

# Anti-IL-10 Therapeutic Strategy Using the Immunomodulator AS101 in Protecting Mice from Sepsis-Induced Death: Dependence on Timing of Immunomodulating Intervention<sup>1</sup>

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The role of IL-10 in experimental sepsis is controversial. The nontoxic immunomodulator, ammonium trichloro(dioxoethylene-o,o')tellurate (AS101) has been previously shown to inhibit IL-10 expression at the transcriptional level. In this study, we show that in mice subjected to cecal ligation and puncture (CLP), treatment with AS101 12 h after, but not before, CLP significantly increased survival of septic mice. This was associated with a significant decrease in serum IL-10 and in IL-10 secretion by peritoneal macrophages 24–48 h after CLP. At that time, the ability of these cells to secrete TNF- $\alpha$  and IL-1 $\beta$  was restored in AS101-treated mice. The increased survival of AS101-treated mice was due to the inhibition of IL-10, since cotreatment with murine rIL-10 abolished the protective activity of AS101. AS101 increased class II Ag expression on peritoneal macrophages, severely depressed in control mice, while it did not affect the expression of class I Ags. This was accompanied by a significant elevation in the level of IFN- $\gamma$  secreted by splenocytes. Moreover, AS101 ameliorated bacterial clearance in the peritoneum and blood and decreased severe multiple organ damage, as indicated by clinical chemistry. Furthermore, myeloperoxidase levels in the liver and lung of AS101-treated mice, an indirect means of determining the recruitment of neutrophils, were significantly decreased. We suggest that nontoxic agents such as AS101, with the capacity to inhibit IL-10 and stimulate macrophage functions, may have clinical potential in the treatment of sepsis, provided they are administered during the phase of sepsis characterized by immune suppression. *The Journal of Immunology*, 2002, 169: 384–392.

**S**epsis syndrome is an acute systemic response to a variety of noxious insults, particularly bacterial infection. This syndrome is accompanied by immunologic processes that are activated and regulated. The development of immunomodulatory therapeutic approaches is therefore one of the principal areas emphasized in developing modern forms of treatment for sepsis.

The role of cytokines in the pathogenesis of sepsis is complex, since both deficient and excessive immune responses have been associated with this syndrome (1). In models of severe systemic infection or inflammation produced by i.v. administration of high doses of bacteria or bacterial products such as endotoxin, excessive production of proinflammatory cytokines significantly contributes to organ failure and death, as reflected by findings that neutralization of either TNF or IL-1 activity markedly reduced mortality in these systemic challenge models (2, 3). However, in experiments in which an initially localized infection was induced, including pneumonia and peritonitis, the local activity of proinflammatory cytokines appeared important for antibacterial host defense at the site of infection (4–6). Together these data suggest that proinflammatory cytokines are, on the one hand, required locally for effec-

tive antibacterial effector mechanisms, but are, in contrast, potentially toxic when secreted into the circulation. Similarly, several animal studies indicated an important role for IL-10, the prototypical antiinflammatory cytokine, in the pathogenesis of bacterial infection. The administration of neutralizing anti-IL-10 Abs increases endotoxin-induced mortality in mice, while the exogenous application of IL-10 can prevent septic shock in mice by inhibiting the synthesis of TNF- $\alpha$ , IL-1, IFN- $\gamma$ , and other proinflammatory mediators (7, 8). IL-10 gene-deficient mice demonstrated multiple organ failure and an enhanced mortality after endotoxin injection or during *Escherichia coli* peritonitis (9, 10), despite accelerated bacterial clearance (10). Nevertheless, during murine pneumonia, treatment with anti-IL-10 Abs inhibited bacterial outgrowth in lungs and improved survival (11, 12). However, during septic peritonitis induced by cecal ligation and puncture (CLP),<sup>3</sup> neutralizing IL-10 was associated with an increased mortality (13, 14). In these experimental systems in which IL-10 or anti-IL-10 Abs were administered, the protective or detrimental effects were induced only if the treatments were applied shortly before or immediately after LPS, CLP, or bacterial challenge. On the basis of these observations, a strategy for inhibiting the synthesis of proinflammatory cytokines was developed in an attempt to treat septic patients. At present, it appears that this approach has not met with the expected successes. Sepsis-induced mortality was not significantly improved in any of the phase II/III studies that have been completed (15, 16).

Recent studies demonstrated that systemic levels of both the proinflammatory cytokines and IL-10 are correlated directly with

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<sup>3</sup> Abbreviations used in this paper: CLP, cecal ligation and puncture; ALT, alanine transaminase; AS101, ammonium trichloro(dioxoethylene-o,o')tellurate; AST, aspartate transaminase; BUN, blood urea nitrogen; MPO, myeloperoxidase; PEC, peritoneal exudate cell; rmIL-10, murine rIL-10.

severity of illness (17). Several investigations on human infections indicated that elevated IL-10 predicts a poor prognosis in the presence of sepsis (18, 19). Recent data of Song et al. (20) suggest that at later time points after the onset of infection, IL-10 may have a net detrimental effect on host antimicrobial clearance mechanisms. In a cecal ligation model, anti-IL-10 Ab delivered at the time of CLP was ineffective in preventing mortality. However, when administered 12 h after CLP, this treatment was protective (20). These data indicate the critical importance of timing of manipulations that affect IL-10 activity in sepsis.

A biphasic immunologic response in sepsis has been recently demonstrated: an initial hyperinflammatory phase, followed by a hypoinflammatory one. The latter stage is associated with an immunodeficient stage, which is characterized by monocyte deactivation (21). In this state, monocytic TNF secretion capacity as well as HLA-DR Ag expression and Ag-presenting capacity are all severely depressed, while, at least temporarily, the capacity to produce IL-10 is preserved. This switch to predominant release of antiinflammatory mediators is associated with a high risk of fatal outcome.

The nontoxic immunomodulator ammonium trichloro(dioxoethylene-*o,o'*)tellurate (AS101) first developed by us has been shown to have beneficial effects in diverse preclinical and clinical studies. Most of its activities have been primarily attributed to the direct inhibition of the antiinflammatory cytokine IL-10, followed by the simultaneous increase of specific cytokines. These include IL-1 $\alpha$ , TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-12, and GM-CSF (22–24). These immunomodulatory properties were found to be crucial for the clinical activities of AS101, demonstrating the protective effects of AS101 in parasite- and viral-infected mice models (25), in autoimmune diseases (26), and in a variety of tumor models in which AS101 had a clear antitumoral effect (27–29). AS101 has also been shown to have protective properties against lethal and sublethal effects of irradiation and chemotherapy (24, 27–31). These activities were also due to the increased production of proinflammatory cytokines and were associated with only minimal toxicity, thus enabling the use of the compound as an adjuvant to chemotherapy in phase II studies (32).

