Background: Sepsis is a major health problem that can be investigated in experimental animal models.

Results: Its etiology is divided into an early therapeutically reversible phase displaying a robust inflammatory response followed by a late therapeutically irreversible stage with reduced innate immune response.

Conclusion: Mortality is associated with a late immune dysfunction.

Significance: This study provides information for the potential window for treatment.

Sepsis is a major health problem in the United States with high incidence and elevated patient care cost. Using an animal model of sepsis, cecum ligation, and puncture, we observed that mice became rapidly hypothermic reaching a threshold temperature of 28 °C within 5–10 h after initiation of the insult, resulting in a reliable predictor of mortality, which occurred within 30–72 h of the initial procedure. We also observed that the inflammatory gene expression in lung and liver developed early within 1–2 h of the insult, reaching maximum levels at 6 h, followed by a decline, approaching basal conditions within 20 h. This decrease in inflammatory gene expression at 20 h after cecal ligation and puncture was not due to resolution of the insult but rather was an immune dysfunction stage that was demonstrated by the inability of the animal to respond to a secondary external inflammatory stimulus. Removal of the injury source, ligated cecum, within 6 h of the initial insult resulted in increased survival, but not after 20 h of cecal ligation and puncture. We concluded that the therapeutic window for resolving sepsis is early after the initial insult and coincides with a stage of hyperinflammation that is followed by a condition of innate immune dysfunction in which reversion of the outcome is no longer possible.
CLP can be divided into two phases: an early hyperinflammatory response and late innate immune dysfunction. These two phases also correspond to therapeutically reversible and irreversible stages of sepsis, respectively.

EXPERIMENTAL PROCEDURES

Experimental Animal Models—C57BL6/J (B6) mice were obtained from Jackson Laboratories. Male B6 mice (8 weeks old) were starved for 16 h before any intervention. CLP was performed as previously described (13). Male mice were anesthetized with isoflurane via a vaporizer at 1.5–2.5 minimum alveolar concentration. Under sterile conditions, a 2-cm incision was made in the lower abdominal region, and the cecum was exposed. The distal portion of the cecum was ligated 1.5 cm from the end with a 4–0 silk suture and punctured once with a 16-gauge needle. The cecum was replaced in the peritoneal cavity and squeezed to place a small portion of its content (bacteria and feces) into the peritoneum. Then the peritoneal wall and skin were closed with double sutures. The mice were resuscitated with a subcutaneous injection of sterile saline (1 ml). As a control, mice were sham operated as described above, except that the cecum was neither ligated nor perforated. Nonoperated mice were also used as a second control. After the procedure, the mice had an accessible source of water and food ad libitum. Although we did not quantitate food and water uptake after CLP, we observed a small gain in weight after CLP with respect to the beginning of the experiment, which was no different from sham operated mice. This observation is consistent with published data (19). For cecum excision, mice 3, 6, 8, or 20 h after CLP were anesthetized with isoflurane (1.5–2.5 minimum alveolar concentration), the initial incision was opened again under sterile conditions, and the ligated cecum was removed by cutting between two sutures. The peritoneum was rinsed three times with sterile saline, and the peritoneal wall and skin were closed with double sutures. As indicated, the mice were slightly anesthetized with isoflurane and injected with LPS (15 mg/kg) or an equal volume of saline. During the time of the initial surgery, a small thermal probe was placed under the skin during the procedure. The mice were placed in individual cages, and their temperature was continuously recorded using an external receiver (VitalView data acquisition system). These animal protocols have been reviewed and approved by the University of California San Diego Institutional Animal Care and Use Committee according to the National Institutes of Health guidelines.

