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HOST RESPONSE AND INFLAMMATION

Why Antibiotic Treatment Is Not Enough for Sepsis Resolution: an Evaluation in an Experimental Animal Model

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ABSTRACT Sepsis remains a major health problem at the levels of mortality, morbidity, and economic burden to the health care system, a condition that is aggravated by the development of secondary conditions such as septic shock and multiple-organ failure. Our current understanding of the etiology of human sepsis has advanced, at least in part, due to the use of experimental animal models, particularly the model of cecum ligation and puncture (CLP). Antibiotic treatment has been commonly used in this model to closely mirror the treatment of human septic patients. However, whether their use may obscure the elucidation of the cellular and molecular mechanisms involved in the septic response is questionable. The objective of the present study was to determine the effect of antibiotic treatment in the outcome of a fulminant model of CLP. Various dosing strategies were used for the administration of imipenem, which has broad-spectrum coverage of enteric bacteria. No statistically significant differences in the survival of mice were observed between the different antibiotic dosing strategies and no treatment, suggesting that live bacteria may not be the only factor inducing septic shock. To further investigate this hypothesis, mice were challenged with sterilized or unsterilized cecal contents. We found that exposure of mice to sterilized cecal contents also resulted in a high mortality rate. Therefore, it is possible that bacterial debris, apart from bacterial proliferation, triggers a septic response and contributes to mortality in this model, suggesting that additional factors are involved in the development of septic shock.

KEYWORDS antibiotics, sepsis, cecal ligation and puncture, inflammation, infection

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Sepsis remains a devastating condition affecting over 750,000 people per year in the United States, with a mortality rate of 30 to 50%, which is aggravated by the development of septic shock and multiple-organ failure (1, 2). The hospital costs associated with the treatment of septic patients are extreme, exceeding over $20 billion per year (3, 4). The limited efficacy of the available supportive interventions as well as the lack of specific, targeted therapies contribute to unacceptably high morbidity and mortality rates and economic burdens. The etiology of sepsis is a dynamic process, including variable host responses modulated by an array of confounding factors and unstandardized supportive interventions. Many of these confounding factors include patient age, sex, genetic background, environment, immune status, medical comorbidities, economic status, lack of insurance, timing of recognition, and aggressiveness of resuscitation, all of which contribute to the clinical outcome of sepsis (5). A recent definition of sepsis indicates that this condition is a life-threatening organ dysfunction

condition caused by a dysregulated host response to infection and injury (6), limiting the capacity to restore homeostasis (7) and resulting in a stage of immune dysfunction or anergy (8, 9). Currently, patients presenting with sepsis of various severities are treated with "sepsis bundles," reflexive, quality-directed, multipronged strategies aimed at the early correction of objective metrics (10). Supportive interventions, including volume resuscitation, vasopressors, mechanical ventilation, and antibiotics, provide survival benefit when combined in a timely fashion during early recognition (4, 10–13).

The molecular and cellular mechanisms involved in the response to sepsis remain poorly understood. Indeed, studies of these molecular and cellular mechanisms in the clinical setting are limited, in particular due to the variabilities of responses and severities, which are exacerbated by the heterogeneity of the patient population. Thus, well-controlled animal models are a useful alternative for the understanding of the cellular and molecular mechanisms underlying sepsis (14). However, it is clear that an animal model might not completely replicate the human response to sepsis. Indeed, experimental animal models should be considered tools directed at elucidating cellular and molecular mechanisms to improve our understanding of the etiology of sepsis. The experimental animal model of cecal ligation and puncture (CLP) has been the gold standard for the study of sepsis over the last 30 years (15). The limitations of the mouse model are not restricted to the species-specific differences in the inflammatory cascade but are also related to methodological differences in the use of this model, including the area of ligation; the size of the perforation; and the use of antibiotics, fluid resuscitation, analgesics, and vasoactive medications, etc., which may reflect variability among results from various research groups. Antibiotics are perhaps the most variable intervention regarding timing, dosage, and even antibiotic class. In this study, we investigate the impact of antibiotic treatment on survival in a mouse model of severe surgical sepsis induced by CLP. We found that in a fulminant model of CLP, antibiotic treatment has no impact on improving outcomes, suggesting that the septic response is the product of more than infection and microbial proliferation.

RESULTS

Antibiotics do not improve survival in a model of severe sepsis. We investigated the impact of antibiotic treatment in a model of fulminant sepsis that results in approximately 80% mortality within 72 h of the insult without antibiotic administration. Male CD-1 mice were subjected to CLP (1.5-cm ligation and 16-gauge needle perforation), followed by fluid resuscitation. In addition, mice were treated with the antibiotic imipenem (25 mg/kg of body weight), which has broad-spectrum coverage of enteric bacteria, in different dosage protocols. The dose of imipenem is consistent with those used in previous studies (16, 17). Perioperative administration of imipenem to mice (single dose at the time of CLP) did not lead to any significant difference in survival within 72 h after CLP in comparison with mice that did not receive antibiotics (Fig. 1A). Repeated intervals of subcutaneous imipenem dosing (1 h before and 3 h and 6 h after CLP) did not improve survival within 72 h after CLP (Fig. 1B). Furthermore, the administration of imipenem at the time of CLP as well as 4, 24, and 48 h after CLP did not result in better survival than that of mice after CLP that were not treated with the antibiotic (Fig. 1C). Moreover, the direct delivery of imipenem to the ligated cecum (the site of bacterial presence) and subsequent subcutaneous injection of the antibiotic 2 or 4 h after CLP also failed to improve survival within 72 h (Fig. 1D). Thus, all antibiotic treatment protocols failed to reduce mortality in this model of sepsis.