Given the ability of AS101 to decrease monocytic IL-10 production (23) and thereby stimulate the production of monocyte-activating cytokines, increase monocyte B-7 expression, followed by amelioration of Ag-presenting capacity (33), we examined its potential role in protecting mice from the lethal effects of sepsis. For this purpose, we used the model of CLP, which resembles the clinical syndrome of septic peritonitis, with spillage of polymicrobial flora into the peritoneal cavity, leading to systemic bacteremia and endotoxemia (34).

## Materials and Methods

### Mice

Male and female BALB/c mice were bred at Bar Ilan University from strains obtained from The Jackson Laboratory (Bar Harbor, ME). Two-month-old male mice were used for experiments.

Animal experiments were performed in accordance with approved institutional protocols and approved by the Institutional Animal Care and Use Committee.

### AS101

AS101 was supplied by M. Albeck from the chemistry department in Bar Ilan University, in a solution of PBS, pH 7.4, and maintained at 4°C. Before use, AS101 was diluted in PBS, and the appropriate concentrations in 0.2 ml vol were administered to mice by i.p. injections.

### Cecal ligation and puncture

Animals were anesthetized using sodium pentobarbital (40 mg/kg) administered i.p. CLP was performed by making a midline incision ~2.5 cm in

length to expose the cecum. A 3-0 silk ligature was placed at the base of the cecum without causing bowel obstruction. The cecum was then punctured twice with a 21-gauge needle. The cecum was gently squeezed to assure patency of the two holes as well as to express fecal material into the peritoneal cavity. In the control animals (sham), the cecum was located, but neither ligated nor punctured. The abdominal incision was then closed in two layers using 3-0 Prolene and 4-0 Dexon, respectively. All animals were fluid resuscitated with 1 ml normal saline by s.c. injection.

### IL-10 and anti-IL-10 Abs

Murine rIL-10 (rmIL-10; BD PharMingen, San Diego, CA) was injected s.c. at 5  $\mu$ g/injection once daily starting 12 h following CLP. Anti-IL-10 (JESS 2A5; BD PharMingen)-neutralizing Abs were injected i.p. at 250  $\mu$ g/mouse at 12 h post-CLP. Rat anti-mouse IgG1 served as control Abs.

### Treatment regimen

For survival experiments, PBS or AS101 were injected i.p. daily at various concentrations starting either 24 h before or at 7 or 12 h following CLP until the end of experiment. For serum cytokine evaluation, AS101 at 10  $\mu$ g/mouse or PBS was injected once 12 h following CLP.

### Cell purification

Spleens were removed and placed in PBS. Spleen cells were passed through stainless steel mesh nets, treated with hypotonic solution to lyse erythrocytes, and washed three times. Cell counts were obtained using a hemocytometer. Viability, as assessed by trypan blue exclusion method, was always found to be >95%.

Adherent splenocytes were obtained following adherence of cells for 1 h in plastic dishes in RPMI 1640 culture medium supplemented with 10% FCS.

Peritoneal macrophages were obtained by peritoneal washing with PBS and adherence of cells for 1 h.

### Induction of cytokine secretion *in vitro*

Splenocytes ( $5 \times 10^6$ /ml) were suspended in enriched RPMI 1640 culture medium supplemented with 10% FCS and seeded in 24-well culture plates in the presence of 2.5  $\mu$ g/ml Con A (for IFN- $\gamma$ ). Adherent splenocytes or peritoneal macrophages were incubated with 1  $\mu$ g/ml LPS (for IL-1 $\beta$ , IL-10, and TNF- $\alpha$ ). The cultures were incubated at 37°C for 24 h. Supernatants were collected and evaluated for cytokine content.

### Quantitation of cytokine levels

The R&D Systems (Minneapolis, MN) IL-1 $\beta$ , TNF- $\alpha$ , IL-10, and IFN- $\gamma$  ELISA kits were used for the quantitative measurement of these cytokines either in mice sera or in spleen and peritoneal exudate cell (PEC) supernatants.

### Immunofluorescence staining

Peritoneal cells were incubated with FITC and PE directly labeled Abs against MHC molecules on ice for 60 min and then washed. The mAbs used included FITC-labeled mouse IgG2a anti-mouse H-2D<sup>d</sup>, rat IgG2a anti-mouse I-A<sup>d</sup>/I-E<sup>d</sup>, rat IgG2a anti-mouse I-A<sup>d</sup>, and PE-labeled rat IgG1 anti-mouse CD14 (BD PharMingen); purified rat IgG2 Abs (Genzyme, Cambridge, MA) were used as isotype controls. For each sample,  $10^4$  cells were analyzed on a FACScan (BD Biosciences, Mountain View, CA) evaluating the percentage of double-stained CD14 and class I- or class II-positive cells.

### Determination of bacterial CFU in the CLP model

Mice were killed 24 h following CLP. The peritoneal cavities were washed with 2 ml sterile PBS, and the lavage fluids were harvested under sterile conditions. A total of 10  $\mu$ l peritoneal lavage fluids and peripheral blood from each mouse was placed on ice and serially diluted with sterile PBS. A total of 10  $\mu$ l of each dilution was aseptically plated on tryptose soy agar blood agar plates (Difco, Detroit, MI) and incubated overnight at 37°C, after which the number of colonies was counted. Data were expressed as CFU per microliter.

### Clinical chemistry

Serum levels of aspartate transaminase (AST), alanine transaminase (ALT), amylase, blood urea nitrogen (BUN), and creatinine were measured using standardized techniques.

### Measurement of myeloperoxidase

Myeloperoxidase (MPO) in tissue extracts was measured by ELISA kit (Calbiochem-Novabiochem, La Jolla, CA), according to the manufacturer's instructions.

### Preparation of organ extracts

A total of 0.1 g excised organs was placed in 1 ml PBS containing complete protease inhibitors (Calbiochem) and homogenized. The homogenates were subsequently freeze-thawed twice. The homogenates were centrifuged at  $6000 \times g$  for 20 min at  $4^{\circ}\text{C}$ , and the cleared supernatants were used for measurement of MPO. The levels of MPO in organ extracts were expressed as ng/mg protein.