Measurement of mRNA Levels Using Quantitative Real Time PCR—Levels of mRNA were measured by quantitative real time RT-PCR (qRT-PCR). Tissues were harvested, flash frozen in liquid nitrogen, and stored at −80 °C. The samples were then suspended and homogenized in TRIzol reagent (Invitrogen), and total RNA was isolated. RNA was treated with DNase I (DNA-free kit; Ambion, Austin, TX) and reverse-transcribed to cDNA using a high capacity reverse transcription kit (Applied Biosystems, Foster City, CA). Newly synthesized cDNA was further diluted and stored at −20 °C. Samples of cDNA were amplified by a 7500 fast real time PCR System (Applied Biosystems) using the QuantiTect SYBR Green PCR kit (Qiagen) with the following QuantiTect validated primer sets (Qiagen): TNF-α (QT00104006), IL-1β (QT01048355), IL-6 (QT00098875), IL-10 (QT00106169), IFN-β (QT00249662), HMGB1 (QT00247786), CD14 (QT00246190), and TLR4 (QT00259042). Standards corresponding to each target gene were added in each PCR. The results for each sample were normalized by copy number of GAPDH (QT01658692; Qiagen), used as a marker of cDNA inputs. All of the results were expressed as copy numbers of target gene per copy numbers of GAPDH.

Myeloperoxidase Assay—Myeloperoxidase activity was measured by a modification of method described by (14). Samples of liver (150 mg) and the left side of the lung were flushed with PBS and homogenized for 30 s in phosphate buffer, pH 7.4. Homogenates were centrifuged at 10,000 × g for 10 min at 4 °C, and the resulting pellets were resuspended in phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. The samples were then subjected to three cycles of freezing and thawing, sonication (three times for 10 s for lung and five times for 10 s for liver), and centrifugation at 10,000 × g for 5 min at 4 °C. The supernatant (25 μl) was mixed with phosphate buffer, pH 6.0, containing O-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (0.0005%), and absorbance at 460 nm was measured immediately at regular intervals (15 s) for 5 min. Protein concentration was measured for each sample by the BCA protein assay, and data (myeloperoxidase activity) were expressed as change in absorbance/min/mg of protein.

Statistical Analysis—All of the data were analyzed using GraphPad Prism software (GraphPad Prism Software, San Diego, CA). Significance was analyzed using one-way or two-way ANOVA followed by Newman-Keul’s multiple comparison test. A p value of < 0.05 was considered statistically significant. Statistical significance was analyzed by log rank test for comparison of mortality rates.

RESULTS

Early Decrease in Core Body Temperature Is Predictor of Mortality—A central objective of this study was to correlate the inflammatory process with the final outcome (mortality) from sepsis induced by CLP. Prior studies have shown that hypothermia is a premorbid condition in experimental sepsis, which can be used as a marker for death after CLP (15). To monitor core body temperature, a small probe was inserted under the skin of the abdomen at the time of CLP intervention to monitor core body temperature using a telemetric system (VitalView data acquisition system). The continuous readout in body temperature was recorded for each individual animal. C57BL/6J (B6) male mice were subjected to a fulminating variant of the CLP model (1.5-cm cecum ligation, 16-gauge needle single puncture) to study the events associated with the response to sepsis in a relatively short time period. This study was performed in the absence of antibiotic treatment. Therefore, this model reflected the natural response to sepsis with minimal support therapy to establish the base line response. We observed a rapid decline in core body temperature within the first few hours after CLP (a typical temperature profile is presented in Fig. 1A). Based on these observations, we found, with 100% accuracy, that animals that reached a core body temperature of 28 °C died.
within 72 h of the initial insult. The animals reached the 28 °C threshold core body temperature within 5–10 h after CLP, but mortality was only observed between 30 and 72 h of the insult. The survival rate at 72 h after CLP was 16.4%, and mice that were predicted to die based on the decrease in core body temperature to 28 °C was 14.6% (Fig. 1B). These observations suggest that reaching a core body temperature threshold of 28 °C is an accurate and early predictor of mortality. These observations were further corroborated using a different mouse strain (A/J), which displayed a different mortality rate after CLP (13, 14). A/J mice were subjected to CLP (1.5-cm cecum ligation, 16-gauge needle single puncture), and both core body temperature and mortality were monitored (Fig. 1C). In this case, all of the animals that died displayed a core body temperature below 28 °C, whereas survivals were above 28 °C. Again, the survival rate of actual mortality was 36.8%, and predicted deaths based on reaching 28 °C were 35.9% (Fig. 1D). Finally, we tested a different animal model that also resulted in significant animal death. Mice (B6) were injected with *Escherichia coli* LPS (15 mg/kg), resulting in 100% mortality. All of the mice dis-
played a very rapid decrease in core body temperature to below 28 °C within 10 h of injection (Fig. 1E), whereas mortality was observed after several hours (Fig. 1F). Thus, the 28 °C threshold temperature appears to be a reliable early predictor of mortality, well ahead of the actual death.