Imipenem administration after CLP has a disparate effect on the inflammatory response. A hallmark of the inflammatory response after CLP is the expression of cytokines. We have found that cytokine mRNA levels in various organs, such as liver and lung, are good indicators of the initial inflammatory response, reflecting the early response rather than the terminal effect at the level of cytokines in circulation (9). Cytokine gene expression was induced very rapidly in the livers and lungs of CD-1 mice after CLP (Fig. 2). Based on this kinetic information, we measured cytokine mRNA levels 3 h after CLP, corresponding to the maximum response. Samples of liver, lung, and

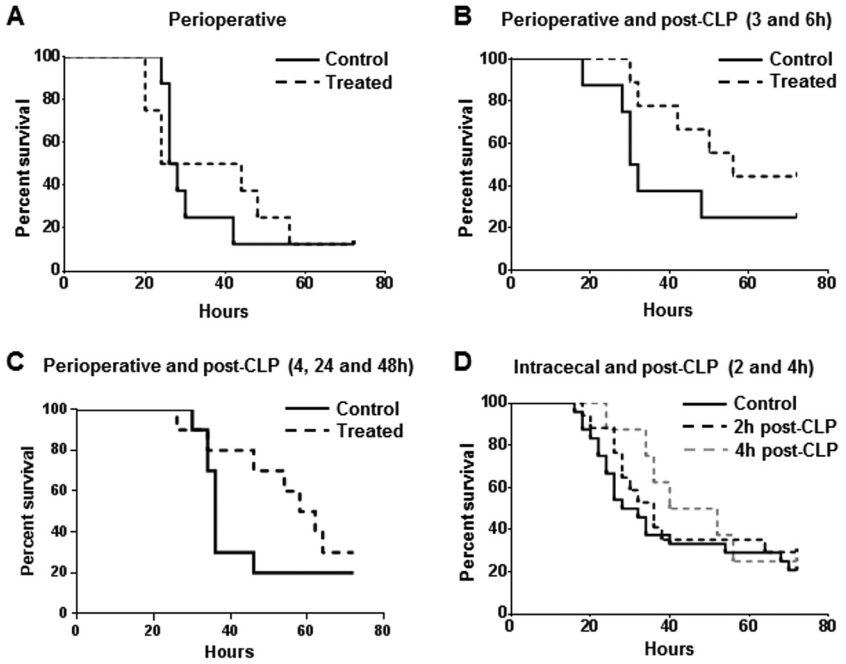

FIG 1 Antibiotic treatments do not improve survival in a model of CLP-induced sepsis. Male CD-1 mice were subjected to CLP (1.5-cm cecum ligation and 16-gauge needle perforation) and resuscitated by the injection of 1 ml saline subcutaneously. Mice were treated with imipenem (25 mg/kg) in a variety of dosing strategies. (A) Mice ($n = 8$) were treated perioperatively with imipenem or an equal volume of PBS subcutaneously. (B) Mice ($n = 9$) were treated with three subcutaneous injections of imipenem or PBS, perioperatively and postoperatively, 3 and 6 h post-CLP. (C) Mice ($n = 9$) were given three subcutaneous injections of imipenem or PBS perioperatively and postoperatively 4, 24, and 48 h after CLP. (D) Mice received one intracecal injection of imipenem before puncture and a postoperative subcutaneous dose 2 h ($n = 18$) or 4 h ($n = 8$) post-CLP or an equal volume of PBS at each time point. Survival and core body temperature were continuously monitored for 72 h after CLP. Statistical significance was analyzed by a log rank test. Neither group showed a significant P value of <0.05 ($P = 0.85$ [A], $P = 0.18$ [B], $P = 0.26$ [C], and $P = 0.38$ [2 h] or $P = 0.59$ [4 h] [D]).

spleen obtained after 3 h of CLP from mice treated or not with imipenem (25 mg/kg) were analyzed for cytokine gene expression. As a control, sham-operated mice were injected with imipenem (25 mg/kg) or an equal volume of phosphate-buffered saline (PBS) and were also analyzed for cytokine expression. We did not observe significant differences in tumor necrosis factor alpha (TNF-α) mRNA levels in livers (Fig. 3A), lungs (Fig. 3D), and spleens (Fig. 3G) of mice treated or not treated with imipenem after CLP. TNF-α mRNA levels were elevated after CLP in mice that were treated or not treated with imipenem in comparison with sham-operated animals that were also injected with the antibiotic, displaying minimal levels of the cytokine (Fig. 3A, D, and G). In contrast, interleukin-6 (IL-6) and IL-10 mRNA levels were elevated in the lungs of mice treated with imipenem in comparison with PBS-injected mice after CLP (Fig. 3E and F). However, imipenem had no effect on sham-operated mice (Fig. 3E and F), suggesting that the elevation of IL-6 and IL-10 mRNA levels is not a direct effect of the antibiotic but rather is a result of the combination of the antibiotic and CLP. No differences in the levels of these two cytokines were observed in liver (Fig. 3B and C) and spleen (Fig. 3H and I) samples obtained from mice that were injected or not injected with imipenem after CLP or sham operation.

