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Sepsis Chronically in MARS: Systemic Cytokine Responses Are Always Mixed Regardless of the Outcome, Magnitude, or Phase of Sepsis

Marcin F. Osuchowski,*† Florin Craciun,* Katrin M. Weixelbaumer,† Elizabeth R. Duffy,* and Daniel G. Remick*

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Despite improvements in supportive care, sepsis continues as a life-threatening condition in patients of all ages (1, 2). In sepsis, infection leads to a systemic immune reaction termed the systemic inflammatory response syndrome (SIRS). The historical consensus characterized the early phase of sepsis by a prompt increase of circulating proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6 (3). Because this strong proinflammatory response was believed to be responsible for early septic deaths (3), inactivation/removal of cytokines during raging SIRS was the focus of experimental and clinical intensive care research over the last decades. Yet, dismal failure of numerous large-scale anti-inflammatory treatment trials (4) was recently followed by the failure of eritoran tetrasodium (a TLR-4 antagonist) (5) and withdrawal of drotrecogin α (recombinant human activated protein C) (6), the only existing drug specifically indicated for treatment of sepsis. Because of these drawbacks, the understanding of the traditional concept of the proinflammatory versus anti-inflammatory immune response in sepsis has been rapidly evolving in recent years. For example, both experimental (7, 8) and clinical (9–11) findings demonstrated that proinflammatory and anti-inflammatory cytokines are released in early sepsis, and signs of immunosuppression are already manifested in the acute stage of sepsis. Therefore, it is evident that sepsis does not progress along a preset disease pattern, but needs to be perceived as a highly dynamic biological process (12, 13).

Because the trajectory of the systemic immunoinflammatory response in sepsis can alternate between hyperactivity and immunosuppression, an uncorrected, escalating deviation from homeostasis in either direction may result in death. Effective corrective measures should either blunt hyperactive responses or boost the suppressed responses before the window of therapeutic opportunity closes. Given this, the future of sepsis treatment lies in a more individual approach to septic patients (14–16); the same drug may be beneficial, noneffective, or even harmful depending on the patient’s immunological status. A notion of using biomarkers (such as cytokines) and their temporal response patterns for identification of homogenous cohorts with the greatest projected benefit from specifically tailored immunomodulatory therapeutics seems especially attractive (17–20). The temporal evolution of the immunoinflammatory response in septic patients is central to both of the earlier concepts. A precise characterization of these changes,
relatively straightforward immediately after the onset of sepsis in nonimmunocompromised patients, is much more problematic during the later phases of the disease, and remains largely unexplored. This creates a dangerous dissonance because advances in the intensive care unit (ICU) have considerably reduced incidences of acute (early) mortality (5). Improved survival throughout the initial stages of sepsis often translates into higher mortality in the later stages of the disease (21–23). In other words, the treatment did not cure the disease, it only delayed death. In the blood, late mortality has been typically associated with increased levels of anti-inflammatory cytokines termed compensatory anti-inflammatory response syndrome (CARS). This has been postulated to cause a protracted dampening of immune functions in chronically ill septic patients (12, 24). Apart from the proposed shift in the profile of circulating cytokines, this late-occurring “immune paralysis” is also reflected by deregulation of cellular compartment, for example, reduced macrophage Ag presentation (7, 25), increased lymphocyte apoptosis (9, 26, 27), and altered leukocyte recruitment (28), all of which increase patients’ susceptibility to secondary complications.

Despite its relevance, the evolution of immunoinflammatory signaling in chronic sepsis and its contribution to late mortality have not been widely investigated. In this study, we characterized the protracted evolution of the chronic prelethal inflammatory response to specifically compare the outcome-based profiles of the proinflammatory and anti-inflammatory cytokines and leukocytes in the blood. In addition, we sought to identify whether a global outcome-dependent pattern of cytokine responses occurring in acute (early) and chronic (late) cecal ligation and puncture (CLP) sepsis defines their key similarities and differences. These findings would provide insight into whether the same basic mechanisms drive the disease process in both acute and chronic sepsis.

Materials and Methods

Animals

ICR outbred mice (Harlan-Sprague Dawley, Frederick, MD) with an average weight of 22 g were used (n = 97). To eliminate sex-related variability, we included only female mice in this study. 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/dark diurnal cycle. Standard rodent chow and water were provided ad libitum. All experiments were carried out in accordance with the National Institutes of Health guidelines and the Boston University Animal Care and Use Committee.

Sepsis model

To ensure adequate reproducibility, we performed surgeries on separate groups of mice (typical n = 10/experiment). The CLP model is widely accepted (29) and used by many to study the immunopathology of sepsis (30–32). We used a medium-grade CLP severity (18-gauge needle, double puncture) to emulate a typical 30% mortality rate occurring in chronic patients with abdominal sepsis (33). The original CLP protocol was followed (34), and previously described modifications were implemented (8) including broad-range antibiotic (imipenem, 25 mg/kg) therapy with 1 ml/mouse fluid resuscitation (Lactated Ringers, 1 ml/mouse) administered (twice daily) only during the first 5 d post-CLP. All animals were followed for 28 d or until death, whichever occurred first. Sham surgeries were not performed because we were comparing the response in dying (DIE) mice with long-term survivors, rather than just cataloguing the response to sepsis.