Expression of Inflammatory Genes Occurs Very Early after CLP—Based on the rapid decrease in core body temperature after CLP, we investigated how early the inflammatory process could be detected following the initial insult. The expression of genes involved in the inflammatory process (i.e., cytokines) was measured at the mRNA level rather than by secreted protein in circulation. This approach is relevant, because it provides a more sensitive read-out of the inflammatory process because the detection limit of qRT-PCR is considerably more sensitive than methods to measure protein, such as ELISA. In addition, this approach allows us to study the kinetic of the inflammatory process after CLP at very early stages of the process when the concentration of cytokines in circulation may be very low. Finally, measuring mRNA levels allows us to study the production of inflammatory molecules in specific organs rather than in circulation, providing more mechanistic information. The expression of four cytokine genes was measured in lung and liver harvested from mice after CLP (1.5-cm cecum ligation, 16-gauge needle single puncture) or sham operation and compared with nonoperated (NO) control mice. We observed a very rapid inflammatory response within the lung, peaking within 2–3 h after CLP for TNF-α, IL-1β, and IL-10, whereas the peak of IL-6 was observed at 6 h in comparison with NO or sham operated mice (Fig. 2A). Similar observations were made in liver samples in which TNF-α, IL-1β, and IL-6 peaked within 2–3 h and IL-10 after 6 h of CLP in comparison with NO or sham operated mice (Fig. 2B). In both organs, the inflammatory response was followed by a rapid decline in gene expression beginning after 6 h of CLP and reaching basal levels similar to sham operated mice within 20 h of CLP (Fig. 2). Further analysis until 30 h of CLP, which corresponded to a time period very close to the initial mortalities after the insult, revealed that the majority of cytokine expression levels were still suppressed in the liver (Fig. 2B). In the lung, both IL-1β and IL-6 displayed minimal expression levels, whereas the expression of TNF-α was slightly but significantly increased in comparison with sham operated mice. On the contrary, IL-10 expression was dramatically elevated with respect to sham operated animals (Fig. 2A).

As another marker of the inflammatory process, we measured the infiltration of PMNL (myeloperoxidase assay) in the lung and liver after CLP. Again, a rapid and significant increase in the infiltration of PMNL in lung was observed within 2–3 h followed by a rapid decline but was still significantly different from sham operated mice after 20 or 30 h of CLP (Fig. 3A). PMNL infiltration in liver was also rapid, reaching maximal levels within 6 h, followed by a decrease that was significantly different from sham operated mice (Fig. 3B). The characterization of the inflammatory process after CLP was further analyzed by measuring the expression of receptors involved in the innate immune response, CD14 and TLR4. The expression of CD14 (mRNA) levels also increased in the lung after CLP, reaching maximum levels within 3–4 h, which was followed by a rapid fall (after 6 h), reaching levels similar to sham operated mice within 20 h, followed by a second increase at 30 h of CLP (Fig. 4A). In the liver, CD14 expression also increased within 4–6 h of CLP, followed by a reduction within 20–30 h of CLP, which was significantly different from sham operated mice (Fig. 4B). In contrast, TLR4 expression was not significantly increased after CLP, and it was similar to sham operated mice (Fig. 4A). All together, these observations suggest that the induction of the inflammatory response by CLP was very rapid and can be divided into two periods: an early hyperinflammatory phase (<6 h) and a hypoinflammatory phase (>20 h), which could be partially modified within 30 h of the insult.