Bacterial debris contributes to mortality after CLP. As antibiotics did not impact survival in our mouse model, we set out to investigate the contribution of live bacteria to the outcome of CLP. We developed an alternative model of CLP to assess the contribution of the gut flora to the response to sepsis. The ceca of male CD-1 mice ($n = 14$) were ligated and excised, and the cecal contents were extracted and pooled from all mice. The cecal contents were then divided into two equal portions. One portion was

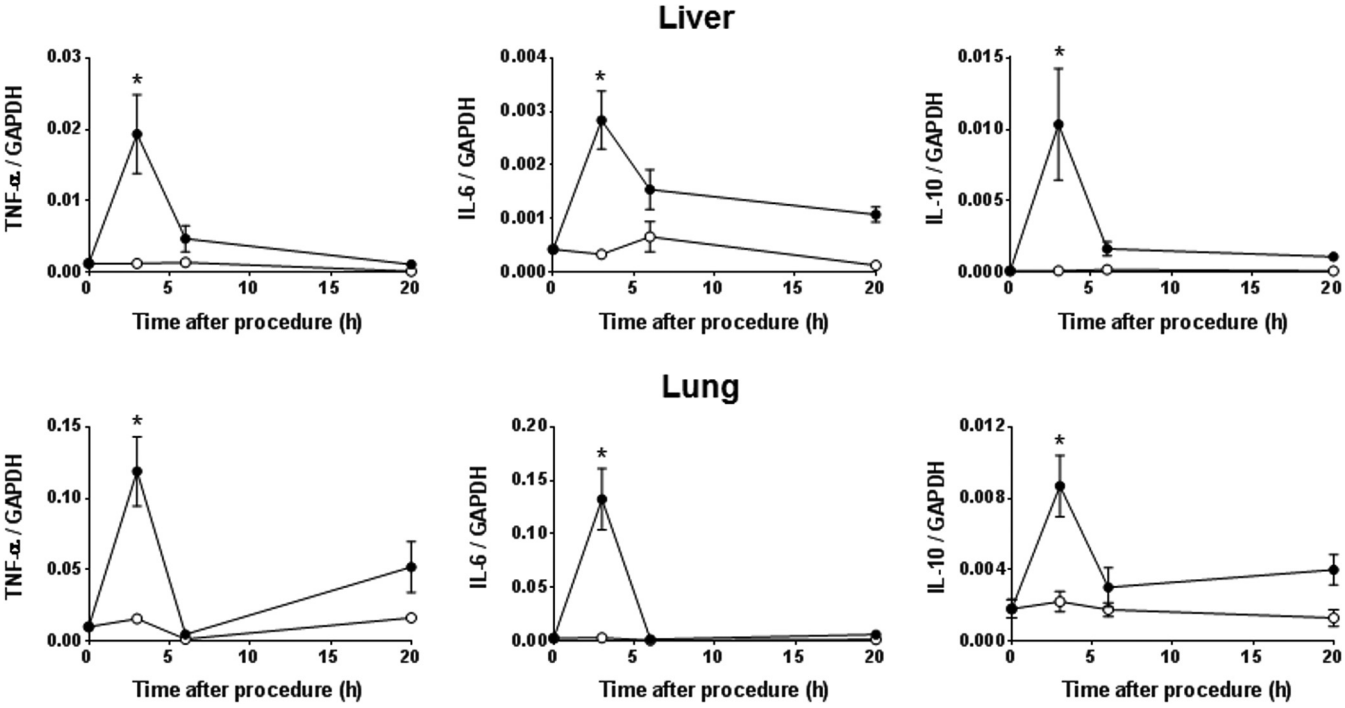

FIG 2 Kinetic expression of inflammatory mediators in the liver and lung of CD-1 mice following CLP. Male CD-1 mice were subjected to CLP (1.5-cm cecum ligation and 16-gauge needle perforation) or sham operation. Liver and lung samples were collected after perfusion with PBS 3, 6, and 20 h after CLP ($n = 5$ per time point) or sham operation ($n = 5$ per time point). Nonoperated mice ($n = 5$) were used to assess basal expression levels of inflammatory mediators (time zero). Total RNA was isolated and cDNA was prepared as described in Materials and Methods. Levels of TNF-α, IL-6, and IL-10 mRNAs in liver and lung were measured by qPCR. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used to normalize data to cDNA inputs. Results are expressed as means ± standard errors of the means, and statistical analysis for comparisons between groups was performed by two-way ANOVA followed by Tukey's multiple-comparison test. * indicates a P value of <0.05.

sterilized by UV radiation, and the other was sham irradiated as a control. UV radiation treatment resulted in the complete death of all bacteria within the cecal material, as assessed by growth on agar plates. New CD-1 mice ($n = 7$ per group) were then subjected to cecum ligation without puncture and injected with one-seventh of the sterilized (UV) or nontreated (NT) cecal contents in the peritoneal cavity, which was subsequently closed. As a control, mice were subjected to cecum ligation without perforation or the addition of cecal contents. Survival was monitored for 72 h. Mice treated with nonsterilized cecum contents showed 100% mortality within 26 h, whereas mice treated with sterilized cecal contents also showed 100% mortality but within 44 h of the procedure (Fig. 4). In both cases, the results were statistically significant in comparison with the results for mice with cecum ligation (no perforation) and no addition of cecal contents. Moreover, there was a significant difference between the times corresponding to 50% mortality between groups of mice treated (24 h) or not treated (40 h) with sterilized cecal contents. In a similar experiment, we measured cytokine expression levels in liver and lung samples 3 h after the procedure. Whereas significant reductions in TNF-α, IL-6, and IL-10 mRNA levels were observed in the livers of mice treated with UV-sterilized cecal contents as opposed to mice treated with nonsterilized cecal material (Fig. 5A), there were no differences in the levels of these cytokines in lung samples between both treatments (Fig. 5B). Data from these experiments suggest that live bacteria are not required for inducing mortality in this experimental model, but other factors may contribute to the induction of the inflammatory response.

