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Circulating Cytokine/Inhibitor Profiles Reshape the Understanding of the SIRS/CARS Continuum in Sepsis and Predict Mortality[1]

Marcin F. Osuchowski,* Kathy Welch,† Javed Siddiqui,* and Daniel G. Remick[2]*

Mortality in sepsis remains unacceptably high and attempts to modulate the inflammatory response failed to improve survival. Previous reports postulated that the sepsis-triggered immunological cascade is multimodal: initial systemic inflammatory response syndrome (SIRS; excessive pro-, but no/low anti-inflammatory plasma mediators), intermediate homeostasis with a mixed anti-inflammatory response syndrome (MARS; both pro- and anti-inflammatory mediators) and final compensatory anti-inflammatory response syndrome (CARS; excessive anti-, but no/low proinflammatory mediators). To verify this, we examined the evolution of the inflammatory response during the early phase of murine sepsis by repetitive blood sampling of septic animals. Increased plasma concentrations of proinflammatory (IL-6, TNF, IL-1β, KC, MIP-2, MCP-1, and eotaxin) and anti-inflammatory (TNF soluble receptors, IL-10, IL-1 receptor antagonist) cytokines were observed in early deaths (days 1–5). These elevations occurred simultaneously for both the pro- and anti-inflammatory mediators. Plasma levels of IL-6 (26 ng/ml), TNF-α (12 ng/ml), KC (33 ng/ml), MIP-2 (14 ng/ml), IL-1 receptor antagonist (65 ng/ml), TNF soluble receptor I (3 ng/ml), and TNF soluble receptor II (14 ng/ml) accurately predicted mortality within 24 h. In contrast, these parameters were not elevated in either the late-deaths (day 6–28) or survivors. Surprisingly, either pro- or anti-inflammatory cytokines were also reliable in predicting mortality up to 48 h before outcome. These data demonstrate that the initial inflammatory response directly correlates to early but not late sepsis mortality. This multifaceted response questions the use of a simple proinflammatory cytokine measurement for classifying the inflammatory status during sepsis. *The Journal of Immunology,* **2006, 177: 1967–1974.**

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Despite rapid progress in health care over the past decades, sepsis continues as a major life-threatening condition in acute care patients. Although overall survival rates improved, between 1979 and 2000, the total sepsis-related mortality rose from 22 to 44 per 100,000 population (1), accounting for ~9% of the overall annual mortality in the United States alone (2, 3). Given this substantial mortality and economic costs, a better understanding of the basic immune alterations in sepsis may help to direct therapy.

Modulation of inflammatory signaling during sepsis has been considered as a possible means to improve survival in sepsis and prevent septic shock. Despite promising results of anti-inflammatory interventions in animal models (4–6) and human phase I and II trials (7, 8), phase III clinical trials generally failed to show any success (9–11) and in some cases even appeared detrimental (12). It has been postulated that the immune response in sepsis represents the interplay of two contrasting phenomena related to the inflammatory status of the septic patient. The early systemic inflammatory response syndrome (SIRS)[3] is characterized by excessive production of proinflammatory mediators (hyperinflammatory status). This early response is then progressively suppressed by the development of the compensatory anti-inflammatory response (hypoinflammatory status) syndrome (CARS) (13–15). This hypothesis was further supplemented by another acronym: the mixed anti-inflammatory response syndrome (MARS) representing temporary homeostasis between diminishing SIRS and ascending CARS (15, 16). However, these definitions are largely nonspecific and although undeniably useful in clinical and research settings, they require in-depth experimental verification before they can be accepted as an accurate portrayal of the complex immune fluctuations encountered in sepsis.

Retrospective statistical analyses of the unsuccessful phase III sepsis trials revealed a strong relationship between the treatment effect and risk of death, and attributed this discrepancy in mortality risk between preclinical testing and human clinical trials as a potential factor contributing to the failure of anti-inflammatory therapies (10, 17). The benefits of the anti-inflammatory intervention on survival were apparent in studies conducted with a high risk of death (18). Because the aggressive anti-inflammatory (or alternative) therapy would predominantly benefit patients with unfavorable outcome, the patient stratification before the implementation of nonstandard anti-inflammatory treatment is therefore critical. Despite certain shortcomings (15), plasma remains one of best sources for measurement of sepsis-related mediators enabling a rapid characterization of the patient's inflammatory status. The increase in plasma levels of several inflammatory mediators is proportional to the sepsis severity and correlates with mortality (15). Using the cecal ligation and puncture (CLP) model of murine sepsis, we have previously demonstrated the robust prognostic value

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[3] Abbreviations used in this paper: SIRS, systemic inflammatory response syndrome; MARS, mixed anti-inflammatory response syndrome; CARS, compensatory anti-inflammatory response syndrome; CLP, cecal ligation and puncture; ED, early death; LD, late death; SUR, survivor; IL-1ra, IL-1 receptor antagonist; ROC, receiver operating characteristic curve; AUC, area under the curve; CI, confidence interval; SR, soluble receptor.

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of IL-6 for determining lethality (19–21). Studies in septic patients further support such prognostic potential based on IL-6 and other (e.g., TNF-α, IL-1) cytokines (22–24).

