Sepsis is a major cause of morbidity and mortality in our hospitals (2). A severe and uncontrolled systemic inflammatory response triggered by an invading microbe may lead to evolving multiorgan dysfunction (7). Occasionally, despite appropriate treatment and support of the septic patient, death may still ensue if irreversible damage occurs to vital organs. The onset of this irreversible shock is not easily defined clinically, nor are the mechanisms regulating this transition known. Cytokines may be important mediators in the development of this lethal multiorgan damage. For example, inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and IL-6, are elevated in the sera of both adult and pediatric septic patients (6, 9, 20, 29) and affect symptoms such as hypotension, fever, and the production of acute-phase proteins (21). However, the contribution of these proinflammatory cytokines in directly mediating mortality from sepsis is not clear, since therapies utilizing neutralizing antibodies or soluble receptor antagonists against TNF-α and IL-1 fail to show a significant benefit in outcome for patients with sepsis (5, 13). Possible explanations for the clinical failure of these cytokine-targeted therapies may be that the antibodies or antagonists were administered too late or that other unopposed inflammatory mediators continue to support the disease process.

In contrast to humans, the use of anticytokine therapies against TNF-α (4) and IL-1 (22) in a murine model of endotoxemia leads to dramatic improvement in animal mortality. When lipopolysaccharide (LPS), the endotoxin from gram-negative bacteria, is injected into susceptible animals a rapid systemic inflammatory cascade is initiated in the absence of bacteremia. The lack of ongoing injury after a single administration of endotoxin and the absence of bacteremia in this model highlight its artificial nature and may explain why many clinical trials, based on observations with this model, have failed. The kinetics and magnitude of cytokine production in LPS-induced sepsis are also dramatically different from that seen in a more clinically relevant model of peritonitis with bacteremia induced by cecal ligation and puncture (CLP) (25), which closely mimics the human disease of septic shock (14). In sepsis induced by CLP, neutralizing antibodies to TNF-α are not protective against mortality (10), and C3H/HeJ mice that are resistant to the lethal effects of LPS are still susceptible to mortality from CLP (19). These differences between the LPS and CLP models suggest that the immunology of sepsis is far more complex than the cascade of events activated by endotoxin and that the exact role of proinflammatory cytokines as mediators of mortality is unclear.

Besides stimulating the synthesis of proinflammatory cytokines, the septic response in patients and animal models also results in the production of antiinflammatory mediators, such as IL-10 (8, 11, 30, 31). IL-10 is a 35-kDa homodimeric protein, produced primarily by monocytes, T cells, and B cells, that inhibits the production of proinflammatory cytokines in vitro (16). IL-10 is completely protective in the LPS model of sepsis (3, 15), but in the CLP model the administration of neutralizing antibodies to IL-10 at the time of CLP only partially exacerbates mortality (28, 31). However, inhibition of IL-10 12 h after CLP actually improves survival (28) and partially rescues surgery after lethal CLP is lost 10 h earlier in IL-10−/− mice. We subjected IL-10−/− mice to CLP and monitored the progression of sepsis, the onset of irreversible shock, and mortality. Onset of lethality in IL-10−/− mice occurred significantly earlier than in IL-10+/+ mice and was associated with 15-fold-higher serum levels of TNF-α and IL-6. Consistent with these findings, the efficacy of rescue surgery after lethal CLP is lost 10 h earlier in IL-10−/− mice than in IL-10+/+ mice. Treatment with recombinant human IL-10 5 h after CLP significantly improved survival and lengthened the therapeutic window for rescue surgery in both strains of mice. These results demonstrate that IL-10 controls the onset of irreversible septic shock after CLP.
the progression of sepsis and control the onset of irreversible shock and mortality in the CLP model.

MATERIALS AND METHODS

Mice. Inbred C57Bl/6J mice deficient for the IL-10 gene (IL-10−/−) and wild-type littermates (IL-10+/−) were used for experimentation between the ages of 8 to 12 weeks. The mice were bred and maintained in microisolator cages in our specific-pathogen-free barrier facility under a 12-h light-dark cycle. While IL-10−/− mice are known to develop colitis, in our facility no signs or symptoms of colitis are observed until 14 weeks of age. All experiments described were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals, and approval was obtained from the Institutional Animal Care and Use Committee of Case Western Reserve University.