Recovery from CLP Can Only Be Achieved during Initial Time Periods of Response—Based on the inflammatory phases observed and the rapid decline in core body temperature after CLP, we were interested in defining how they related to the possible therapeutic window after CLP. We thought that removal of the ligated/perforated cecum, which is the source of infection and injury, was probably the most dramatic intervention to resolve sepsis. Previous studies have shown that, indeed, cecum excision improved survival after CLP (16–19). We excised the ligated cecum at 3 or 20 h after CLP, because these time points represent the two different inflammatory phases observed after the insult. The mice were subjected to CLP, and then they were randomized into three groups at 3 h or 20 h after CLP. In one group, the ligated cecum was removed (CLP + cecum removal). The second group was subjected to sham operation (CLP + sham). The third group was not operated a second time (CLP + NO). Removal of the ligated cecum at 3 h after CLP showed a statistically significant improvement of the survival rate in comparison with animals in which the cecum was not removed but were sham operated or nonoperated a second time (Fig. 5A). In contrast, excision of the cecum at 20 h after CLP did not improve the survival rate (Fig. 5B). Subsequent experiments in which the cecum was removed at 6 or 8 h after CLP showed 80 and 30% survival rates, respectively (Fig. 5C). Based on these observations, we defined two distinct phases of sepsis: therapeutically reversible (<6 h) and therapeutically irreversible (>20 h), which coincide with the two inflammatory stages observed above.

The Inflammatory Response during Therapeutically Irreversible Phase of Sepsis Is Reduced—The therapeutically irreversible phase of sepsis corresponds to a condition in which the inflammatory response is already reduced with respect to the early hyperinflammatory and therapeutically reversible phase. This condition may resemble a stage of immunosuppression or immunoparalysis, which has been previously described during sepsis (12). Therefore, we decided to investigate this possible immunosuppressive stage by using a functional assay. We evaluated the response to LPS, which induces a strong inflammatory response, at a late stage of sepsis (20 h after CLP), in which mortalities were still not observed. The mice were subjected to CLP, sham operation (SO), or NO. These animals were challenged with LPS (15 mg/kg) at 20 h after the initial procedure, and cytokine levels (qRT-PCR) in lungs and liver were measured 2 h after LPS injection. As a negative control, a group of mice that were neither subjected to CLP nor injected with LPS...
were included. We observed that TNF-α and IFN-β mRNA levels were induced after LPS injection in mice that were initially NO or SO in comparison with mice that were not injected with LPS. In contrast, LPS-induced TNF-α and IFN-β mRNA levels in both lung and liver samples were significantly reduced in mice that were subjected to CLP first in comparison with SO or NO after LPS injection (Fig. 6). LPS-induced IL-6 and IL-10 mRNA levels were highly increased in lung of mice that were CLP pretreated in comparison with mice that were SO or NO (Fig. 6B). No statistical differences were observed in IL-6 and IL-10 mRNA levels in liver after LPS injection between animals that were initially subjected to CLP, SO, or NO (Fig. 6B). The expression of a late sepsis marker, HMGB1, was also evaluated. Significant increases in HMGB1 mRNA levels were observed in all animals injected with LPS regardless of whether or not they were initially subjected to CLP, sham operation, or not operated at all in comparison with the absence of LPS injection (Fig. 6). These observations suggest that the innate immune