In previous studies, a simple plasma measurement served as the basis to define the status of the immune response during ongoing sepsis (25) and patients are considered hyperinflammatory if proinflammatory cytokines are found at elevated levels. We now report that the early phase of lethal sepsis is characterized by the overexpression of both proinflammatory as well as anti-inflammatory cytokines. The rapid concurrent release of these pro- and anti-inflammatory mediators represents a central feature of the inflammatory response at the onset of sepsis. Defining the inflammatory status on the basis of a more complete plasma cytokine profile yields accurate prognostic information even if these values do not clearly define subjects into the traditional SIRS or CARS categories. In the current studies, we show that early mortality predictions based on plasma biomarkers during the early phase (days 1–5) of sepsis can be highly accurate, supporting an individualized, prognostic-based therapy as a potentially new strategy in the treatment of sepsis.

Materials and Methods

Animals

Female ICR mice (Harlan Sprague Dawley) with an average weight of 22 g were used. The mice were acclimated to the laboratory environment for at least 48 h before surgery and housed in a temperature-controlled room with a 12-h light-12-h dark diurnal cycle after the procedure. All experiments were in accordance with National Institutes of Health guidelines and the University of Michigan Animal Care and Use Committee.

Sepsis model

The murine model of CLP used in the study closely emulates polymicrobial human sepsis and has been extensively used by others to investigate the immunopathology of sepsis (19–21, 26, 27). The 18-gauge needle size was used to produce ~50% mortality during early sepsis. We followed the original CLP protocol (28) with previously described modifications (19).

Sampling

To collect peripheral blood, the distal tail was clipped (~2 mm) and 20 μl of blood was drawn into a pipette rinsed with EDTA (169 mM tripotassium salt). Mice were not sacrificed at the time of sampling. Blood was collected 6 h after CLP and at 24-h intervals for the first 5 days. The last individual cytokine measurement represents an animal that died within the 24 h after sampling. Samples were immediately diluted 1/10 in PBS with a 1/50 dilution of EDTA, centrifuged (5 min/1000 × g in 4°C), the plasma was removed and stored at −20°C until analysis.

Sequential ELISA

We developed a sequential ELISA to allow measurement of multiple cytokines from small volumes (29). The assay dramatically increases the number of biomarkers which may be measured in one sample by repeatedly reusing this sample in multiple successive cycles (30). The assay was based on the standard ELISA previously optimized for cytokine detection (31) and used commercially available matched Ab pairs (R&D Systems) as detailed elsewhere (29).

Statistical analysis

The Kaplan-Meier 28 day-survival curve (see Fig. 1) was plotted using Prism 4 (GraphPad Software). For early deaths (ED; dead by day 5), every data point shown in Figs. 2–4 represents the average concentration taken from the animals that died within the next 24 h, indicating a different group of animals at each time point. For the late death group (LD; dead after day 5) and survivors (SUR, lived to 28 days), the average concentration values at each time point are based on repetitive measurements taken from the same animals. The cytokine levels measured for the LD group and SUR largely overlapped, they were therefore pooled for comparison to the ED group. To determine whether the data for the LD and SUR groups could be pooled ($p > 0.1$ pooled, $p \leq 0.1$ separate) and for comparisons of cytokine values between the ED and the pooled (or separate) values for LD and SUR, the nonparametric Wilcoxon rank-sum test (rather than t tests) was used because neither the original or log-transformed cytokine values were normally distributed. The values for the LD and SUR groups were pooled for all cytokines at all time points except: IL-10 at each time point, IL-1β and IL-1 receptor antagonist (IL-1ra) at 48 and 72 h only. For all figures (except 4) and tables, $n = 90$ for IL-6, TNF-α, KC, and IL-1ra and $n = 80$ for the remaining cytokines. Fig. 4 is based on $n = 80$. When the ED group was compared with remaining groups separately (data not pooled), the higher p value is listed on the graph.

The receiver operating characteristic curve (ROC curve) was used to evaluate the prognostic accuracy (defined by the area under the curve; AUC) of the analyzed cytokines and to determine the sensitivity and specificity (in percent) at selected cutoff values. The 95% confidence intervals (CI) for the AUC values were estimated using the conservative bootstrap bias-corrected and accelerated method to obtain more accurate intervals (32, 33). The accuracy of the ROC-AUC test is: 0.9–1 = excellent, 0.8–0.9 = good, 0.7–0.8 = fair, 0.6–0.7 = poor and <0.6 = not useful. The inflammatory cytokines in Fig. 4 were normalized (each mediator individually) to the cutoff value listed in Table II (24 h cutoff) for each cytokine. This cutoff value represents the threshold for predicting the survival of the animal. The normalized values were added for each animal across all pro- (IL-6, TNF-α, IL-1β, KC, MIP-2, MCP-1, and eotaxin) and anti-inflammatory (IL-1ra, IL-10, TNF soluble receptors (SR) I and II) cytokines. Because the CLP-dependent activation of IL-6R resulted in its decrease rather than an increase, these values were excluded from the anti-inflammatory segment. Data are expressed as the mean ± SEMs wherever applicable. All statistical analyses were performed using SAS release 9.1.2 on Windows.