Genotyping of IL-10−/− mice. The genotype of each mouse was determined by PCR analysis from tail clip DNA (12). Briefly, tail clips 0.25 cm in length were digested with proteinase K (Gibco-BRL, Gaithersburg, Md.) in tail buffer (50 mM Tris buffer, pH 8.0; 100 mM EDTA; 100 mM NaCl; 1% sodium dodecyl sulfate) overnight. The following day, digested tails were extracted twice with phenol (Gibco-BRL), followed by an extraction once with 24:1 chloroform-isooamyl alcohol (Sigma, St. Louis, Mo.). DNA was precipitated with 100% isopropanol, washed in 70% ethanol, air dried, and dissolved in 100 μl of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). A total of 1 μl of DNA was added to a 25-μl PCR mixture containing 1× Taq extender buffer (Stratagene, La Jolla, Calif.), 0.25 mM deoxynucleoside triphosphates (Gibco-BRL), 2 U of Taq polymerase (Boehringer Mannheim, Indianapolis, Ind.), and 2 U of Taq Extender (Stratagene). Then, 1 μl of a three-primer cocktail (1.6 μg/ml) was added (primer 1, 5′-CCCTCTAGTATAAAAGGGGGA-3′; primer 2, 5′-CAGTTGTCATAGGGC-3′; primer 3, 5′-CTTCAACCCCTAAAACCTCCTG-3′). PCR amplification conditions were 94°C for 5 min, 94°C for 1 min, 55°C for 5 min, and 72°C for 1 min for 40 cycles, followed by 72°C for 5 min. The products were analyzed on a 2.0% agarose gel in 0.5× Tris-borate-EDTA buffer. Samples were loaded in buffer containing bromophenol blue and xylene cyanol. The gel was stained with 1 μg of ethidium bromide/ml for 30 min and then destained in distilled water for an additional 30 min. The product size for the wild-type and disrupted IL-10 genes were 700 and 600 bp, respectively.

Cecal ligation and puncture. Anesthesia in the mice was induced with an intraperitoneal injection of 2.5% tribromoethanol at 0.20 ml/kg in 0.5 ml of normal saline twice daily by subcutaneous injection (Merck, West Point, Pa.)/kg in 0.5 ml of normal saline twice daily by subcutaneous injection. The colon was maintained. The antimesenteric border of the cecum was punctured once with either a 25- or 18-gauge needle, and the cecum was gently digested with proteinase K (Gibco-BRL, Gaithersburg, Md.) in tail buffer (50 mM Tris, 1 mM EDTA; pH 8.0). A total of 1 μl of DNA was added to a 25-μl PCR mixture containing 1× Taq extender buffer (Stratagene, La Jolla, Calif.), 0.25 mM deoxynucleoside triphosphates (Gibco-BRL), 2 U of Taq polymerase (Boehringer Mannheim, Indianapolis, Ind.), and 2 U of Taq Extender (Stratagene). Then, 1 μl of a three-primer cocktail (1.6 μg/ml) was added (primer 1, 5′-CCCTCTAGTATAAAAGGGGGA-3′; primer 2, 5′-CAGTTGTCATAGGGC-3′; primer 3, 5′-CTTCAACCCCTAAAACCTCCTG-3′). PCR amplification conditions were 94°C for 5 min, 94°C for 1 min, 55°C for 5 min, and 72°C for 1 min for 40 cycles, followed by 72°C for 5 min. The products were analyzed on a 2.0% agarose gel in 0.5× Tris-borate-EDTA buffer. Samples were loaded in buffer containing bromophenol blue and xylene cyanol. The gel was stained with 1 μg of ethidium bromide/ml for 30 min and then destained in distilled water for an additional 30 min. The product size for the wild-type and disrupted IL-10 genes were 700 and 600 bp, respectively.