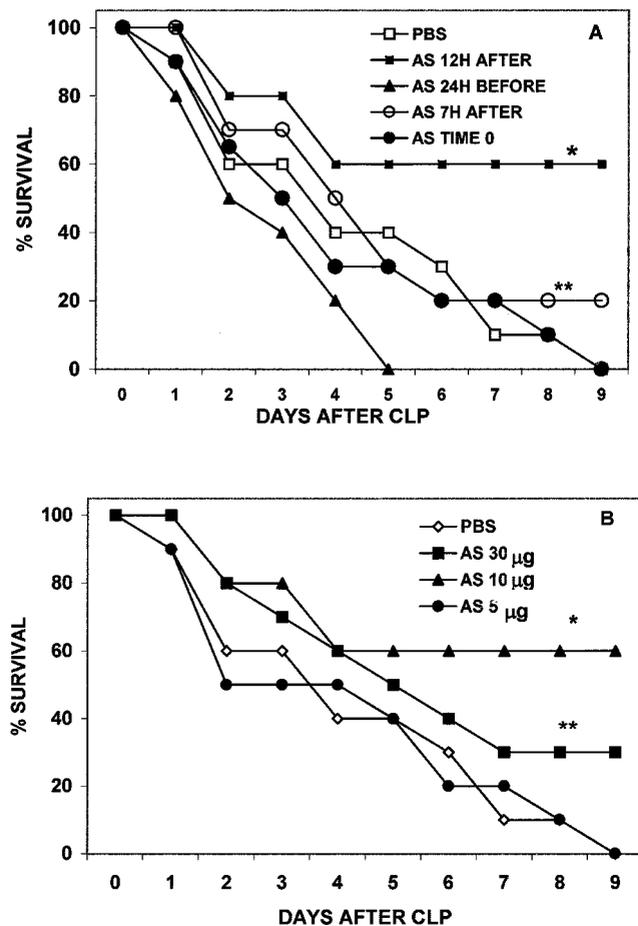
### Statistical analysis

Survival curves were statistically analyzed by comparing the cumulative percentage of survival using the Gehan-Wilcoxon test. For comparisons of means of the various groups, the pairwise *t* test was used.

## Results

### Enhanced survival of AS101-treated mice following induction of sepsis

We first determined whether AS101 may rescue mice from sepsis-induced death. Peritonitis induced by CLP with a 21-gauge needle results in 100% lethality at day 9 (Fig. 1). Treatment with AS101 had a salutary effect only if injected after sepsis induction (Fig.



**FIGURE 1.** Enhanced survival of AS101-treated mice following induction of sepsis. AS101 at  $10 \mu\text{g}/\text{injection}/\text{mouse}$  or PBS were injected to mice at various time points before and after CLP (A); AS101 at various doses or PBS were injected 12 h after CLP (B) and thereafter once daily until the end of experiments. Survival was monitored for 9 days. Results shown represent a total of 30 mice/group. \*,  $p < 0.01$  increase vs PBS; \*\*,  $p < 0.05$  increase vs PBS.

1A). The optimal dose of AS101 was  $10 \mu\text{g}/\text{mouse}$ , injected 12 h following CLP and thereafter every day until the end of experiments (Fig. 1). Under these conditions, 60% of treated mice survived ( $p < 0.01$ ; increase vs CLP + PBS). This concentration of AS101 was previously found by us to be optimal in various preclinical studies in which AS101 had beneficial effects. A concentration of  $30 \mu\text{g}/\text{mouse}$  AS101 was less effective, although this dose also significantly increased the rate of survival ( $p < 0.05$ ). A lower concentration of  $5 \mu\text{g}/\text{mouse}$  was ineffective (Fig. 1B). Injection of AS101 7 h post-CLP was also effective, although to a lesser extent (20%;  $p < 0.05$ ). No significant difference was observed when AS101 was injected at the time of injury (time zero). Notably, treatment with AS101 24 h before CLP decreased survival, compared with PBS-treated mice. Thus, it appears that AS101 could protect mice from sepsis-induced death only if injected after, but not before, or at the time of sepsis induction.

### Plasma levels of cytokines

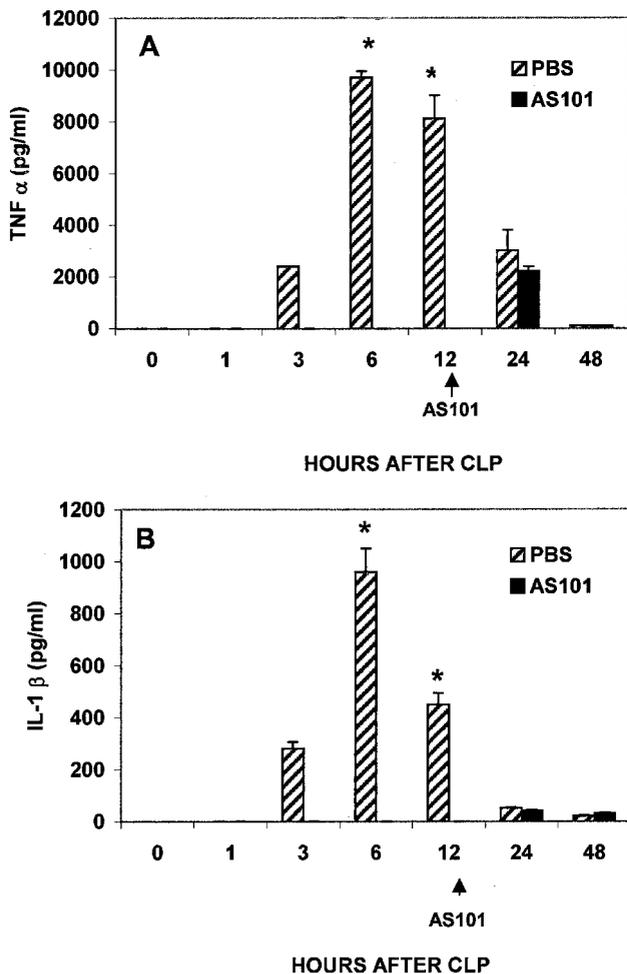
Organ injury observed in sepsis is due to the explosive release of cytokines into the plasma. We therefore sought to determine the plasma levels of cytokines following CLP and their response to AS101 treatment. Two of the classic cytokines produced in the initial period of an inflammatory insult are  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$ , which are followed by overproduction of  $\text{IL-10}$ . It can be seen that  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  were undetectable in the serum at 1 h post-CLP (Fig. 2). Maximal levels of these cytokines were found at 6 and 12 h. The inflammatory response was not persistent and it gradually declined, until at 24–48 h after CLP it was only minimal. Evaluation of plasma levels of proinflammatory cytokines in the AS101-treated mice revealed no significant differences in plasma  $\text{TNF-}\alpha$  and  $\text{IL-1}\beta$  levels between AS101- and PBS-treated mice (Fig. 2).