Results

Mortality after CLP-dependent sepsis

A total of 90 mice were subjected to CLP on day 1 and monitored for 28 days. The early phase of sepsis was defined as the first 96 h after CLP, days 1–5. We selected the genetically heterogeneous ICR outbred strain of mice to generate a more diverse profile of immune responses, and more closely simulate the human population. The mortality at day 5 post-CLP reached 38%, and the overall mortality at the end of the study (day 28) was 62% (56/90 mice, dead/total, Fig. 1). It has been postulated that the early, post-CLP mortality results primarily from SIRS, whereas the late mortality is predominantly CARS dependent (15, 34). The mortality related to the acute phase of sepsis (ED, days 1–5) was separated from the late phase lethality (LD, days 6–28) based on the distribution of deaths found in our previous studies (19, 21, 35–37). This pattern was confirmed in the current experiment: 61% of total deaths (34/56 mice) in the study occurred during the first 5 days, whereas only 22 mice died between days 6 and 28 (24% mortality rate, Fig. 1). The single day mortality was highest between days 2 and 3 ($n = 15$).

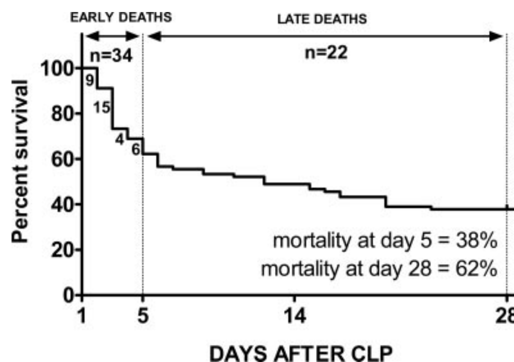

FIGURE 1. Twenty-eight-day mortality after CLP-dependent sepsis. CLP was performed with an 18-gauge needle to produce ~50% mortality during the early phase of sepsis. Mortality was monitored for 28 days. The deaths were separated into two groups: early (days 1–5) or late (days 6–28). Total $n = 90$.

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Proinflammatory cytokine profiles in ED, LD, and SUR

It has been reported that mice subjected to CLP display high levels of proinflammatory cytokines such as IL-6, TNF-α, or MIP-2 (38, 39). We developed a sequential ELISA method (29) which enabled us to monitor changes of multiple inflammatory mediators from a single, 20-μl blood sample. The graphs in Figs. 2 and 3 were generated by averaging the individual cytokine profiles from all animals. Importantly, mice were not sacrificed for any of the data in this experiment and each animal was followed separately for 28 days or until death. Based on survival outcome, mice were retrospectively divided into three groups: dead by day 5, dead after day 5, and alive at 28 days (SUR). The proinflammatory mediators profiled in the study (Fig. 2) represent cytokines (IL-6, TNF-α, and IL-1β), CXC chemokines (KC and MIP-2), and CC chemokines (MCP-1 and eotaxin). Dividing the mice into the ED, LD, and SUR provided significant insights into potential mechanisms of mortality. Mice dying in the early phase of sepsis had significantly higher plasma cytokine levels for virtually all proinflammatory mediators (Fig. 2, *A–F*) when compared with the SUR and LD groups. The cytokine increase was especially robust for IL-6 (Fig. 2*A*) such that levels in the ED group were 20-fold higher compared with the LD and SUR at the 24-h time. In LD group, the post-CLP cytokine levels were nearly superimposable with those animals who survived to 28 days. These results indicate that while the ED are directly correlated to significant production of proinflammatory cytokines, the late mortality was not correlated with the proinflammatory status during the initial phase of sepsis. These experimental data corroborate the widely held hypothesis that ED are due to an exuberant, proinflammatory response, but the next set of data show that these proinflammatory measurements fail to disclose the full story.

Anti-inflammatory cytokine profiles in ED, LD, and SUR

Although the proinflammatory mediators correlate with sepsis mortality, elevations in the levels of anti-inflammatory cytokines may also indicate a poor outcome (40–42). In our study, changes in the cytokine inhibitors mirrored those observed with the proinflammatory cytokines. Specifically, the temporal change of cytokine inhibitors in the LD and SUR were significantly lower than those mice dying in early sepsis (Fig. 3). There was an increase in the plasma levels of IL-1ra, IL-10, and TNF SR I and II (Fig. 3,