Cecal removal (CR) surgery. After CLP some mice underwent a second rescue surgery, wherein the necrotic cecum was excised to remove the infecting nidus. Before the septic mice were anesthetized for their second laparotomy, they were further fluid resuscitated by a subcutaneous injection of 0.5 ml of normal saline. A 30% lower dose of tribromoethanol was administered by intraperitoneal injection (Merck, West Point, Pa.)/kg in 0.5 ml of normal saline, whereas control mice received vehicle alone. The total daily fluid administered to the mice after CLP remained the same by an appropriate decrease in the volume of the subsequent dose of antibiotic. The dose of IL-10 used here has previously been shown to be protective in the LPS model of sepsis (15).

Data analysis. The overall survival for lethal and sublethal injury was described by using Kaplan-Meier plots. The difference between the survival curves of the two strains of mice (Logrank test, P < 0.0001) after CLP with an 18-gauge needle (n = 30 mice per strain). The time of death within 10-h intervals was recorded, and the data are expressed as the cumulative percentage of mice still alive within each interval. The overall mortality in the IL-10−/− mice was significantly more rapid when the survival curves between both strains of mice were compared (Logrank test, P < 0.0001), but the mortality at 120 h was statistically indistinguishable between the two strains (FET, P = 0.20).

Treatment of mice with IL-10. In specific experiments, 1 μg of recombinant human IL-10 (rhIL-10 [a gift from Schering-Plough Research Institute, Kenilworth, N.J.]) was administered after CLP by subcutaneous injection in 0.25 ml of normal saline, whereas control mice received vehicle alone. The total daily fluid administered to the mice after CLP remained the same by an appropriate decrease in the volume of the subsequent dose of antibiotic. The dose of IL-10 used here has previously been shown to be protective in the LPS model of sepsis (15).

RESULTS

Endogenous IL-10 delays the onset of lethality induced by CLP and protects against mortality. To test our hypothesis that IL-10 functions as a temporal regulator of the septic response, we compared the time of onset of mortality and the subsequent kinetics of disease progression between IL-10-deficient mice on a C57Bl/6J background (IL-10−/−) and their wild-type littermates (IL-10+/−) after CLP with an 18-gauge needle (Fig. 1). Onset of lethality in IL-10−/− mice occurred at 10 h after CLP, but in IL-10+/− mice it was delayed until 30 h, by which time the IL-10−/− mice had a significant 67% greater mortality (Fisher’s exact test [FET], P < 0.0001). The overall mortality in the IL-10−/− mice was also significantly more rapid when we compared the survival curves between both strains of mice (Logrank test, P < 0.0001). However, the modest 15% difference in long-term survival at 120 h after CLP...
between IL-10/−/− and IL-10/+/+ mice was not statistically significant (FET, \( P = 0.20 \)). These results indicate that the absence of endogenous IL-10 leads to a more rapid onset of death but does not affect the overall mortality.

**Delay of disease onset by IL-10 is similarly observed after a sublethal injury.** The more rapid onset of mortality in the IL-10/−/− mice compared to IL-10/+/+ animals after CLP with an 18-gauge needle may be due to the severity of the insult. We therefore evaluated a sublethal injury with a 25-gauge needle to puncture the cecum and then monitored survival (Fig. 2). The onset of mortality at 10 h after CLP in IL-10/−/− mice was identical between a lethal and sublethal injury. In IL-10/+/+ mice the onset of mortality after sublethal injury was delayed for 60 h, by which time there was a 35% significantly greater mortality in the IL-10/−/− mice (FET, \( P < 0.01 \)).