FIGURE 2. The expression of inflammatory genes occurred very early after CLP. Male B6 mice (n = 50) were subjected to CLP, 1.5-cm cecum ligation and 16-gauge perforation (closed squares), or sham operation (open squares). At different time points after CLP (n = 5) or sham operation (n = 5), the mice were sacrificed, lung (A) and liver (B) were harvested, and total RNA was isolated. The nonoperated group corresponds to time 0. The levels of mRNA for TNF-α, IL-6, IL-1β, and IL-10 were measured by qRT-PCR. The mRNA levels were established by comparison with a standard curve and expressed as copy numbers. The values were normalized to GADPH mRNA levels. Statistical analysis for the time course of CLP was performed by one-way ANOVA, and comparison between CLP and sham operation was measured by two-way ANOVA. *, p < 0.05 CLP versus sham operated at each time point. Notice that cytokine gene expression is observed very rapidly after CLP in both organs.
and molecular bases of sepsis has been derived mainly from studies using experimental animal models. In this regard, CLP is considered the gold standard experimental animal model to study sepsis. Although this animal model does not totally resemble human sepsis, it has important clinical features, such as the presence of a necrotic focus (ligated cecum) and a source of polymicrobial infection (leaking of bacteria from the gut into the peritoneum) resembling many symptoms observed in critically ill patients within the surgical intensive care unit. Using the CLP model, we characterized the events associated with the early response during sepsis.

Previous studies have proposed that hypothermia could be a good predictor of mortality induced by sepsis (15). We investigated this possibility by monitoring the core body temperature of mice after CLP using a telemetric system. Indeed, we observed a rapid decline of core body temperature within 5–8 h after CLP. Moreover, we found that all mice that reached a threshold of 28 °C (less than 10 h after CLP) died between 30–72 h of the initial insult. This assumption was validated using other mouse strains that displayed different mortality rates after CLP or after a different lethal insult (injection of LPS). Therefore, we concluded that reaching a temperature of 28 °C is an accurate predictor of mortality during sepsis, well ahead of the actual death. Although hypothermia has been documented in response to various pathogenic infections and injection of LPS (21–23), the causes for this decrease in temperature are not well understood. Thermogenesis mediated by prostaglandins and other products released by macrophages have been proposed as the causes for the decrease in temperature during infections (24). Other studies have correlated hypothermia with the presence of TNF-α (25). Recently, a possible role for heat shock factor 1, the main inducer of the heat shock response, has also been proposed as a key regulator of changes in temperature (26). The concept of whether or not hypothermia may be beneficial as pretreatment during trauma has been proposed but remains controversial (27). Our data may argue in disfavor of hypothermia as a pretreatment, because we showed a good correlation between a reduction of core body temperature and a poor outcome during septic and endotoxic shock.

Based on the very rapid decline in core body temperature, we hypothesized that the inflammatory response associated with sepsis could also occur very rapidly. Consequently, we investigated the inflammatory process at very early time periods after CLP. Expression of inflammatory molecules, such as cytokines, was studied at the mRNA level by qRT-PCR in two critical organs, lung and liver, after CLP. We observed a rapid decline of expression, reaching low levels comparable with sham operated mice. Maximum cytokine mRNA levels were observed within 3–6 h after CLP, which was followed by a rapid decline of expression, reaching low levels comparable with sham operated mice within 20 and 30 h after CLP with the exception of TNF-α, IL-10, which displayed an increase at 30 h of CLP, but only in the lung. This increase in pulmonary IL-10/TNF-α expression was parallel to elevation of CD14 levels. The infiltration of PMNL into both lungs and liver was also elevated within 2–6 h after CLP, followed by a rapid decline. The decrease in PMNL in the organs after infiltration is likely

**FIGURE 3.** Lung and liver PMNL infiltration was observed very rapidly after CLP. Male C57Bl/6 mice (n = 7) were subjected to CLP, 1.5-cm cecum ligation and 16-gauge needle perforation (closed squares), or sham operation (open squares). At different time points after CLP or sham operation, the mice were sacrificed, and lung (A) and liver (B) were harvested and flushed with PBS. Tissue samples were homogenized, and myeloperoxidase activity (measurement of PMNL infiltration) was measured. The data are expressed as absorbance change per min and per mg of protein. Statistical analysis for the time course of CLP was performed by oneway ANOVA, and comparison between CLP and sham operation was measured by two-way ANOVA. *, p < 0.05 CLP versus sham operated at each time point. Notice that PMNL infiltration in the lung preceded infiltration into the liver, but both reached maximum very early.