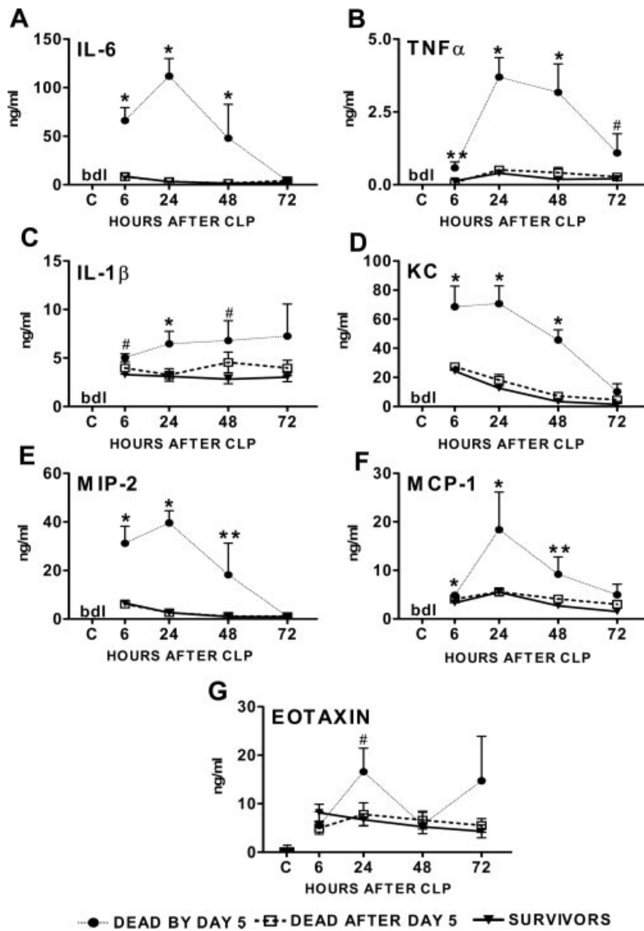

FIGURE 2. Temporal profiles of proinflammatory cytokines based on the outcome. Mice were divided into three groups based on outcome: ED (dead by day 5), LD (dead after day 5), and SUR (alive at 28 days). Data are presented as the mean ± SEM. For the ED, n varies between time points: at 6 h $n = 9$, at 24 h $n = 15$, at 48 h $n = 4$, and at 72 h $n = 5$, for IL-6, TNF-α, KC, and MCP-1. For MIP-2 and eotaxin, n is lower at 6 h ($n = 7$) and 24 h ($n = 14$) and 73 h ($n = 5$) time points. For the LD and SUR groups, the n is consistent at all time points: $n = 22$ and $n = 34$, respectively, for IL-6, TNF-α, KC, and MCP-1; $n = 20$ and $n = 30$, respectively, for MIP-2 and eotaxin. *, $p < 0.0001$; **, $p < 0.005$; #, $p < 0.05$ comparing ED to the pooled results from the LD and SURs. Bdl, below detectable limit. *A*, IL-6; *B*, TNF-α; *C*, IL-1β; *D*, KC; *E*, MIP-2; *F*, MCP-1; *G*, eotaxin.

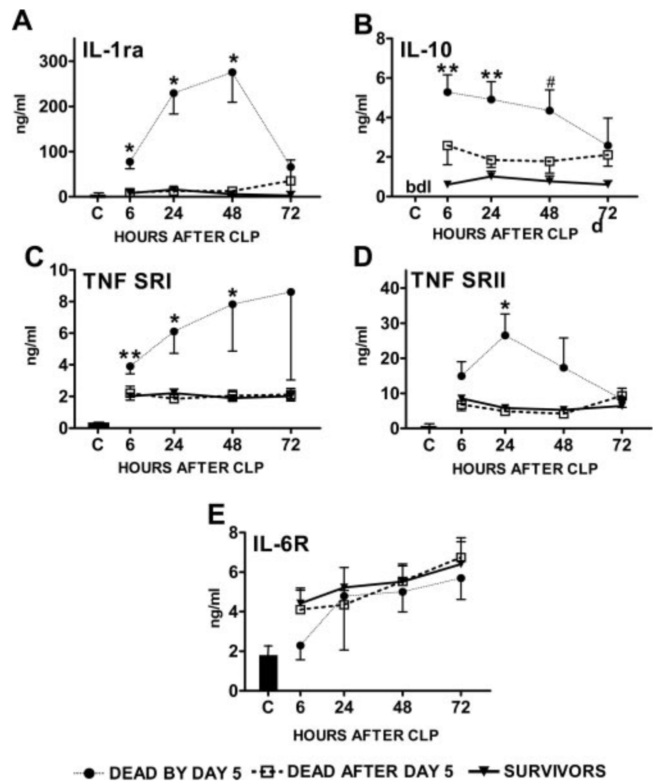

FIGURE 3. Temporal profiles of anti-inflammatory cytokines based on the outcome. The anti-inflammatory cytokines were graphed to demonstrate the development of the cytokine inhibitor response during the early phase of sepsis. Mice were divided into three groups based on outcome: ED (dead by day 5), LD (dead after day 5), and SUR (lived 28 days). Data were analyzed similar to Fig. 2 and are presented as mean ± SEM. For the ED, n varies between time points: at 6 h $n = 7$ ($n = 9$ for IL-1ra), at 24 h $n = 14$ ($n = 15$ for IL-1ra), at 48 h $n = 4$ and at 72 h $n = 6$ ($n = 5$ for IL-1ra). For the LD and SUR, n is consistent at all time points: $n = 22$ ($n = 20$ for IL-1ra) and 34 ($n = 30$ for IL-1ra), respectively. *, $p < 0.0001$; **, $p < 0.005$; #, $p < 0.05$ comparing ED to the pooled results from the LD and SUR. Bdl, below detectable limit. *A*, IL-1ra; *B*, IL-10; *C*, TNF SRI; *D*, TNF SRII; *E*, IL-6R.