**Kinetics of proinflammatory cytokine production after CLP.** An IL-10 deficiency has greater impact on mortality earlier in the disease process, suggesting that IL-10 functions in the CLP model of sepsis to regulate the timeline of systemic inflammation. We therefore predicted that the absence of IL-10 would alter the kinetics of production of the proinflammatory cytokines TNF-\( \alpha \) and IL-6 after CLP. IL-10/−/− mice produced significantly greater levels of TNF-\( \alpha \) and IL-6 in serum compared to IL-10/+/+ animals at each time point from 5 to 20 h (ANOVA, \( P \leq 0.0001 \)) after CLP with an 18-gauge needle (Fig. 3). In comparison, IL-10/−/− and IL-10/+/+ mice that underwent sham surgery had no detectable TNF-\( \alpha \) in their serum and only a small peak of IL-6, to \(< 1 \) ng/ml, at 5 h after laparotomy (data not shown). There was no significant change from 5 to 20 h after CLP in the concentrations of either TNF-\( \alpha \) or IL-6 in the sera of both strains of mice (ANOVA, \( P > 0.07 \)). The 15-fold-greater concentrations of TNF-\( \alpha \) and IL-6 measured in the sera of IL-10/−/− mice after CLP may contribute to the earlier onset of lethality.

**IL-10 levels in serum are elevated after CLP.** The lower TNF-\( \alpha \) and IL-6 levels seen after CLP in IL-10/+/+ mice compared to IL-10/−/− mice are not unexpected. Therefore, we investigated the kinetics of serum IL-10 production in IL-10/+/+ mice after lethal CLP to determine whether it paralleled the proinflammatory cytokine profile. Levels of IL-10 in serum were maximal at 5 h after CLP and remained elevated until 20 h after injury (Fig. 4), matching the kinetics of proinflammatory cytokine production. As expected, IL-10/−/− mice did not have any detectable IL-10.

**Absence of IL-10 shortens the therapeutic window for rescue surgery after lethal CLP.** Our findings thus far indicate that IL-10 regulates the onset of mortality, suggesting that IL-10 may also modulate the transition from early reversible sepsis to late irreversible shock. It was shown previously that the lethality of CLP could be reversed by a second surgery to remove the ligated necrotic cecum (1). Therefore, we defined the transition from reversible sepsis to irreversible shock in the CLP model as the time when rescue surgery by removal of the
necrotic cecum is no longer effective. At the time of CR all mice exhibited similar signs of murine sepsis, such as ruffled fur, periorbital exudates, tremor, and lethargy. IL-10−/− mice could be partially rescued by CR at 5 h after lethal CLP (18-gauge needle), with 60% surviving long term compared to 3% after CLP alone (FET, \( P < 0.001 \); Fig. 5). At 10 h CR was ineffective in IL-10−/− mice, with the 20% survival being not significantly different to that after CLP alone (FET, \( P = 0.15 \)), whereas IL-10+/+ mice that underwent CR at 10 h still showed 100% survival compared to the 17% survival with no intervention (FET, \( P < 0.0001 \)). However, by 20 h CR in IL-10+/+ mice was no longer efficacious (FET, \( P = 1.0 \)). These findings show that in lethal sepsis induced by CLP the success of rescue surgery is time dependent. Furthermore, the earlier failure of CR in IL-10−/− mice suggests that IL-10 regulates the transition from reversible sepsis to irreversible shock.

FIG. 4. The IL-10 concentrations in serum are elevated and maintained from 5 to 20 h during sepsis initiated by CLP. Groups of IL-10+/+ mice underwent lethal CLP and had sera collected at the indicated times (n = 8 mice per time point). IL-10 concentrations in serum were determined by ELISA. The data are shown as the log transformation of the mean ± the standard deviation.

FIG. 5. Rescue surgery with CR must occur earlier in IL-10−/− mice to improve survival. Groups of IL-10+/+ (■, n = 10) and IL-10−/− (▲, n = 10) mice underwent CR at 5, 10, 15, and 20 h after CLP, and the survival at 120 h was recorded. Compared to CLP-treated animals with no additional intervention, CR at 5 h in IL-10−/− mice and at 5, 10, and 15 h in IL-10+/+ mice led to improved survival. However, intervention in IL-10−/− mice at 10 h and in IL-10+/+ mice at 20 h was not successful (FET, \( P \geq 0.15 \)). ND, not done.