Since UV radiation kills bacteria but is unlikely to affect the conformation of proteins within the cecal material, we repeated the experiment described above, using heat inactivation of cecal contents at 100°C for 30 min. This treatment also killed all bacteria within the cecal material and is likely to denature proteins as well.

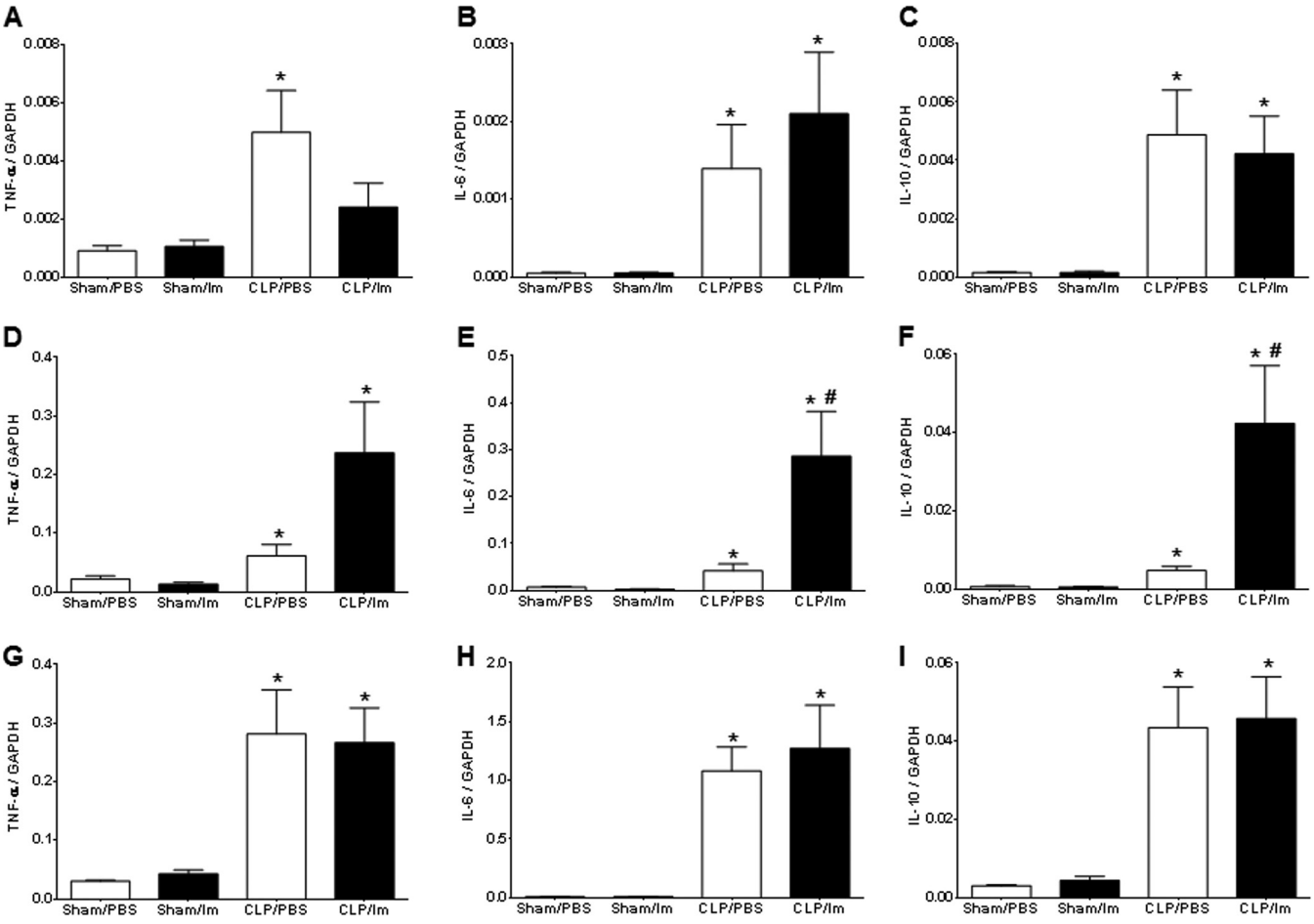

FIG 3 Imipenem differentially affects the expression of inflammatory mediators in liver, lung, and spleen following CLP. Male CD-1 mice ($n = 6$ in each group) were subjected to CLP (1.5-cm cecum ligation and 16-gauge needle perforation) or sham operation and injected perioperatively with imipenem (Im) (25 mg/kg) or an equal volume of PBS. Liver, lung, and spleen samples were collected after perfusion with PBS 3 h after CLP operation. Total RNA was isolated and cDNA was prepared as described in Materials and Methods. Levels of TNF-α (A, D, and G), IL-6 (B, E, and H) and IL-10 (C, F, and I) mRNAs in liver (A to C), lung (D to F), and spleen (G to I) were measured by qPCR. The GAPDH housekeeping gene was used to normalize data to cDNA inputs. Results are expressed as means $\pm$ standard errors of the means, and statistical analysis for comparisons between groups was performed by one-way ANOVA followed by Tukey's multiple-comparison test. *, $P < 0.05$ for CLP versus sham operation; #, $P < 0.05$ for CLP-imipenem versus CLP-PBS.