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A–D). The concentration of IL-6R showed a unique trend with lower levels in the ED group (Fig. 3*E*). As in the case of the proinflammatory cytokines, these intergroup differences were already significant at the 6-h time point. The mortality during early sepsis coincided especially well with the levels of IL-1ra and the increase at 24 h was ~11-fold higher when compared with the other two groups. The profiles of IL-1ra, TNF SR I and II, and IL-6R in mice dying after day 5 were virtually identical with the corresponding proinflammatory cytokine profiles in surviving animals. These results emphasize the tight correlation between the activation of anti-inflammatory signaling and mortality in the acute stages of sepsis. The evidence from Figs. 2 and 3 clearly indicates that the early inflammatory response accurately predicts early mortality but lacks prognostic value for deaths occurring in the late phase of the disease. This suggests that the late outcome is not "preprogrammed" during the early phase of sepsis.

Plasma pro- and anti-inflammatory cytokines predict early mortality

Using the murine CLP model, we (21) and others (43) previously demonstrated that an increase in plasma IL-6 6 h after initiation of sepsis predicts early phase mortality. We wished to confirm these findings in the outbred ICR mice and to examine other potential biomarkers, including proinflammatory cytokines, chemokines, and anti-inflammatory cytokines (Table I), for their use in predicting outcome. As in our previous results (21), the mortality during the first 5 days was examined. ROC curves correlated the plasma

Table I. *Prediction of early sepsis mortality (day 5) based on cytokine values obtained 6 h after CLP*

Cytokine	Parameter	Value[a]
Proinflammatory cytokines		
IL-6[b]	AUC-ROC	0.92 (0.81–0.96)
	Cutoff (pg/ml)	13,000
	Sensitivity (%)	82
	Specificity (%)	86
TNF-α[b]	AUC-ROC	0.63 (0.52–0.75)
	Cutoff (pg/ml)	850
	Sensitivity (%)	Not relevant
	Specificity (%)	Not relevant
IL-1β[c]	AUC-ROC	0.63 (0.42–0.72)
	Cutoff (pg/ml)	4,900
	Sensitivity (%)	Not relevant
	Specificity (%)	Not relevant
Proinflammatory chemokines		
KC[b]	AUC-ROC	0.91 (0.81–0.96)
	Cutoff (pg/ml)	36,000
	Sensitivity (%)	82
	Specificity (%)	95
MIP-2[c]	AUC-ROC	0.88 (0.76–0.94)
	Cutoff (pg/ml)	14,000
	Sensitivity (%)	72
	Specificity (%)	91
MCP-1[b]	AUC-ROC	0.88 (0.78–0.94)
	Cutoff (pg/ml)	3,220
	Sensitivity (%)	72
	Specificity (%)	87
Eotaxin[c]	AUC-ROC	0.50 (0.38–0.59)
	Cutoff (pg/ml)	4,400
	Sensitivity (%)	Not relevant
	Specificity (%)	Not relevant
Anti-inflammatory cytokines		
IL-1ra[b]	AUC-ROC	0.87 (0.76–0.93)
	Cutoff (pg/ml)	17,000
	Sensitivity (%)	71
	Specificity (%)	84
IL-10[c]	AUC-ROC	0.85 (0.72–0.93)
	Cutoff (pg/ml)	1,950
	Sensitivity (%)	84
	Specificity (%)	82
TNF SRI[c]	AUC-ROC	0.56 (0.46–0.70)
	Cutoff (pg/ml)	3,000
	Sensitivity (%)	Not relevant
	Specificity (%)	Not relevant
TNF SRII[c]	AUC-ROC	0.63 (0.51–0.75)
	Cutoff (pg/ml)	14,000
	Sensitivity (%)	Not relevant
	Specificity (%)	Not relevant
IL-6R[d]	AUC-ROC	0.67 (0.55–0.78)
	Cutoff (pg/ml)	2,200
	Sensitivity (%)	64
	Specificity (%)	76

[a] Sensitivity/specificity were not listed if UAC <0.7 (except IL-6R).
[b] $n = 62$ for alive and $n = 28$ for dead.
[c] $n = 55$ for alive and $n = 25$ for dead.
[d] A decrease not an increase of IL-6R was correlated with mortality.

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cytokine levels taken 6 h after CLP with the status on day 5 (dead or alive), to test the prognostic accuracy and to select the optimal sensitivity/specificity based on the selected cutoff values. The ROC method is especially useful in the evaluation of patient diagnosis/prognosis and in the treatment decision making, because the cutoff guidelines may be adjusted to each therapy (33). In an aggressive treatment which may harm false-positive subjects, the separation would be based on a cutoff ensuring 100% specificity.

The complete evaluation for each cytokine including the ROC-generated AUC with CI and sensitivity/specificity at given cutoff values, is listed in Table I. The selected cutoffs offer the optimal balance between sensitivity and specificity and can be modified to improve either parameter. Based on the 6-h measurement, excellent prognostic accuracy was shown for both IL-6 (AUC = 0.92) and KC (AUC = 0.91). Slightly lower accuracy was noted for MIP-2, MCP-1, IL-1ra, and IL-10 (all with AUC $\geq$0.85). The remaining cytokines failed to yield satisfactory predictive values. These results show that a single measurement of selected cytokines or inhibitors at the onset of sepsis can accurately predict the short-term outcome. This rapid diagnostic approach could be especially helpful during the initial evaluation of the critically ill patient, such as in the emergency room setting. Concerning the SIRS to CARS paradigm, 6 h after the onset of sepsis the plasma levels of the anti-inflammatory mediators (IL-1ra and IL-10) predicted mortality nearly as well as the prototypically proinflammatory cytokines.