Since every inflammatory response stimulates not only the synthesis of proinflammatory mediators, but also, with a slight delay, the production and secretion of antiinflammatory cytokines such as  $\text{IL-10}$ , we chose to evaluate the concentrations of this cytokine in the plasma of sepsis-induced mice. Moreover, we determined whether AS101 can inhibit the production of this cytokine.

Quantitation of  $\text{IL-10}$  plasma levels revealed a gradual increase in plasma content of this antiinflammatory cytokine that peaked at 24 h following CLP. These levels remained elevated until 96 h after CLP (Fig. 3), although they gradually decreased (data not shown). AS101 injected 12 h after CLP promptly and significantly ( $p < 0.01$ ) inhibited  $\text{IL-10}$  levels at both 24 and 48 h (Fig. 3). At 72–96 h,  $\text{IL-10}$  levels in the AS101 group were negligible (data not shown).

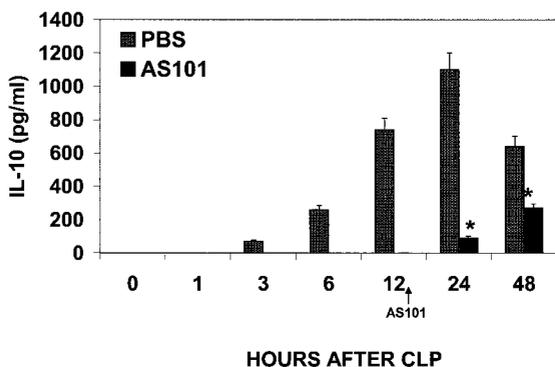
### Role of $\text{IL-10}$ inhibition in the increased survival of AS101-treated mice

The primary effect of AS101 has been shown previously to involve the direct inhibition of  $\text{IL-10}$  in human and mouse macrophages, at the transcription level (23). Moreover, most of the beneficial activities of AS101 observed in preclinical studies have been primarily attributed to the direct inhibition of  $\text{IL-10}$  (25–27). We therefore examined the role of  $\text{IL-10}$  inhibition in the increased survival of AS101-treated mice subjected to CLP. To this end, we injected AS101-treated mice with  $\text{rmIL-10}$  ( $5 \mu\text{g}/\text{injection}$ ) once daily starting 12 h after the induction of CLP. Fig. 4 shows that treatment with  $\text{rmIL-10}$  significantly reduced the salutary effect of AS101. The percentage of survival decreased from 60 to 10% ( $p < 0.01$ ). Treatment of PBS-treated mice with  $\text{rmIL-10}$  starting 12 h following CLP resulted in enhanced mortality, 100% of mice

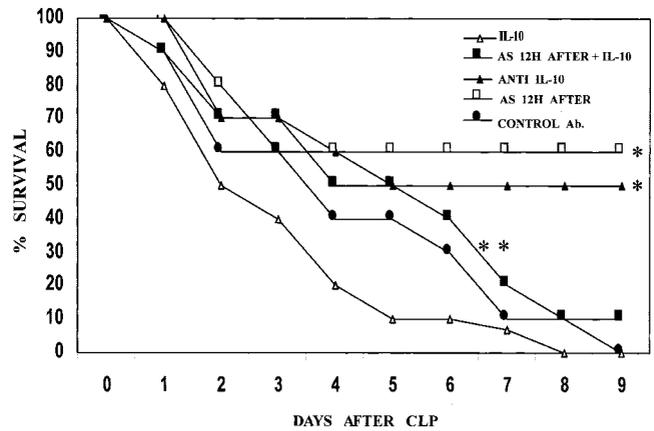


**FIGURE 2.** Kinetics of serum TNF- $\alpha$  (A) and IL-1 $\beta$  (B) levels after CLP. AS101 at 10  $\mu$ g or PBS was injected into mice 12 h after CLP. Serum TNF- $\alpha$  and IL-1 $\beta$  levels were quantitated at different time points after CLP. The results represent means  $\pm$  SE of three experiments including 18 mice/group. \*,  $p < 0.01$  increase vs time 0 and 3 h.

dying 7 days following CLP. Alternatively, injection of neutralizing anti-IL-10 Abs 12 h after CLP resulted in 50% survival ( $p < 0.01$  vs control Ab).



**FIGURE 3.** Kinetics of serum IL-10 levels after CLP. AS101 at 10  $\mu$ g/injection or PBS was injected into mice 12 h after CLP. Serum IL-10 levels were quantitated at different time points after CLP. The results represent means  $\pm$  SE of three experiments including 18 mice/group. \*,  $p < 0.01$  decrease vs PBS.



**FIGURE 4.** AS101 enhances survival of sepsis-induced mice by inhibition of IL-10. AS101 at 10  $\mu$ g/injection/mouse or PBS was injected into mice 12 h after CLP with or without 5  $\mu$ g rmIL-10 and thereafter once daily until the end of experiments. One group was injected with PBS and neutralizing anti-IL-10 Abs. Rat anti-mouse IgG1 served as control Abs. Survival was monitored for 9 days. Results represent a total of 10 mice/group. \*,  $p < 0.01$  increase vs control Ab; \*\*,  $p < 0.01$  decrease vs AS101.

*Prevention of macrophage deactivation*

Recently, it has been suggested that septic patients demonstrate a state in which monocytic TNF secretion capacity as well as HLA-DR Ag expression are severely depressed, while the capacity to produce IL-10 is at least temporarily preserved (35). This predominantly antiinflammatory state, in contrast to the assumption of persistent hyperinflammation, was associated with a high risk of death from persistent infection and multiple organ failure.

Table I shows that peritoneal macrophages obtained from CLP-induced mice gradually lose the ability to secrete TNF- $\alpha$  and IL-1 $\beta$ . This loss of function is significantly reflected at 24–48 h following CLP. At 48 h, peritoneal macrophages completely lose their ability to secrete those proinflammatory cytokines (Table I). This capacity is significantly restored ( $p < 0.01$ ) in AS101-treated mice at all time points following AS101 injections. Similar results were obtained when TNF- $\alpha$  and IL-1 $\beta$  in supernatants from adherent splenocytes were quantitated (data not shown). In contrast to the loss of macrophage ability to secrete TNF- $\alpha$  and IL-1 $\beta$ , the capacity of PEC cells to secrete IL-10 at 24–48 h was preserved. Nevertheless, PEC cells from AS101-treated mice secreted significantly decreased amounts of IL-10 at 24–48 h post-CLP ( $p < 0.01$ ) (Table I).