In both cases, 100% mortality was observed for mice treated with heat-inactivated (HI) or nontreated (NT) cecal contents. However, mice that were treated with non-heat-inactivated cecal contents died more rapidly than did mice treated with heat-inactivated and nontreated cecal contents (Fig. 6). Analysis of cytokine gene expression levels after this procedure showed no differences in TNF-α, IL-6, and IL-10 mRNA levels in liver samples, but the levels of these cytokines were elevated in lung samples of mice treated with HI cecal contents, as opposed to mice treated with NT cecal material (Fig. 7).

DISCUSSION

Sepsis is a multifactorial condition modulated by several components, including the age, sex, genetic background, and physical condition of the patient (18). Therefore, it is not surprising that therapeutic interventions directed at neutralizing single target molecules have failed to alleviate this detrimental condition (19). Therapy for sepsis remains dominated by aggressive supportive treatment in conjunction with source control, in which the use of antibiotics is a primary intervention. Although there is no doubt that antibiotic therapy has saved the lives of many critically ill patients, the timing of antibiotic treatment may be crucial for its success. Indeed, the Surviving Sepsis campaign calls for the timely administration of broad-spectrum antibiotics

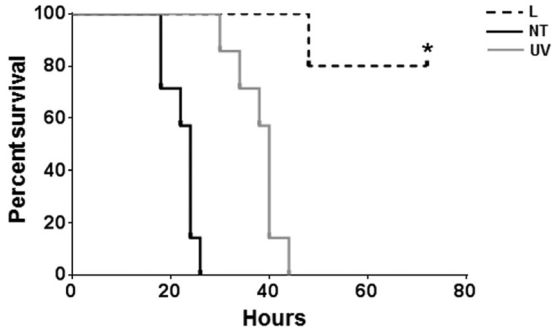

FIG 4 Sterilization of cecal contents by UV radiation does not prevent mortality. The ceca of male CD-1 mice ($n = 14$) were ligated and excised, and the cecum contents were extracted from all mice and pooled. Half of the pooled cecum content was sterilized by UV radiation (500 kJ for 4 h), and the other half was sham irradiated. CD-1 mice ($n = 7$ per group) were subjected to cecum ligation (no puncture), and one-seventh of the sterilized (UV) ($n = 7$) or nonsterilized (NT) ($n = 7$) cecum contents was introduced into the peritoneal cavity, which was subsequently closed. As a control, mice were subjected to cecum ligation without perforation and without the addition of cecal contents (L) ($n = 5$). Survival and core body temperature were continuously monitored for 72 h after CLP. Statistical significance was analyzed by a log rank test, and * denotes a P value of <0.05.

(http://www.survivingsepsis.org/). Clinical studies indicated that early therapy interventions such as antibiotic treatment increased survival from injury (20–24), although data from other studies do not sustain these findings completely (25, 26). Recently, a large clinical study confirmed that the early identification and treatment of sepsis, including the use of antibiotics within the first 3 h of admission, improved outcomes substantially (27). However, concerns have also been raised regarding the prolonged use of antibi-

