IL-6, IL-8, and IL-12 p40 in baboons, which is in line with *in vitro* studies (2–6). Hence, these data indicate that IL-10 exerts inhibitory effects on the induction of proinflammatory cytokines during primate endotoxemia.

IL-12 is a heterodimeric cytokine consisting of two covalently linked subunits of 35 (p35) and 40 (p40) kDa (31). The p35 subunit most likely mediates IL-12 signal transduction, while the p40 subunit is required for receptor binding. IL-12 is biologically active only in the heterodimeric form (31). Importantly, the production of IL-12 during endotoxemia is at least in part responsible for the development of tissue injury and death (32). We found a transient increase in IL-12 p40 after *i.v.* injection of LPS in baboons, which was inhibited by rHuIL-10. Presumably, endogenously produced IL-10 also inhibits IL-12 production during endotoxemia, as IL-10 and IL-12 p40 levels inversely correlated in bacteremic baboons (21). In the latter study, low levels of heterodimeric IL-12 (in the 100 pg/ml range) could be detected in plasma (21). Using the same p35/p40 heterodimer detecting ELISA, we found IL-12 levels of maximally 6 pg/ml in baboons infused with LPS only, while in baboons treated with rHuIL-10, IL-12 heterodimer could not be detected (data not shown). Together these results suggest that the p40 subunit is preferentially released after a bacterial challenge, while the intact IL-12 heterodimer can only be detected during severe bacteremia.

Sepsis is associated frequently with changes in the hemostatic mechanism (9). Previous studies in humans and nonhuman primates have established that low dose LPS induces activation of the coagulation system via the extrinsic tissue factor/factor VII-mediated pathway, as demonstrated by a complete prevention of coagulation activation by treatment with either anti-tissue factor or anti-factor VII/VIIa Abs (14–16, 33). Furthermore, LPS-induced IL-6 may play a role in this inflammatory response, as indicated by the finding that anti-IL-6 strongly attenuated coagulation activation in endotoxemic chimpanzees (34). LPS-induced stimulation of the fibrinolytic system appears to be mediated primarily by TNF, since anti-TNF abrogated this response in endotoxemic primates (16). In the present study, LPS-induced activation of the coagulation system was reflected by a rise in plasma concentrations of TAT complexes. IL-10 could be expected to inhibit this coagulant response via several mechanisms. First, IL-10 can inhibit the expression of tissue factor by monocytes stimulated with LPS *in vitro* (11, 12). Second, IL-10 infusion resulted in a significant inhibition of LPS-induced release of IL-6. However, IL-10 infusion did not influence coagulation activation. Furthermore, the fibrinolytic response to *i.v.* LPS remained unaltered. This latter finding contrasts with an earlier report demonstrating that complete inhibition of LPS-induced TNF activity in chimpanzees is associated with a strong inhibition of fibrinolytic activation (16).

Elastase is a potent proteinase derived from the azurophilic granules of neutrophils. It circulates in complex with its major inhibitor  $\alpha_1$ -antitrypsin. The plasma concentrations of elastase/ $\alpha_1$ -antitrypsin complexes have been used as indicators of neutrophil degranulation *in vivo*, and correlate with mortality rates in patients with sepsis (16, 26, 34, 35). The role of cytokines and chemokines in LPS-induced neutrophil degranulation in primates has not been firmly established, although anti-TNF modestly inhibited this response in endotoxemic chimpanzees (16). In accordance with findings in humans and chimpanzees (16, 34, 35), LPS elicited an increase in the plasma levels of elastase/ $\alpha_1$ -antitrypsin complexes, a response that was attenuated significantly by rHuIL-10. Although IL-10 may influence neutrophil functions directly (36, 37), it has, to our knowledge, not been reported to affect neutrophil degranulation. The *in vivo* effect of IL-10 on neutrophil degranulation

could have been mediated indirectly, at least in part, via inhibition of TNF production (16, 38). It is unlikely that the decrease in elastase/ $\alpha_1$ -antitrypsin complexes is a mere reflection of the reduced granulocytosis in IL-10-treated baboons, since these two inflammatory changes have previously been shown to not be linked in chimpanzees with low grade endotoxemia (16, 39).

Infusion of LPS led to a transient decrease in the expression of surface receptors for TNF on circulating granulocytes, consistent with earlier findings in humans and baboons challenged with LPS and live bacteria, respectively (28, 29). This down-regulation of the availability of cellular TNF receptors to induce signal transduction may represent a mechanism to protect the host against excessive activity of TNF. It has been suggested that IL-10 may antagonize TNF effects on cells not only by inhibiting the production of this proinflammatory cytokine, but also by down-modulating its cellular receptors. Indeed, IL-10 has been found to reduce monocyte surface expression of TNF receptors (39, 40). The present study demonstrates that infusion of rHuIL-10 does not influence the decrease in granulocyte TNF binding during endotoxemia. Unfortunately, TNF binding by monocytes could not be established reliably, since monocytes transiently disappeared from the circulation after administration of LPS (data not shown).

IL-10 has been implicated as an anti-inflammatory cytokine that may be useful as an adjunct to current sepsis treatment strategies by virtue of its capacity to inhibit the production of several proinflammatory cytokines. We report in this work that infusion of rHuIL-10 into baboons, at a dose sufficient to markedly inhibit cytokine production, does not affect the coagulant and fibrinolytic responses to LPS, and only modestly attenuates neutrophil degranulation. It thus appears that in primate sublethal endotoxemia, the most potent anti-inflammatory effect of IL-10 is inhibition of proinflammatory cytokine synthesis. It should be noted, however, that inhibition of proinflammatory cytokine production and/or activity during bacterial infection may be associated with an impaired clearance of bacteria from the site of an infection and reduced survival rates, as has been demonstrated in mouse models of pneumonia (42–44). The potential role of exogenous IL-10 in the treatment of bacterial sepsis therefore remains to be established.

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