Data failing to support the hypothesized SIRS to CARS progression in early sepsis

Previous work has postulated that the immunological cascade triggered by sepsis displays a multimodal character (14–16, 44). The initial hyperinflammatory SIRS gradually recedes into a transient phase of MARS homeostasis which progresses into the hypoinflammatory CARS. Although this is a widely accepted paradigm, there are few data to support the underlying hypothesis. Measuring the individual plasma cytokines/inhibitors over time in our study period did not reveal a typical SIRS to CARS progression. One would anticipate the presence of proinflammatory cytokines in the plasma early followed at later time points by elevations in the anti-inflammatory cytokines. In mice dead by day 5, we noted an early rise of anti-inflammatory cytokines that occurred at the same time as the up-regulation of proinflammatory mediators (Fig. 2). To better illustrate the kinetics of inflammatory mediators, all cytokine and cytokine inhibitor values were normalized and grouped as pro- or anti-inflammatory. Comparison of the trajectories generated by either component revealed that the prelethal profiles of pro- and anti-inflammatory mediators demonstrated a strikingly similar temporal pattern (Fig. 4). The mediator surge was apparent as early as 6 h after CLP, spiking at the 24-h time point. These results show that during the early stage of lethal sepsis, there is a simultaneous release of both pro- and anti-inflammatory mediators into the general circulation. If only a single proinflammatory cytokine such as IL-6 were measured, the subject would be considered hyperinflammatory, while if a single anti-inflammatory inhibitor such as IL-1ra were measured, the subject would be classified as hypoinflammatory. Consequently, the classification of SIRS/CARS fluctuations during the ongoing peritonitis based only on the plasma biomarker profiling does not fully reflect the complex nature of the septic inflammatory response.

Prelethal IL-6 and IL-1ra changes in individual animals

To examine the temporal patterns of IL-6 and IL-1ra activation in individual moribund mice, the last two values from the consecutive measurements taken before death (48 and 24 h before death) were plotted for all individuals dying during the early phase of sepsis

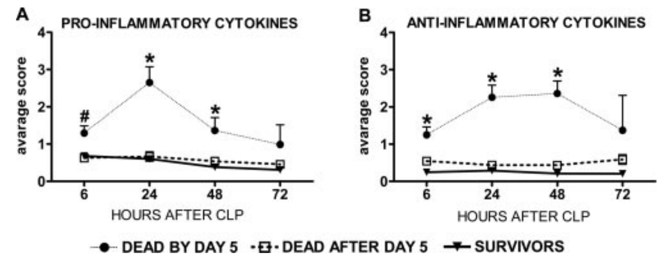

FIGURE 4. Mortality in the early sepsis is preceded by a simultaneous release of pro- (*A*) and anti-inflammatory (*B*) mediators. Combined profiles of pro- (IL-6, TNF-α, IL-1β, KC, MIP-2, MCP-1, and eotaxin) and *B*, anti-inflammatory (IL-1ra, IL-10, TNF SR I, and II) cytokines based on outcome. Data are presented as mean $\pm$ SEM. For the ED group, n varies between time points: at 6 h $n = 7$, at 24 h $n = 14$, at 48 h $n = 4$, and at 72 h $n = 5$. For the LD and SUR groups, n is consistent at all time points: $n = 20$ and $n = 30$, respectively. $*$, $p < 0.0001$ and $**$, $p < 0.005$ comparing ED to the pooled results from the LD and SUR.

(*Fig. 5*). Because the animals that died within 24 h of CLP ($n = 9$) had only one recorded measurement (at 6 h), they were not included in this analysis (but these are displayed in Fig. 2*A* (IL-6) and 3*A* (IL-1ra)). The changes in plasma levels of IL-6 and IL-1ra are presented in Fig. 5. IL-6 and IL-1ra are characteristic of the activation trends of the other pro- and anti-inflammatory mediators and they were selected because they were among the best predictors of early mortality (Table I). In the majority of animals dying during the early phase of sepsis, there was a robust and simultaneous increase (between 48 and 24 h) of IL-6 and IL-1ra before death. This trend was especially clear in animals dying within the initial 3 days after CLP. In a few mice, death was not preceded by a clear increase of IL-6 and IL-1ra and one animal dead by day 4 had a distinct downward trend. Despite the decreasing tendency, however, both IL-6 and IL-1ra remained highly elevated and cytokine concentration changes followed a similar trajectory in all of these individuals. Interestingly, there was an opposite pattern of prelethal cytokine changes in three mice that died by day 5. Their death was preceded by an extremely low/undetectable cytokine levels and this trend was again identical for both IL-6 and IL-1ra (Fig. 5). These results indicate that in each individual animal, the temporal fluctuations of pro- and anti-inflammatory cytokines in plasma occur simultaneously and follow the similar activation trajectory during deaths in the early phase of sepsis.