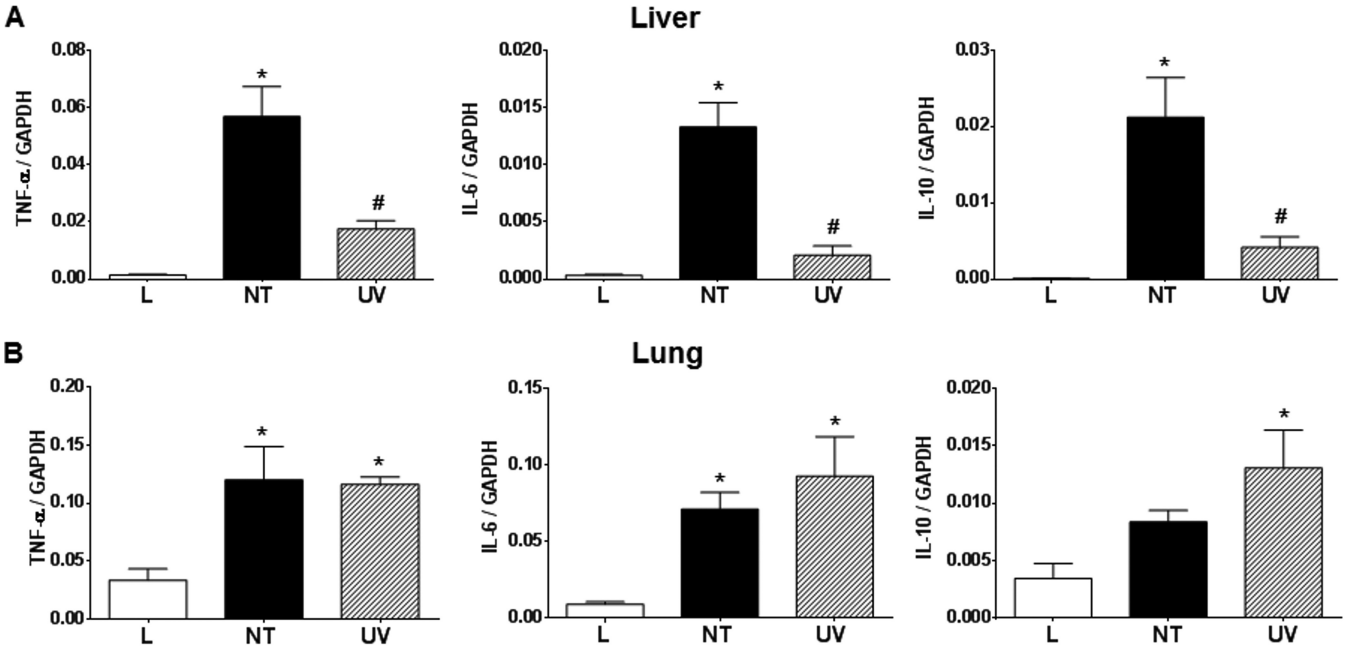

FIG 5 Sterilization of cecal contents by UV radiation differentially affects the inflammatory response. The ceca of male CD-1 mice ($n = 10$) were ligated and excised, and the cecum contents were extracted from all mice and pooled. Half of the pooled cecum content was sterilized by UV radiation (500 kJ for 4 h), and the other half was sham irradiated as a control. CD-1 mice ($n = 5$ per group) were subjected to cecum ligation, and one-fifth of the sterilized (UV) or nonsterilized (NT) cecum contents was introduced into the peritoneal cavity, which was subsequently closed. As a control, mice were subjected to cecum ligation without perforation (L) and without the addition of cecal contents. Liver and lung samples were collected after perfusion with PBS 3 h after the injection of the sterilized or nonsterilized cecum contents. Total RNA was isolated and cDNA was prepared as described in Materials and Methods. Levels of TNF-α, IL-6, and IL-10 mRNAs in liver (A) and lung (B) were measured by qPCR. The GAPDH housekeeping gene was used to normalize data to cDNA inputs. Results are expressed as means $\pm$ standard errors of the means, and statistical analysis for comparisons between groups was performed by one-way ANOVA followed by Tukey's multiple-comparison test. *, $P < 0.05$ for NT or UV treatment versus cecum ligation without perforation; #, $P < 0.05$ for NT versus UV treatment.

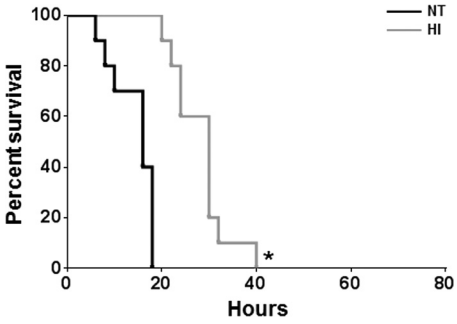

FIG 6 Sterilization of cecal contents by heat inactivation does not prevent mortality. The ceca of male CD-1 mice ($n = 20$) were ligated and excised, and the cecum contents were extracted from all mice and pooled. Half of the pooled cecum content was sterilized by heat inactivation (HI) (100°C for 30 min), and the other half was kept at 25°C as a control. CD-1 mice ($n = 10$ per group) were subjected to cecum ligation, and 1/10 of the sterilized or nonsterilized cecum contents was introduced into the peritoneal cavity, which was subsequently closed. Survival was continuously monitored for 72 h after CLP. Statistical significance was analyzed by a log rank test, and * denotes a P value of <0.05.

otics as well as their administration when it is not necessary due to the increasing incidence of antibiotic-resistant pathogens (28, 29).

Antibiotic treatment has been included in several experimental animal models of sepsis to more closely mirror the supportive care that patients receive in the clinical setting (14). However, it could be argued that it is very difficult to reproduce all supportive interventions included in the care of critically ill patients in an experimental rodent model. The inclusion of antibiotics in an experimental animal model might add an additional variable that could obscure the cellular and molecular mechanisms involved in the septic response. In addition, the inclusion of antibiotics in these experimental animal models has varied tremendously with regard to the type of antibiotic, dose, route, and even dosing schedule. Some studies demonstrated improved outcomes (16, 17, 30, 31) or a delay in mortality (32) after CLP in the presence of antibiotics, whereas other studies did not show an improved survival rate (33). Thus, the efficacy of antibiotic treatment in an experimental murine model remains unclear. In the present study, we evaluated systematically the contribution of antibiotics in an acute model of sepsis induced by CLP. We used imipenem as the antibiotic of choice for its broad spectrum, covering most enteric organisms, and because it was previously used in various studies (16, 17, 30–33). We did not observe any improvement in survival after CLP by using a variety of antibiotic treatment protocols, including periprocedural, preprocedural, multiple-interval-dosing postprocedural, and even intracecal protocols. We also observed that mice treated with imipenem after CLP displayed elevated levels of IL-6 and IL-10 in the lungs in comparison with those in septic mice in the absence of antibiotic treatment. However, this increase in cytokine levels after CLP and imipenem treatment appears to be due to a combination of both factors rather than a direct effect of the antibiotic itself, since imipenem did not increase cytokine levels when administered to sham-operated mice. Regardless of this observation, previous studies showed an effect of antibiotics on cytokine expression in human monocytes (34) and polymorphonuclear neutrophils (35) and under culture conditions and in whole blood *ex vivo* (36). Consequently, we concluded that the use of antibiotics in this acute model of sepsis is not necessary and may add another confounding factor to an already complex response. However, we do not want to discourage the use of antibiotics in low-injury long-term experimental animal models.