**DISCUSSION**

Trauma/injury is the third overall cause of mortality in the United States, surpassed only by cardiovascular disease and cancer. Moreover, it is the main killer in people under 40 years of age. One of the consequences of trauma/injury is the incidence of sepsis, which is often followed by the development of septic shock, multiple organ failure, and death (2, 3, 20). The severe impact of sepsis and septic shock on society is at several levels, including the enormous number of lives that are lost every year and the tremendous burden to the health care system (1). Despite an increasing understanding of the biology associated with sepsis and septic shock, there are still no current predictors or diagnostics that can accurately anticipate the development of sepsis and septic shock. Therapy for these conditions remains supportive, and a possible therapeutic window has not been well defined. Our current understanding of the cellular
related to cell death (apoptosis) after activation induced by bacterial or particle uptake and subsequent clearance by resident phagocytic cells (28–30). It is possible that the expression of cytokines that was observed in both lungs and liver was related to the activation and infiltration of PMNL after CLP. However, we cannot discard the possibility that activation of local macrophages contributed to the response that was observed. The current dogma indicates that death from sepsis is the product of an exaggerated inflammatory response, or “cytokine storm,” particularly from counterinflammatory cytokines (5, 10). The results of our studies are consistent with this elevated inflammatory response during sepsis during the initial stages of the insult. However, we did not observe differences between pro- and anti-inflammatory cytokine gene expression levels. The rapid expression of genes involved in the innate immune response was followed by a decline in mRNA levels within 20–30 h after CLP, reaching levels similar to sham operated mice. This decline in the inflammatory response occurred in spite of the fact that the initial insult (necrosis and bacteremia) has not been resolved yet. In addition, we did not observe any early difference in the expression kinetics of both proinflammatory and anti-inflammatory cytokines, which echoed prior observations demonstrating the initial and concurrent presence of typical proinflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines in the plasma after endotoxic or septic shock in mice (13, 31, 32), correlating with a worse outcome (13). We detected an elevation in IL-10 and, to a lesser extent, TNF-α in the lung, but not in the liver, at 30 h after CLP, which was coincidental with the time that initial mortalities could be observed after the insult. In contrast, IL-1β and IL-6 expression did not change at 30 h in comparison with 20 h after CLP in both lung and liver, suggesting a late anti-inflammatory response. In general, a specific cytokine pattern could not be correlated with outcome (mortality or survival).

The expression kinetics of genes involved in the inflammatory process allowed us to divide the response to sepsis into an early hyperinflammatory stage (<6 h after CLP) and a late resting-inflammatory phase (>20 h after CLP). Therefore, we were interested in investigating the potential contribution of these two stages to the outcome from CLP. Previous studies have shown that excision of the ligated cecum after CLP resulted in improved survival (16–19). Thus, we removed the ligated cecum at early (3 or 6 h) or late (20 h) time periods after CLP. We observed that early cecum excision reverted mortality from sepsis (3–6 h after CLP), whereas cecum removal at 20 h did not improve the survival of the animals. Based on these observations, we proposed that the therapeutic window for treatment of sepsis is very early after CLP, which coincides with the hyperinflammatory stage, at least at the level of gene expression. A clinical limitation of our study is that antibiotics were not given after CLP, which is usually the case following the diagnostic of sepsis in patients. However, antibiotic treatment is not given to patients immediately at the time of the insult, but rather when a positive bacterial culture is obtained that is likely to occur several hours or days after the initiating injury. The administration of antibiotics to this experimental animal model may prolong