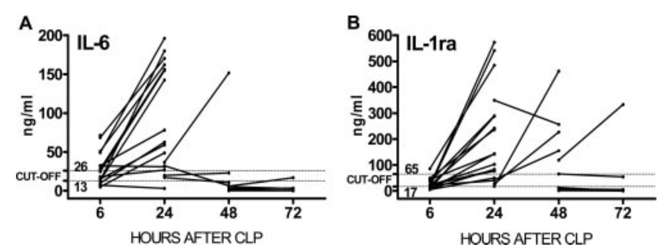

FIGURE 5. Trajectories of prelethal changes in IL-6 (*A*) and IL-1ra (*B*) concentrations in individual animals. Prelethal (within 48 and 24 h of death) cytokine values of each individual animal ($n = 25$ total) are represented by two points connected by a line: the left end (point) of any given line indicates the cytokine value measured 48 h before death, whereas the right end (point) of any given line signifies concentration measured 24 h before death. Mice that died by 24 h ($n = 9$) are not graphed, because only one prelethal (within 24 h) value was obtained. Listed cutoffs are identical with those used for survival prediction in Table II. For the 6–24 h segment, $n = 15$, for 24–48 h $n = 4$, and for 48–72 h $n = 6$.

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Outcome based on cytokine/inhibitor profiles before death

All previous data have been reported on the basis of the time after the onset of sepsis, which helps to define the natural history of the disease. However, as observed in Fig. 1, animals die at different time points after the onset of sepsis. In the clinical situation, the precise onset of sepsis may not be known with certainty. We therefore analyzed the cytokine data using the time of death as the reference point rather than the onset of sepsis. We specifically evaluated the cytokine profiles of those mice who died by day 3 (highest single day mortality). This analysis was performed with an attempt to understand whether cytokine values 24 or 48 h before death would predict subsequent mortality. To determine the diagnostic use of the cytokine values, ROC curves and the total AUC were calculated. Several interesting observations are found within these data (Table II). With the exception of IL-10, all the cytokines values 24 h before death are better predictors of death than those obtained 48 h before death. Both proinflammatory cytokines, chemokines, as well as anti-inflammatory mediators (Table II) may serve as excellent indicators of impending mortality. In general, those components of the inflammatory response such as IL-6, KC, and IL-1ra that predicted mortality when measured at 6 h continue to have excellent accuracy. TNF had little value for predicting ED when measured either 6 h after the onset of sepsis or 48 h before death. However, TNF levels 24 h before death were highly accurate, indicating its dramatic prelethal release.

Table II. *Prognostic values based on selected cutoffs for sepsis-related mortality at 48 and 24 h prior to death*

Cytokine	Parameter	Value[a] (48 h Prior)	Value[a] (24 h Prior)
Proinflammatory cytokines			
IL-6[b]	AUC-ROC	0.86 (0.69–0.95)	0.97 (0.84–0.99)
	Cutoff (pg/ml)	13,000	26,000 (13,000)
	Sensitivity (%)	80	93 (93)
	Specificity (%)	82	97 (88)
TNF-α[b]	AUC-ROC	0.600 (0.45–0.74)	0.95 (0.86–0.99)
	Cutoff (pg/ml)	Not relevant	12,000
	Sensitivity (%)	Not relevant	80
	Specificity (%)	Not relevant	88
IL-1β[c]	AUC-ROC	0.59 (0.45–0.73)	0.77 (0.56–0.90)
	Cutoff (pg/ml)	Not relevant	4,900
	Sensitivity (%)	Not relevant	89
	Specificity (%)	Not relevant	79
Proinflammatory chemokines			
KC[b]	AUC-ROC	0.87 (0.62–0.95)	0.90 (0.79–0.96)
	Cutoff (pg/ml)	33,100	33,000
	Sensitivity (%)	70	87
	Specificity (%)	93	90
MIP-2[c]	AUC-ROC	0.89 (0.76–0.96)	0.98 (0.92–0.99)
	Cutoff (pg/ml)	14,000	14,000
	Sensitivity (%)	79	93
	Specificity (%)	90	95
MCP-1[b]	AUC-ROC	0.79 (0.66–0.89)	0.85 (0.74–0.92)
	Cutoff (pg/ml)	3,220	4,400
	Sensitivity (%)	73	73
	Specificity (%)	56	56
Eotaxin[c]	AUC-ROC	0.55 (0.39–0.70)	0.69 (0.47–0.82)
	Cutoff (pg/ml)	Not relevant	4,400
	Sensitivity (%)	Not relevant	Not relevant
	Specificity (%)	Not relevant	Not relevant
Anti-inflammatory cytokines			
IL-1ra[b]	AUC-ROC	0.81 (0.66–0.90)	0.96 (0.90–0.99)
	Cutoff (pg/ml)	17,000	65,000 (17,000)
	Sensitivity (%)	73	87 (100)
	Specificity (%)	83	94 (70)
IL-10[c]	AUC-ROC	0.80 (0.57–0.90)	0.78 (0.59–0.91)
	Cutoff (pg/ml)	1,900	3,200 (1,900)
	Sensitivity (%)	86	79 (86)
	Specificity (%)	76	80 (61)
TNF SRI[c]	AUC-ROC	0.50 (0.37–0.63)	0.80 (0.63–0.90)
	Cutoff (pg/ml)	Not relevant	3,000
	Sensitivity (%)	Not relevant	71
	Specificity (%)	Not relevant	80
TNF SRII[c]	AUC-ROC	0.64 (0.49–0.78)	0.81 (0.65–0.93)
	Cutoff (pg/ml)	Not relevant	14,000
	Sensitivity (%)	Not relevant	64
	Specificity (%)	Not relevant	91
IL-6R[d]	AUC-ROC	0.70 (0.43–0.85)	0.67 (0.49–0.83)
	Cutoff (pg/ml)	1,700 (2,000)	2,000
	Sensitivity (%)	70 (64)	71
	Specificity (%)	79 (79)	64