Treatment with IL-10 improves survival and delays the onset of mortality and irreversible shock. As IL-10 appears to modulate the therapeutic window for rescue surgery, then treatment with exogenous IL-10 should delay the onset of irreversible shock. We therefore treated both IL-10−/− and IL-10+/+ mice with 1 \( \mu \)g of rhIL-10 after CLP to determine whether the onset of lethality would be delayed and the therapeutic window for rescue surgery extended. The time chosen for administration of the rhIL-10 was 5 h after CLP because this was the earliest time point at which we found maximal IL-10 levels in the sera of IL-10+/+ mice (Fig. 4). In both strains of mice after CLP with an 18-gauge needle, rhIL-10 treatment delayed the onset of lethality compared to mice who received no treatment (Fig. 6 and Table 1). Furthermore, for both IL-10−/− and IL-10+/+ mice treated with rhIL-10 5 h after CLP overall survival was significantly improved compared to that of untreated mice of the same strain (Logrank test, \( P \leq 0.007 \)). Consistent with previous reports (17, 24), treatment of

FIG. 6. Treatment with rhIL-10 delayed the onset of lethality and improved long-term survival in both IL-10+/+ and IL-10−/− mice. Groups of IL-10−/− and IL-10+/+ mice underwent CLP with an 18-gauge needle. At 5 h after CLP-treated mice (IL-10−/−, dashed line; IL-10+/+, solid broken line) received 1 \( \mu \)g of rhIL-10 in 0.25 ml of normal saline (n = 20) by subcutaneous injection. Control mice (IL-10−/−, dotted line; IL-10+/+, solid thin line) were injected with normal saline alone (n = 30). The time of death within 10-h intervals was recorded, and the data were expressed as the cumulative percentage of mice alive within each interval. Survival of treated mice in both strains, as determined by the Logrank test, was significantly improved compared to untreated animals (\( P \leq 0.007 \)).

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>rhIL-10 treatment</th>
<th>% Survival at 30 h</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10−/−</td>
<td>−</td>
<td>26</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-10+/+</td>
<td>+</td>
<td>75</td>
<td>0.94</td>
</tr>
<tr>
<td>IL-10+/+</td>
<td>−</td>
<td>80</td>
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* Each group of mice on a C57BL/6J background underwent CLP with an 18-gauge needle.

* Five hours later the mice were injected subcutaneously with 1 \( \mu \)g of rhIL-10 in 0.25 ml of normal saline (n = 20) or vehicle alone (n = 30).

* Statistical differences between groups was determined by using Fisher’s exact test.

TABLE 1. IL-10 Treatment delays the onset of lethality
mice with rhIL-10 at the time of CLP did not lead to a significant improvement in survival (data not shown).

Since the onset of mortality was delayed in IL-10−/− and IL-10+/+ mice treated with rhIL-10 5 h after CLP, we hypothesized that the efficacy of rescue surgery would be significantly improved (Fig. 7). Treatment of IL-10−/− mice with rhIL-10 increased the effectiveness of CR at 10 h from a survival rate of 20 to 80% (FET, P = 0.02). Similarly rhIL-10 treatment in IL-10+/+ mice significantly improved survival after rescue surgery at 20 h from 20 to 90% (FET, P = 0.006). Thus, IL-10 treatment extends the therapeutic window for rescue surgery and delays the onset of irreversible shock.

DISCUSSION

In this report we demonstrate that in sepsis triggered by CLP the transition from reversible sepsis to irreversible septic shock, defined as the time point at which rescue surgery fails, is regulated by IL-10. The absence of IL-10 leads to a more rapid onset of mortality after CLP and an earlier transition from reversible to irreversible sepsis. In contrast, treatment of mice with rhIL-10 after CLP delays the onset of lethality, improves survival, and extends the therapeutic window for rescue surgery. We therefore propose that IL-10 regulates a “point of no return” for recovery from severe sepsis.

The more rapid-onset of mortality in IL-10−/− mice compared to IL-10+/+ mice after CLP is similar to data obtained by using blocking antibodies to IL-10 (28, 31). However, the eventual survival in anti-IL-10-treated mice was not that much lower compared to that of control mice and, in the study by Song et al., was not significantly different (28). Similarly, in our study the ultimate lower survival in IL-10−/− mice was not significantly different to that of IL-10+/+ mice after either lethal or sublethal CLP. Our results, together with these previous reports, indicate that endogenous IL-10 alters the kinetics of the septic response in the CLP model but probably does not dramatically affect long-term survival.