**FIGURE 4. The expression of CD14, but not Tlr4, changed during the time course of CLP.** Male B6 mice (n = 50) were subjected to CLP, 1.5-cm cecum ligation and 16-gauge needle perforation (closed squares), or sham operation (open squares). At different time points after CLP (n = 5) or sham operation (n = 5), the mice were sacrificed, lung (A) and liver (B) were harvested, and total RNA was isolated. Levels of mRNA for CD14 or Tlr4 were measured by qRT-PCR. The mRNA levels were established by comparison with a standard curve and expressed as a copy number. The values were normalized to GADPH mRNA levels. Statistical analysis for the time course of CLP was performed by one-way ANOVA, and comparison between CLP and sham operation was measured by two-way ANOVA. *, p < 0.05 CLP versus sham operated at each time point. Notice that cytokine gene expression is observed very rapidly after CLP in both organs.
the time length in which mortalities are observed, but it may not change necessarily the mortality frequency. Because our concluding hypothesis is that early events after the initiation of sepsis (CLP) are critical for the resolution of the disease, antibiotic treatment may interfere with these early episodes occurring the mechanistic cellular and molecular information that could be gathered from this experimental animal model. Indeed, the selection of a fulminant variant of the CLP model (1.5-cm cecum ligation, 16-gauge needle single puncture) directed at studying early events associated with the response to sepsis in a relatively short time period. Another limitation of our studies is that precise cause of death is not well established, which can be related to several factors, including cardiovascular collapse; organ failure, which could be secondary to dysregulation of the complement cascade; increase in apoptosis; hypoxia; and metabolic dysfunction (4, 5, 33).

It is notable that the expression of genes involved in the inflammatory process is abated at 20 h after CLP during conditions in which the sources of the insult, necrosis and infection, are still present. This observation suggests that the innate immune system may not be capable of responding to the continuous presence of inflammatory stimuli, resulting in a dysfunctional stage. This dysfunctional condition is coincidental with the therapeutically irreversible phase of sepsis. The assumption that the innate immune system is dysfunctional was tested by injecting mice with LPS, a potent inducer of the inflammatory response, at 20 h after CLP. We observed that the expression of the proinflammatory genes TNF-α and IFN-β was reduced after LPS injection in animals that were initially subjected to CLP in comparison with LPS injection in absence of CLP. A similar lack of response to secondary stimuli has been previously reported as tolerance (34). However, we argue that the definition of tolerance suggests a salutary effect, and our observations disagree with this possibility because the outcome from sepsis could not be improved during the stage of innate immune dysfunction. Other investigators have proposed that the initial inflammatory response during sepsis is followed by a stage of immunosuppression (12). Immunosuppression has been established at different levels, including dysfunction of monocytes isolated from critically ill patients (35) and reduced function of T and B cells in experimental animal models of sepsis (36, 37). Indeed, our findings resemble prior studies indicating that immune cells isolated from septic patients do not demonstrate a robust induction of the inflammatory response after ex vivo challenge with LPS (38). Moreover, the stage of immunosuppression has been pointed out as being responsible for the higher incidence of nosocomial infections, such as Pseudomonas aeruginosa, observed in critically ill patients (38–41). These observations have been replicated in experimental animal models (42). Similarly, overwhelming bacteremia in the absence of antibiotics was only observed at late times after CLP (42, 43). The increase in the bacterial load may be related to poor clearance of the microorganisms. Indeed, impairment in the phagocytosis of bacteria has been observed in macrophages isolated from septic animals (44, 45). Overall, macrophage dysfunction has been associated with the development of sepsis (46–48). Therefore, it is possible that part of the therapeutically irreversible stage of sepsis is related to the combination of increased bacterial proliferation and reduced clearance. Other investigators have proposed that immunosuppression is associated with dysfunction of the adaptive immune response (49), resulting in a stage of anergy (36, 50). This stage of immunosuppression may be related to increasing incidences of apoptosis, particularly in cells of the immune system as previously proposed (4, 12, 50). In fact, early autopsies of septic patients demonstrated a high degree of apoptosis in different cell types (12). The increase in immune cell death may be exacerbated by failure of the bone marrow to restore these cells. In other words,
it is possible that mortality associated with sepsis may be related to
the incapacity of bone marrow to achieve immune homeo-
stasis. Prior studies have, indeed, shown bone marrow dysfunction
is present in critically ill patients (51, 52), as well as in
animal models of sepsis (53).