Since antibiotic treatment was ineffective in our experimental animal model, we investigated whether the presence of live bacteria in the cecal contents was necessary for the development of septic shock in mice. Using a variant of the CLP model in which the cecum was ligated but not perforated and the cecum content was delivered directly into the peritoneal cavity, we found that sterilization of the cecal contents did not reverse the mortality associated with our model. Indeed, cecal content sterilization by

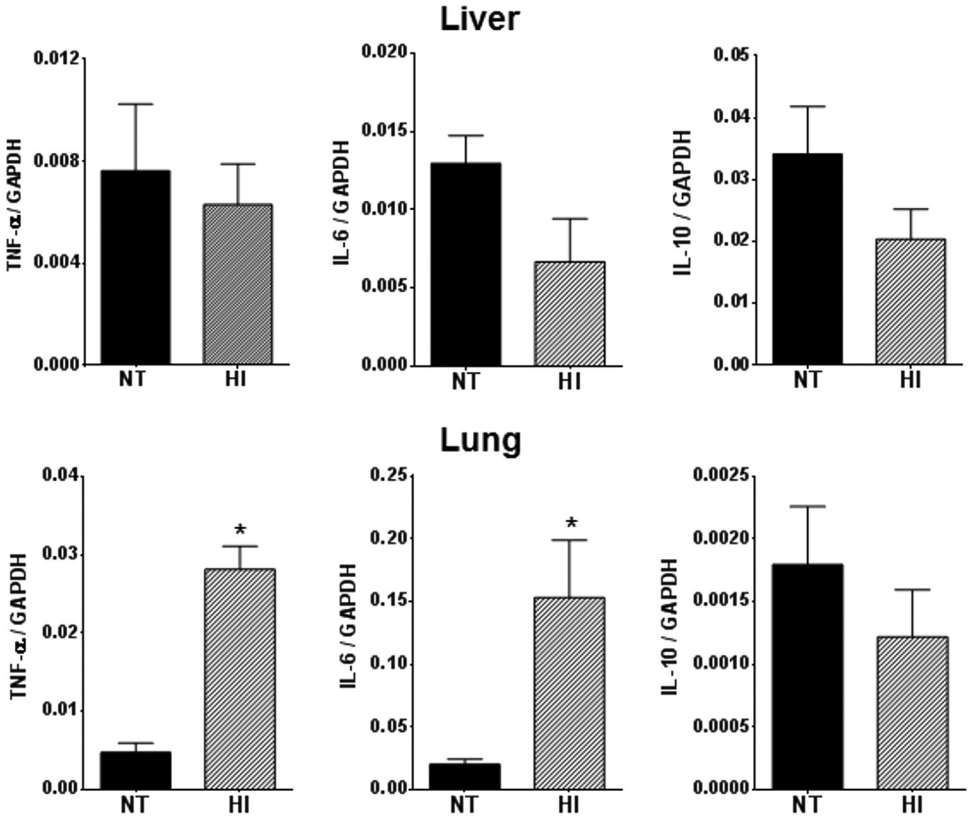

FIG 7 Sterilization of cecal contents by heat inactivation increases the expression of proinflammatory mediators in the lung. The ceca of male CD-1 mice ($n = 10$) were ligated and excised, and the cecum contents were extracted from all mice and pooled. Half of the pooled cecum content was sterilized by heat inactivation (HI) (100°C for 30 min), and the other half was kept at 25°C as a control. CD-1 mice ($n = 5$ per group) were subjected to cecum ligation, and one-fifth of the sterilized (HI) or nonsterilized (NT) cecum contents was introduced into the peritoneal cavity, which was subsequently closed. Liver and lung samples were collected after perfusion with PBS 3 h after injection of the sterilized or nonsterilized cecum contents. Total RNA was isolated and cDNA was prepared as described in Materials and Methods. Levels of TNF-α, IL-6, and IL-10 mRNAs in liver and lung were measured by qPCR. The GAPDH housekeeping gene was used to normalize data to cDNA inputs. Results are expressed as means $\pm$ standard errors of the means, and statistical analysis for comparisons between groups was performed by using an unpaired Student *t* test. * indicates a *P* value of <0.05 for HI versus NT.

UV radiation or exposure to high temperatures (heat inactivation) led to 100% mortality in mice, as was observed for mice receiving the same amount of nonsterilized cecum contents. These observations suggest that septic shock is associated not only with bacterial proliferation but also with the release of bacterial debris or components that activate a robust inflammatory response. Previous studies stated that bacterial lysis leads to endotoxin release, augmenting the inflammatory response (37, 38), particularly after the administration of antibiotics (39). Certainly, the injection of sterile endotoxin triggers a robust inflammatory response and high mortality rates in mice (40). Other studies reported that the profile of mice after administration of sterile *Streptococcus pneumoniae* cell wall material in the cerebrospinal fluid resembled the clinical profile of aseptic meningitis (41). In addition, nucleic acids, particularly mitochondrial DNA, have emerged as additional factors that modulate the inflammatory response (42). Bacterial debris or components have been named pathogen-associated molecular patterns, which are recognized by receptors on immune cells and endothelial cells, called pattern recognition receptors, that activate a robust inflammatory response (43). In this regard, we observed a very interesting pattern of inflammatory responses when mice were challenged with sterilized or nonsterilized cecal contents. Although we observed no differences in cytokine gene expression levels in the lungs of mice exposed to cecal contents that were UV sterilized or not, the response was diminished in mouse livers