Table I. Kinetics of cytokine secretion from PEC cells after CLP<sup>a</sup>

Hours after CLP	TNF- $\alpha$ (pg/ml)	IL-1 $\beta$ (pg/ml)	IL-10 (pg/ml)
PBS 0	4368 $\pm$ 396	6166 $\pm$ 512	236 $\pm$ 26
PBS 12	4653 $\pm$ 502	6645 $\pm$ 624	221 $\pm$ 26
PBS 24	1523 <sup>b</sup> $\pm$ 142	1546 <sup>b</sup> $\pm$ 148	187 <sup>c</sup> $\pm$ 20
AS101 24	3266 <sup>d</sup> $\pm$ 219	4286 <sup>d</sup> $\pm$ 386	121 <sup>e</sup> $\pm$ 10
PBS 48	46 <sup>b</sup> $\pm$ 5	86 <sup>b</sup> $\pm$ 10	182 <sup>c</sup> $\pm$ 16
AS101 48	1984 <sup>d</sup> $\pm$ 160	2018 <sup>d</sup> $\pm$ 194	126 <sup>e</sup> $\pm$ 13

<sup>a</sup> AS101 at 10  $\mu$ g/injection/mouse or PBS were injected into mice 12 h after CLP. At various time points, PEC cells were harvested and cultured with LPS. Supernatants were quantitated for TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 content. The results represent means  $\pm$  SE of three experiments including 18 mice/group.

<sup>b</sup>  $p < 0.01$ .

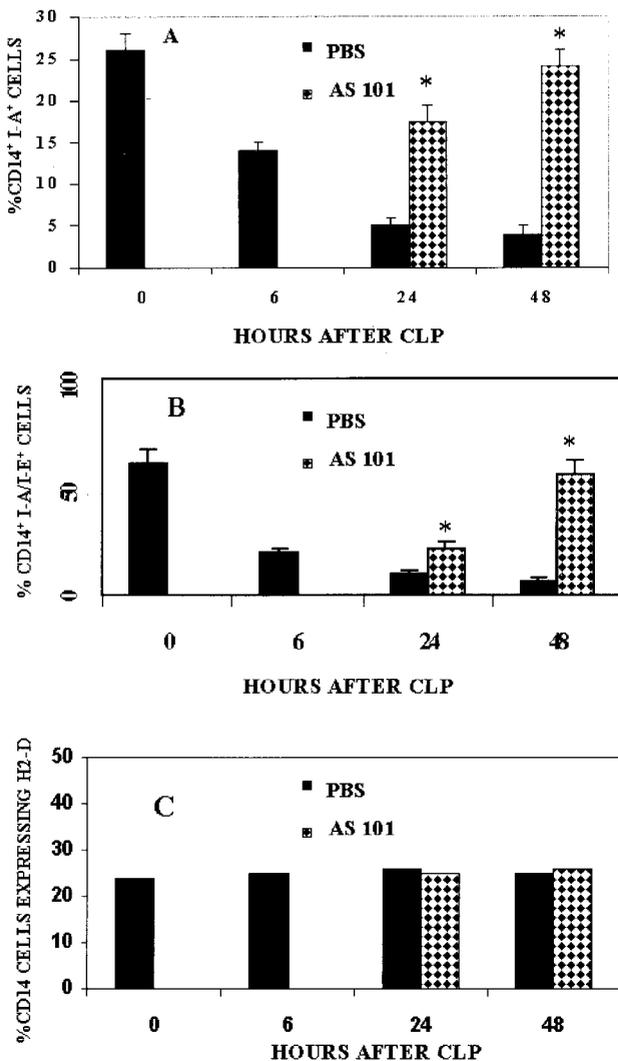
<sup>c</sup>  $p < 0.05$ , decrease vs time zero.

<sup>d</sup>  $p < 0.01$ , increase vs PBS.

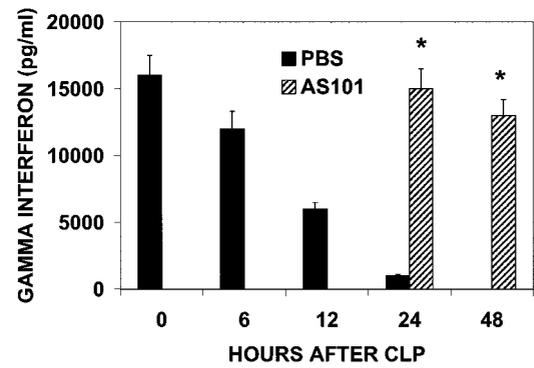
<sup>e</sup>  $p < 0.01$ , decrease vs PBS.

To evaluate the depressed class II monocyte Ag expression, the proportion of both CD14<sup>+</sup>I-A<sup>+</sup> and CD14<sup>+</sup>I-A/I-E<sup>+</sup> cells was determined at different time points following CLP. As can be seen in Fig. 5A, the proportion of CD14<sup>+</sup> peritoneal cells expressing I-A Ag gradually decreased following CLP from 26.2 ± 2 to 4 ± 2% at 48 h post-CLP. Administration of AS101 12 h after CLP significantly restored the percentage of I-A<sup>+</sup>-expressing CD14<sup>+</sup> cells. This was reflected by an increase amounting to 17 ± 1% CD14<sup>+</sup>I-A<sup>+</sup> cells at 24 h post-CLP and 23 ± 2% CD14<sup>+</sup>I-A<sup>+</sup> cells at 48 h after the insult. Similar results were obtained when the proportion of CD14<sup>+</sup> cells expressing both I-A and I-E was evaluated. While the percentage of these cells decreased from 61 ± 5% to 6 ± 1% at 48 h post-CLP, they amounted at that time point to 55 ± 6% in mice treated with AS101 (Fig. 5B).

No differences in the expression of class I Ags on CD14<sup>+</sup> cells were observed at various time points after CLP induction (Fig. 5C). Treatment with AS101 did not alter H-2D Ag expression on CD14<sup>+</sup> cells.