The possible stage of innate immune dysfunction that was
observed in our study was not due to a complete lack of
response to external stimuli. First, the levels of key receptors
involved in the innate immune response, CD14 and Tlr-4,
appeared at normal or elevated levels at 20 h after CLP, sug-
gesting that the capacity to recognize external stimuli is intact.
Expression of TNF-α and IL-10 were elevated at 30 h after CLP
in the lung. Moreover, the expression of IL-6 and the anti-in-
mflammatory cytokine IL-10 following injection of LPS was not
hampered at 20 h after CLP, suggesting that recognition of the
bacterial product is not the limiting factor. Similarly, the
expression of the late septic marker HMGB1 (54, 55) was fully
induced after injection of LPS in mice pretreated with CLP.
IL-10 is well known to down-regulate the expression of TNF-α
(56, 57). Therefore, it is possible that reduced expression of
LPS-induced TNF-α after initial CLP may be due to an excess of
IL-10 during the immunosuppressive stage. However, this pos-
sibility cannot explain the response at the IL-6 level observed
after injection of LPS in CLP pretreated mice because expres-
sion of this cytokine is also reduced by external IL-10 (58).
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dysfunction is related to dysfunction of the TLR4 signal trans-
duction pathway. In this regard, TLR4 is the master molecule
involved in the inflammatory response signal transduction,
activating a variety of pathways (59). Ligands of TLR4, such as
LPS, can differentially activate the TIRAP-MyD88 and the
TRAM-TRIF pathways resulting in the expression of TNF-α or
IFN-β, respectively. Because both TNF-α and IFN-β expres-
sion was depressed following LPS injection in CLP pretreated
mice, it is likely that these two pathways are affected dur-
ing innate immune dysfunction. However, other signaling
branches of these two major pathways, such as those respon-
sible for IL-10 and IL-6 expression, may not be affected during
late sepsis. Previous studies have proposed IL-6 as a premorbid
marker for increased mortality in experimental animal models
of sepsis (60), which may be consistent with our observations.
Similarly, elevated expression of IL-10 in critically ill patients
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Innate Immune Dysfunction during Sepsis

In summary, our observations define two possible stages for
the response to sepsis. First, there is a therapeutically reversible
phase, characterized by an increase in the infiltration of PMNL
into organs and elevated expression of inflammatory genes.
This early stage is followed by a therapeutically irreversible
phase, which resembles a condition previously coined immu-
noparalysis, which we have redefined as innate immune dys-
function. During this final phase, the fate of the organism is
already determined, and no beneficial interventions are possi-
ble. Therefore, therapeutic approaches to ameliorate sepsis
need to be performed at much earlier time points after the ini-
tial insult. This assumption is supported by clinical studies indi-
-cating that early therapy increased survival from injury (65).
Similarly, early aggressive therapy by fluid resuscitation (66–
68) or pentoxifylline administration (69, 70) has been shown to
improve the outcome in experimental animal models of injury.
Moreover, the possibility of early therapy may be related to the
common concept in many trauma centers referred as the
“golden hour,” suggesting that very early interventions are crit-
ical to save lives after injury (71). This concept has been chal-
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FIGURE 6. The inflammatory response during the therapeutically irreversible phase of sepsis is reduced. Male B6 mice (n = 6–7) were subjected to CLP
(1.5-cm cecum ligation and 16-gauge needle perforation), SO, or NO. At 20 h after the initial operative procedure, the mice were injected with LPS (15 mg/kg,
intraperitoneal) and sacrificed after 2 h. Lung (A) and liver (B) samples were harvested, and total RNA was isolated. TNF-α IFN-β, IL-6, IL-10, and HMGB-1 mRNA
levels were measured by qRT-PCR. The mRNA levels were established by comparison with a standard curve and expressed as copy number. The values were
normalized to GADPH mRNA levels. Statistical analysis for the comparison between groups was performed by ANOVA. *p < 0.05 CLP + LPS versus SO + LPS.
Notice that LPS failed to induced the expression of TNF-α and IFN-β after CLP.

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