[a] All values are based on the data from mice dead at the 48 h time point after CLP; sensitivity/specificity were not listed if AUC <0.7 (except IL-6R).
[b] $n = 66$ for alive and $n = 15$ for dead.
[c] $n = 59$ for alive and $n = 14$ for dead.
[d] A decrease not an increase of IL-6R was correlated with mortality.

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Discussion

Although there are many controversies in the field of sepsis, there is uniform agreement that the disease process is highly complex. The septic patient presents with a bewildering array of alterations in their physiologic and inflammatory response. Animal models may be used to help provide some of the necessary information concerning the basic pathophysiologic responses to sepsis (45). The CLP model recapitulates many of the alterations observed in human sepsis. One important aspect of the model is the improvement in survival when fluid resuscitation and antibiotic therapy are provided (35, 43), similar to studies in human septic patients. Several interesting observations may be made from the current data. First, the inflammatory response is dynamic, changing over time. Second, the inflammatory changes are not rapid spikes in plasma levels of cytokines such as observed after endotoxin exposure (46, 47). These data indicate that measuring the inflammatory response every 24 h should be sufficient to profile the inflammatory status of the patient if a number of biomarkers are evaluated.

A concept has been proposed where septic patients begin with SIRS, transition to a MARS, and then progress to a CARS (15, 34). Although our data do not include components of the cellular response, the plasma cytokine data do not support such a simple, linear transition. The inflammatory status of the patient may be defined by the plasma concentrations of either the proinflammatory cytokines such as TNF and IL-6 or the anti-inflammatory cytokines such as IL-1ra or IL-10. Several previous studies have demonstrated that proinflammatory cytokines are elevated in septic patients and predict outcome (25, 48) and other studies have documented the presence of the anti-inflammatory mediators at the onset of sepsis (49). Mice dying within the first 5 days may be considered to have a heightened inflammatory response if this response is defined by the ability of the host to generate potent biological mediators. The determination of whether SIRS is present may be more accurately determined from the capacity of the host to respond rather than measurement of the mediators.

It should be noted that the SIRS and CARS syndromes were not defined only by circulating cytokine concentrations but also by the expression and/or immune responses of various cellular components. Previous work has shown that cells derived from septic patients have a markedly reduced capacity to produce cytokines following an ex vivo stimulation (50, 51). Experimental sepsis models demonstrate similar findings. Ayala et al. (52–54) showed in several reports that 24 h after CLP-induced sepsis, the capacity of septic mouse splenic/peritoneal macrophages to release cytokines was diminished. Specifically, ex vivo stimulation of these cells resulted in suppressed production of IL-2, IFN-γ, and IL-6 while IL-4 and IL-10 synthesis was enhanced after LPS/concanavalin stimulus. Also, CARS-specific decrease of MHC class II expression was reported early (within 24 h) in both experimental murine (55) and human (56) sepsis.

Measuring the plasma levels of both the pro and anti-inflammatory cytokines represents a rapid method for evaluating function rather than performing ex vivo stimulation. The presence of the mediators may have significant prognostic implications not because of their proposed biological function, but rather because the host is capable of producing them.

There are implications for the clinical treatment of patients from our studies. The previous approach of measuring simple physiologic and immune status parameters such as heart rate or white blood cell count (57) is almost certainly not sufficient to direct appropriate therapy to the individual patient. Some patients with sepsis die from an apparent exuberant inflammatory response which injures cells, tissues, and organs, resulting in death. These patients would most likely benefit from a reduction of the inflammatory response. As the recent Lenercept trial shows (58), the 6–72 h (postinjection) serum concentrations achieved with recombinant soluble TNF SR I (p55) during the treatment for human sepsis were substantially higher (by 2–3 logs) than the post-CLP concentration of endogenous TNF SR I observed here and in human sepsis (59, 60). Hence, it is unlikely that the treatment failure was due to the inadequate supplementation of the endogenous pool of TNF SR I with the exogenous TNFR. Given these data, it remains problematic whether attenuation of the early excessive inflammatory response can be adequately achieved by targeting only individual proinflammatory components. We noted a significant improvement in post-CLP survival when the combined (i.e., TNF SR I and IL-1ra) immunotherapy was used (36)

In contrast to the hyperinflammatory patients, other subjects have difficulty defending themselves against the microbial invasion (61). These patients would be unlikely to benefit from therapies directed at blunting the inflammatory response. Similar to virtually all other human diseases, the response to disease changes over time such that the therapy appropriate for the patient on Monday may not be appropriate on Tuesday.

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Disclosures

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