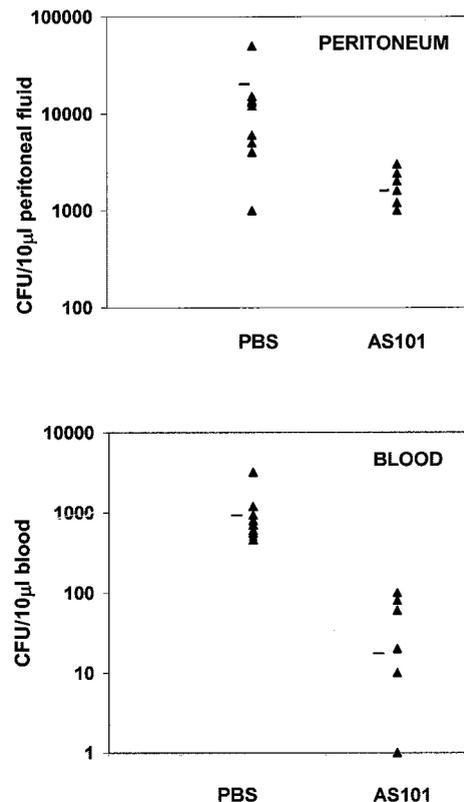
**FIGURE 5.** Treatment with AS101 increases the expression of class II and not class I Ag expression in PEC cells from mice after CLP. AS101 at 10  $\mu\text{g}/\text{injection}/\text{mouse}$  or PBS was injected in mice 12 h after CLP. The percentage of CD14<sup>+</sup> cells expressing class II Ags (I-A, A; or I-A/I-E, B) or class I Ags (H-2D, C) was determined at various time points after CLP. The results represent means  $\pm$  SE of three experiments including 18 mice/group. \*,  $p < 0.01$  increase vs PBS.



**FIGURE 6.** Treatment with AS101 increases the ability of splenocytes from mice after CLP to secrete IFN- $\gamma$ . AS101 at 10  $\mu\text{g}/\text{injection}/\text{mouse}$  or PBS was injected in mice 12 h after CLP. At various time points, splenocytes were harvested and cultured with Con A. Supernatants were quantitated for IFN- $\gamma$  content. The results represent means  $\pm$  SE of three experiments including 18 mice/group. \*,  $p < 0.01$  increase vs PBS.

#### Effect of AS101 on IFN- $\gamma$ production by spleen cells

The increased expression of class II Ags on CD14<sup>+</sup> cells from AS101-treated mice led us to examine the level of IFN- $\gamma$  production and secretion in these mice due to the potent ability of this cytokine to induce class II Ag expression on CD14<sup>+</sup> cells. As can be seen in Fig. 6, Con A-stimulated splenocytes significantly decrease their ability to secrete IFN- $\gamma$  at 24–48 h following CLP. A considerable and significant increase ( $p < 0.01$ ) in the levels of



**FIGURE 7.** AS101 increases the clearance of bacteria after CLP. AS101 at 10  $\mu\text{g}$  or PBS was injected in mice 12 h after CLP. At 24 h after CLP, mice were killed and the peritoneal fluids and sera were harvested. A total of 10  $\mu\text{l}$  peritoneal fluids and sera was serially diluted and plated on tryptose soy agar blood agar plates. The results represent 10 AS101-treated and 9 PBS-treated mice. Line represents mean CFU count.

IFN- $\gamma$  were obtained at all time points following AS101 injections when splenocytes from AS101-treated mice were used (Fig. 6).

#### Augmentation of bacterial clearance in AS101-treated mice

IL-10 has been shown recently to impair bacterial clearance from the peritoneal cavity and to facilitate dissemination of bacteria to distant organs (10). The significant decrease in serum IL-10 levels in CLP-induced mice treated with AS101, as well as the increased survival of these mice led us to evaluate the role of AS101 in the bacterial clearance of mice subjected to CLP. To this end, we examined the bacterial load in the peritoneal fluids and blood of AS101- and PBS-treated mice. No bacteria were recovered from the peritoneum or blood of mice that were not subjected to CLP (data not shown). The peritoneal fluids at 24 h after CLP in PBS-injected mice contained a significant number of bacteria in the peritoneum (Fig. 7). At this time point, the bacterial load in the peritoneum of AS101-treated mice was significantly decreased ( $p < 0.01$ ). At 72 h, AS101-treated mice who shivered and had bristled hair, clinical symptoms of active sepsis, had increased peritoneal bacterial load (13,000–40,000 CFU/10  $\mu$ l). These amounted to 40%. In the remaining mice, the peritoneal bacterial load did not change significantly as compared with that at 24 h after CLP (data not shown). Likewise, the bacterial load recovered from peripheral blood of AS101-treated mice was significantly lower than that in control PBS-treated mice ( $p < 0.05$ ) (Fig. 7).

#### CLP-induced organ injury is ameliorated in AS101-treated mice

Sepsis frequently causes multiple organ failure, a condition that leads to death (36). To understand the mechanism whereby AS101-treated mice were resistant to CLP, experiments were conducted to assess the organ damage induced by CLP and its response to AS101. For this purpose, we measured biochemical markers of liver damage (AST, ALT), pancreas damage (amylase), and renal failure (creatinine, BUN) at 24 h following CLP. Fig. 8 shows that control PBS-treated mice demonstrated biochemical evidence of more severe multiple organ damage than AS101-treated mice, as reflected by higher ALT, AST, amylase, creati-