incubated with UV-sterilized cecal material, as opposed to nonsterilized cecal contents. In contrast, an increase in cytokine expression levels was observed in the lungs after exposure to heat-inactivated cecal contents in comparison with non-heat-inactivated cecal contents, whereas no differences were observed for the liver. The difference between the two sterilization procedures may be related in that UV treatment kills bacteria without the denaturation of proteins but likely affects nucleic acids. On the other hand, heat inactivation not only kills bacteria but also denatures proteins. Although the nature of the factors in the sterilized cecal contents that modulate the inflammatory response is not known, it is possible that the inflammatory response is mediated by lipids, the molecular nature of which is unlikely to be affected by either UV radiation or high-heat exposure. However, other components, such as proteins and nucleic acids, cannot be discarded. Consequently, it is important to elucidate the nature of these factors since their neutralization may result in a second layer of therapeutic interventions to ameliorate the detrimental consequences of sepsis.

Our observations suggest that the development of septic shock is more complex than the proliferation of pathogens and perhaps demonstrate why antibiotic therapy is not totally effective in controlling sepsis. However, we do not argue that the administration of antibiotics is not necessary for the treatment of septic patients. On the contrary, we advocate that additional therapeutic approaches should be combined with the early administration of antibiotics. In addition, our observations may add experimental evidence for the incidence of septic shock in the absence of positive bacterial cultures. Finally, we emphasize the importance of early interventions for the control of sepsis. Previous investigations using experimental animal models indicated that early aggressive fluid resuscitation (44–46) or pentoxifylline administration (47, 48) resulted in a salutary benefit for injury. Moreover, we demonstrated experimentally that the therapeutic window for overcoming sepsis is as early as 6 h after the initiation of sepsis by CLP (9) and in other similar models (49), which may reflect the old concept of the "golden hour" (50).

Although early antibiotic administration, combined with source control and other aggressive forms of supportive care, improves clinical outcomes in septic patients, our results indicate that it does not improve survival in an experimental animal model. The true impact of antibiotics on the inflammatory cascade is not completely understood and needs to be further investigated. However, antibiotics may add an unnecessary confounder in studies of the mechanisms underlying inflammation and sepsis in experimental models. Indeed, the administration of antibiotics might hinder the molecular and cellular mechanisms responsible for poor outcomes in sepsis, as has been shown for the prolonged use of anesthetics (51, 52) and analgesics (53).

MATERIALS AND METHODS

Animal model. Male CD-1 mice (8 weeks old) were obtained from Charles River Laboratories (San Diego, CA, USA) and maintained under pathogen-free conditions at the University of California, San Diego (UCSD), Animal Facility (La Jolla, CA, USA). Experiments were approved by the UCSD Institutional Animal Care and Use Committee. Mice were fasted for 16 h prior to the procedure. Animals were anesthetized with isoflurane, and a 2-cm laparotomy was made to expose the cecum, which was ligated 1.5 cm from the end and perforated by using a 16-gauge needle. The cecum was placed back into the peritoneum cavity, which was closed in one layer with silk sutures. A temperature probe was placed under the skin, and the skin was closed over the probe. The mice were continuously monitored for changes in core body temperature for 72 h after surgery, and mortality was recorded following this period of observation (9). Mice were treated or not treated with imipenem (25 mg/kg), which was selected for its broad-spectrum coverage of enteric bacteria. The dose of imipenem used was also used in previous studies (16). A variety of dosing strategies based on previously described studies was chosen, including subcutaneously perioperative intervals (within 1 h of surgery), subcutaneously perioperative and postoperative intervals (3 and 6 h), and intracecal injection prior to puncture followed by subcutaneously postoperative intervals. Control animals were injected with an equal volume of saline. In some experiments, livers and lungs were harvested for cytokine analysis by quantitative real-time PCR (qPCR) as previously described (9).

Sterilization of cecal contents. To observe the impact of the exposure of the peritoneum to sterile bacterial debris on survival, cecal ligation without puncture was performed, followed by the administration of sterilized versus nonsterilized cecal contents in the peritoneal cavity. Cecal contents were procured from separate mice the day prior to experimentation. The cecum was ligated 1.5 cm from the

tip and excised. The cecal contents from all mice were combined and separated into two groups for sterilization or no sterilization. The fecal mixture was then placed into a 10-cm petri dish, spread thin, and exposed to UV radiation (500 kJ for 4 h). Alternatively, the cecal contents were heat inactivated (100°C for 30 min). The extent of sterilization was assessed by bacterial growth on both plates. The sterilized or sham-sterilized cecal contents were further divided according to the number of mice that were used (one cecum donor and one cecum acceptor). Mice were subjected to laparotomy and cecum ligation (no perforation), the sterilized or nonsterilized cecal contents were added to the peritoneum, and the abdomen was closed in two layers as described above. Body temperature and mortality were monitored for 72 h.

Statistical analysis. All data were analyzed by using GraphPad Prism software (GraphPad Software, San Diego, CA). Significance was analyzed by using Student's unpaired *t* test, one-way analysis of variance (ANOVA), or two-way ANOVA followed by Tukey's multiple-comparison test. A P value of <0.05 was considered statistically significant. Statistical significance for comparisons of survival rates was analyzed by the log rank (Mantel-Cox) test.

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