Increased synthesis of proinflammatory cytokines in IL-10−/− compared to IL-10+/+ mice after CLP is not unexpected since IL-10 is known to suppress the production of TNF-α and IL-6 (16). The 15-fold-greater levels of TNF-α and IL-6 after CLP in the IL-10−/− mice may contribute to their more rapid onset of mortality. It is unlikely that the more rapid lethality of IL-10−/− mice is due to increased bacteremia, since in an Escherichia coli peritonitis model increased lethality of IL-10−/− mice was associated with an accelerated bacterial clearance but greater organ dysfunction (27). Therefore, it could be that the exaggerated proinflammatory cytokine response in the IL-10−/− mice after CLP leads to a more rapid onset of irreversible damage to vital organs and hastens the onset of irreversible shock.

In the CLP model it has been described that survival can be significantly improved by the use of antibiotics (18) and fluid resuscitation (18, 32), both treatments being utilized in our experimental CLP protocol. Removal of the necrotic cecum after CLP has also been shown to improve outcome (1). The results from our therapeutic approach that involves removal of the necrotic cecum at various times after CLP has a striking clinical correlate. In human disease the mainstay of clinical management for bacterial peritonitis is to operate and remove the infectious nidus. With timely intervention this leads to resolution of the peritonitis and recovery of the patient in the majority of cases. However, some patients go on to die from unremitting sepsis and multiorgan failure despite appropriate surgery and supportive care (26). At the time of presentation these critically ill patients appear alike in their level of acuity as those who recover but are presumably further advanced in the disease process. Similarly, as presented here, the efficacy of CR after CLP decreases the longer it is delayed, and the transition into irreversible shock in both IL-10−/− and IL-10+/+ mice does not correlate with any obvious clinical signs nor any changes in the serum cytokine profile. However, our finding that the therapeutic window for rescue surgery is extended by 10 h in IL-10+/+ compared to IL-10−/− mice suggests that IL-10 may have a significant role in regulating the onset of the “point of no return” and irreversible shock.

If IL-10 does control the onset of irreversible shock in severe sepsis, then the treatment of IL-10−/− and IL-10+/+ mice with IL-10 should delay the onset of mortality and lengthen the therapeutic window for rescue surgery. Our data show that for both strains of mice treated with rhIL-10 after CLP the onset of mortality was delayed, and the efficacy of CR was now extended beyond the time at which it was previously ineffective in untreated mice. Thus, the combination therapy of rhIL-10 and late CR after lethal CLP can lead to increased recovery of septic mice.

The modest improvement in survival in IL-10+/+ mice after rhIL-10 treatment alone 5 h after CLP is consistent with a previous report in which a 50% improvement was seen when IL-10 was given as a single dose 6 h after CLP (17). Similarly, our findings that treatment with rhIL-10 at the time of CLP does not increase survival (data not shown) are in agreement with reports showing that initiation of IL-10 treatment at the time of CLP does not improve outcome (17, 24). It may be that IL-10 is not critical at the initiation of the septic response, as evidenced by the ability of early surgical intervention with CR at 5 h to partially reverse CLP-induced lethality in IL-10−/− mice. However, soon after the septic response is initiated, the
absence of IL-10 supports a more rapid onset of mortality, yet the ultimate impact of the absence of IL-10 on long-term survival is not that great. In contrast, exogenous IL-10 administered during the reversible phase of sepsis improves outcome. We also hypothesize that administering IL-10 too late during the irreversible phase of shock has a limited therapeutic benefit, since blocking endogenous IL-10 late after CLP improves survival (28). Therefore, therapy with IL-10 once irreversible shock has begun may be harmful.

Not surprisingly, the role of IL-10 in sepsis is complex, with potentially opposite effects depending on the timing of intervention and whether endogenous versus exogenous IL-10 is manipulated. The findings presented here, however, help to clarify the confusion by demonstrating that IL-10 functions to regulate the onset of irreversible septic shock and the therapeutic window for rescue surgery.

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