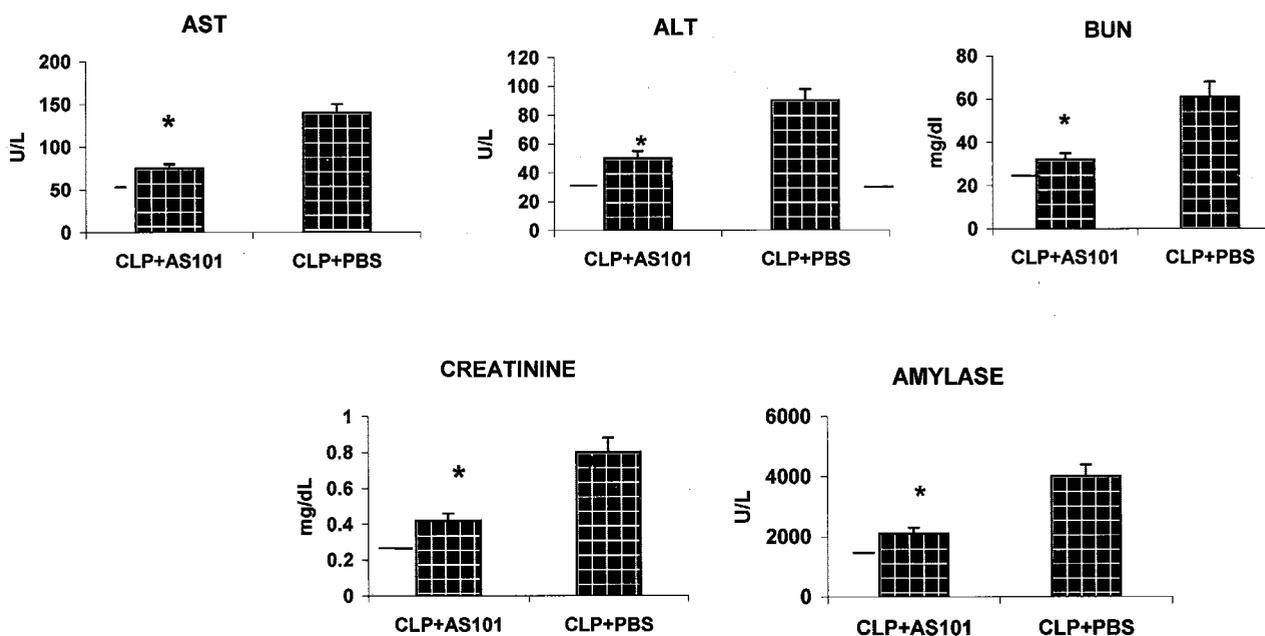
nine, and BUN concentrations (all  $p < 0.05$ ). The levels in AS101-treated mice were comparable with those in mice not subjected to CLP, suggesting that organ injury was avoided in AS101-treated mice. Nevertheless, in 40% of mice in the AS101 group, in which the bacterial load was elevated at 72 h, the levels of AST and ALT increased to 100–120 U/L and 65–80 U/L, respectively, at that time (data not shown), suggesting that in these mice organ damage was only delayed and not entirely avoided.

#### Evaluation of MPO levels in infected organs

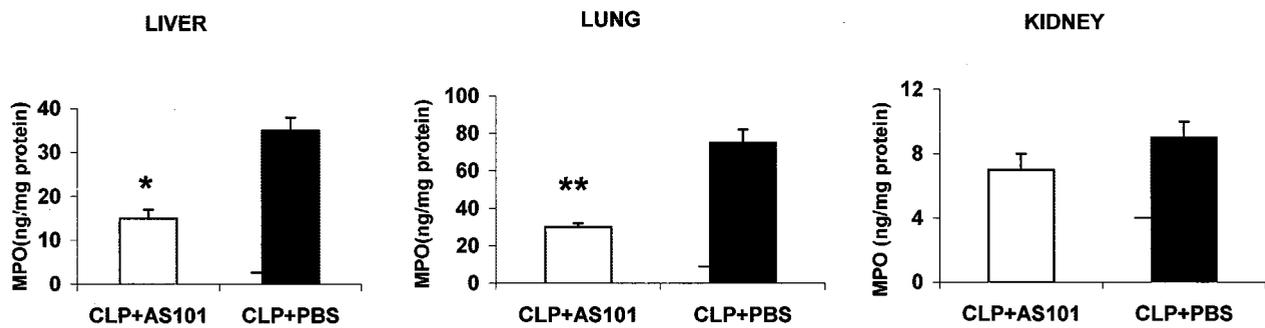
MPO level is an indirect means of determining the recruitment of neutrophils to the infected organs. As can be seen in Fig. 9, MPO levels in the liver and lung, 24 h after CLP, were elevated in PBS-treated mice as compared with AS101-treated mice. This increase was significant in both organs ( $p < 0.01$ ;  $p < 0.05$ ). The level of MPO in both organs in control PBS-treated mice was >10-fold increased as compared with normal untreated mice. MPO levels in the kidney of PBS-treated mice were only slightly elevated as compared with untreated mice (2-fold). AS101 also decreased the level of MPO in the kidney, but not significantly (Fig. 9).

## Discussion

In the present study, we present evidence demonstrating the ability of AS101 to protect from sepsis-induced death. This protection was observed because AS101 was injected after, and not during the initial hyperinflammatory state induced by CLP. Moreover, we show that the increased survival of AS101-treated mice subjected to CLP is due, mostly, to the prompt inhibition of the antiinflammatory cytokine IL-10. As shown in this study, the initial period following the inflammatory insult was characterized by a hyperinflammatory response, as reflected by high serum levels of TNF- $\alpha$  and IL-1 $\beta$ , 6 h after CLP. Nevertheless, the proinflammatory response was not consistent and it gradually decreased over 24–48 h. This was followed by an immune suppressed state characterized by overproduction of IL-10. Treatment with AS101 12 h following CLP did not result in important changes in serum TNF- $\alpha$  or IL-1 $\beta$



**FIGURE 8.** Treatment with AS101 ameliorates organ injury induced by CLP. AS101 at 10  $\mu$ g/injection/mouse or PBS was injected into mice 12 h after CLP. At 24 h after CLP, mice were killed, and the amounts of AST, ALT, BUN, creatinine, and amylase in sera were measured. The results represent means  $\pm$  SE of 10 mice/group. Line represents mean data obtained from sham-operated mice ( $n = 4$ ). \*:  $p < 0.05$  decrease vs PBS.



**FIGURE 9.** Treatment with AS101 decreases MPO levels in organs after CLP. Twenty-four hours after CLP induction, mice were killed and the liver, lung, and kidney were resected. The organs were extracted, and the amounts of MPO were measured. The results represent means  $\pm$  SE of 10 mice/group. Line represents mean data obtained from sham-operated mice ( $n = 4$ ). \*,  $p < 0.01$ ; \*\*,  $p < 0.05$  increase vs AS101.

levels. However, the antiinflammatory response was profoundly decreased in AS101-treated mice, as reflected by profound suppression of serum IL-10 levels. The switch to an antiinflammatory state in late sepsis was also reflected by the relatively preserved ability of peritoneal macrophages from untreated CLP-induced mice to secrete IL-10, while their ability to secrete TNF- $\alpha$  and IL-1 $\beta$  was totally lost. Treatment of mice with AS101 decreased IL-10 secretion by macrophages and restored their ability to produce and secrete proinflammatory cytokines.

AS101 has previously been shown to directly inhibit IL-10 production by both mice and human macrophages, at the transcriptional level (23). This property accounted for most of the beneficial effects of AS101 in preclinical studies associated with IL-10 overexpression.

In some of those models, such as systemic lupus erythematosus, the beneficial effect of AS101 was directly due to the inhibition of the high levels of IL-10, resulting in decreased production of autoantibodies and delayed development of lupus-like autoimmunity (26). In other models such as tumor-bearing (27) or parasite- and viral-infected mice (25), the salutary effect of AS101 was due to its ability to switch predominant type II responses to type I responses, thus augmenting the cell-mediated immune response to cancer, viral, and parasitic infections. Moreover, inhibition by AS101 of IL-10 production by macrophages results in up-regulation of the costimulatory molecule B7-1, and increased Ag-presenting ability of these cells (33). Most importantly, AS101 decreases IL-10 production only in models associated with overproduction of this cytokine, resulting in nearly normal IL-10 levels, followed by increased, yet normal, levels of proinflammatory and Th1 cytokines. This probably accounts for AS101's lack of toxicity, enabling the use of the compound as an adjuvant to chemotherapy in phase II studies (32).

The cytokine network plays a pivotal role in the orchestration of inflammatory responses to bacterial infection. The balance between pro- and anti-inflammatory cytokines critically influences the function of immunocompetent cells and the resistance against infection. While proinflammatory cytokines such as IL-12 and TNF- $\alpha$  have been shown to enhance bacterial clearance in several bacterial infection models including septic peritonitis (37, 38), antiinflammatory cytokines such as IL-10 and IL-13 have been shown to play a protective role in this model of sepsis, by down-regulating the excessive inflammatory response (13, 39), while IL-10 was not successful as a therapeutic strategy in a cecal ligation model of intraabdominal sepsis (40). The development of an adequate immune response to a bacterial challenge relies on the complex interplay between the innate and the specific immune system, reflected by intact monocyte/T cell/neutrophil interactions. Monocyte and macrophage functions include the recognition, up-

take, and killing of invading organisms, and Ag presentation to initiate an immune response. The early stages of such a response may be mediated by secretion of proinflammatory mediators such as TNF, IL-1, IL-12, and a direct Ag-presenting capacity reflected by the expression of HLA class II and CD80/86. These monocyte functions are up-regulated by immunostimulatory cytokines such as IFN- $\gamma$  and suppressed by IL-10. This functional monocytic depression is closely associated with a loss of type I T cell response, which can be characterized by an increase in Th cell production of IL-4 and IL-10 and a decreased IFN- $\gamma$ -producing Th cell phenotype. Indeed, Song et al. (20) have recently shown that IL-10 is involved in the immune dysfunction in splenic lymphocytes from septic animals, as reflected by their decreased ability to release IL-2 and IFN- $\gamma$ . Furthermore, the role of IL-10 in depressing monocyte functions important in sepsis was recently demonstrated (41). These data are in line with our results showing that treatment with AS101, through inhibition of IL-10, restores monocyte functions, as reflected by preservation of their ability to secrete TNF- $\alpha$  and IL-1 $\beta$  and by the prompt increase of their class II Ag expression. Moreover, treatment with AS101 restores the ability of lymphocytes to secrete IFN- $\gamma$ , a known up-regulator of monocyte functions. Taken together, it appears that sepsis is characterized by a biphasic immunologic response: an initial systemic proinflammatory mediator response that is the motor for developing the anti-inflammatory response causing immune cell dysfunction. Therefore, a number of investigators have suggested that inhibition of this proinflammatory response may provide a survival benefit. However, this often does not take into consideration that the degree of proinflammation produced in response to a polymicrobial insult, such as CLP, is typically modest compared with that seen in lethal endotoxemia. A number of studies have documented that the levels of proinflammatory mediators such as TNF and IL-1 produced in CLP may actually be required to initiate a protective immune response and that anti-TNF Ab therapy fails to prevent lethality after CLP (42, 43).

Our results, demonstrating the beneficial effects of AS101 in CLP-induced sepsis, illustrate not only the significance of the immunosuppressive mediator IL-10 in the development of sepsis, but also the importance of correct timing of the potential intervention. Most studies demonstrating the beneficial effect of IL-10 in sepsis either injected IL-10 before or immediately after the induction of sepsis, or used IL-10 knockout mice to demonstrate the role of endogenous IL-10 in sepsis. In the first case, IL-10 was injected in mice in which an excessive hyperinflammatory state dominated. Therefore, this treatment balanced hyperinflammation. However, in IL-10 KO mice, some degree of hyperinflammation exists before the induction of sepsis. In this case, after the insult, hyperinflammation is more excessive than in normal mice subjected to

sepsis. Alternatively, we show in the present study that the response of sepsis-induced mice to treatment with an agent that decreases IL-10 levels depends on the time of therapeutic intervention. When AS101 was injected before CLP, a significant decrease in the percentage of survival was obtained. Only when AS101 was injected at the time in which the anti-inflammatory response predominated was the resulting decrease in IL-10 beneficial to the septic mice. Treatment with AS101 at that stage of sepsis eventually restored the balance between the pro- and anti-inflammatory arms of the cytokine network, ameliorating the bacterial clearance on the one hand and diminishing organ damage in contrast. AS101 protects 60% of mice from sepsis-induced death. In the remaining 40%, the level of bacterial load and that of organ damage is only delayed and is increased later on until the mice die. These data are in line with studies (20) that showed that treatment with anti-IL-10 Abs during the immune suppressive state (12 h after CLP) markedly increased survival. Our data are also consistent with studies (44) showing that inhibition of mitogen-activated protein kinase p38 *in vivo* results in suppression of IL-10 and in increased survival, provided the inhibitor is injected 12 h post-CLP.

Our results may explain why clinical trials with septic patients aimed at down-regulating proinflammatory cytokines using Abs against TNF- $\alpha$ , antagonists of IL-1, or platelet-derived growth factor have proved to be uniformly disappointing. Not only have these agents been found to be ineffective, but they may also increase mortality. It appears that these trials all addressed the proinflammatory phase of sepsis when there was no evidence that the proinflammatory phase was dominant.

Taken together, clinical and experimental evidence suggests that septic patients are not necessarily in an overactivated immunological state. Therefore, any immunomodulatory therapy should be based on measurable immune functions to determine which patients may benefit from such therapies. We suggest that the use of the nontoxic compound AS101, currently undergoing phase II clinical trials, has potential in the clinical treatment of sepsis if given during the immune suppressive state of septic patients.

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