Study design

In the first, chronic sepsis phase, part of the study (see Figs. 1–5), the experiment investigated the protracted immunoinflammatory responses in between days 6 and 28 post-CLP. The selection of the 5-d cutoff was justified based on dissimilar mechanism(s) of death between the acute (days 1–5 post-CLP) versus chronic sepsis (16, 35, 36). Death was used as a reference time point for all DIE mice. Consequently, data are plotted in an inverted fashion: the 24-h time point represents the last individual (and/or average) parameter value in an animal that died within 24 h of sampling (see Table I, all figures, and all supplemental tables and figures), whereas 48- and 72-h time points (also present in Figs. 2–4 and Supplemental Fig. 2) represent measurements taken within 48 and 72 h of death, respectively. For comparisons, all chronic DIE mice (irrespective of the day of death) were pooled (based on the sequence of their before-death time points) and retrospectively matched with two (randomly selected) surviving (SUR; alive at day 28) animals of the same post-CLP day (see Supplemental Fig. 1 schematic). After selection, three consecutive (i.e., 72- to 24-h SUR time points) daily measurements were taken from the same SUR mice to match the predeath measurements from a DIE mouse of each (one DIE/two SUR per experiment; e.g., for a death on day 13, both SUR and DIE values from days 11, 12, and 13 were tallied).

In the second part of the study, the current data from the chronic sepsis experiment were combined with the historical data recorded in the previous acute (8) and chronic (35) sepsis experiments (Fig. 7, Table I, and all supplemental tables). Only the 24 h before death time points (regardless of sepsis phase) from all three studies were combined for comparison of inflammatory responses in acute versus chronic sepsis. Data merger was justified by identical experimental protocol in all three studies, high reproducibility of measurements, and improved statistical power of such combined analysis.

Sampling

All animals that survived the period of acute sepsis (days 1–5) were sampled daily in the chronic phase of sepsis (days 6–28). Blood (20 μl) was collected from all animals by facial vein (vena submandibularis) puncture (alternating cheeks daily) using a 23-gauge needle for an optimal sampling precision (37). Repetitive daily sampling over 22 d (days 6–28 post-CLP) was safe: it affected neither 28-d mortality nor any of the recorded parameters (i.e., hemoglobin, cell blood counts, circulating cytokines) (38). All samples were drawn into a pipette rinsed with EDTA and then immediately diluted 1:10 in PBS with 2% (v/v) EDTA. After centrifugation (1000 × g, 5 min), plasma was removed and stored at −80 °C until analysis. All in all, 15 chronic DIE mice (of 21 total) were included in the final analysis; 1 mouse was excluded from the study because of a sampling injury, and irregularity in the sampling schedule eliminated the remaining 5. Fig. 1 shows the temporal distribution of sampled animals.

Cytokine immunoassays

In this study, we used a validated microarray immunoassay methodology with a capacity to simultaneously measure multiple mouse cytokines (39). All targets contained on our microarray platform were shown to be in and/or directly implicated in the septic inflammatory sequelae (40, 41). Other classical biomarkers such as procalcitonin and C-reactive protein were not assessed because of the sample volume constraints (20 μl/mouse) and/or lack of commercial murine assays.

Cytokine data from two previous studies that were used for comparisons between acute and chronic sepsis (Figs. 7, Table I, and all supplemental tables) were obtained by either the same microarray immunoassay (previous chronic sepsis study [35]) or a sequential ELISA (previous acute sepsis study [8]) method described elsewhere (42). In all three studies (regardless of the assay used), the same standard ELISA-based and previously optimized Ab pairs were used (43). Reliability of the presented comparisons was assured, as the microarray used and classical ELISA immunoassays display a virtually perfect correlation and detect the same relative levels of cytokines (with the same degree of variability) in the blood plasma samples (39).

In brief, the primary (capture) Abs were spotted on the bottoms of 96-well microtiter plates (this study and Osuchowski et al. [8]) or nitrocellulose pads (35). Next, samples were incubated with a biotinylated secondary Ab, then streptavidin-conjugated to HRP or an infrared fluorophore, and plates were read with the ELISA plate reader (Bio-Tek Instruments, Winooski, VT) or the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE), respectively.

Hematology

After blood collection, the cell pellet was immediately resuspended in 480 μl Hemavet solution (CDC Technologies, Oxford, CT). A complete blood count including differential was performed with a Hemavet 1500 (CDC Technologies).

Statistical analysis

Twenty-eight–day survival (Fig. 1) was plotted using the Kaplan–Meier analysis; 1 mouse was excluded from the study because of a sampling injury, and irregularity in the sampling schedule eliminated the remaining 5. Fig. 1 shows the temporal distribution of sampled animals.

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Statistical analysis

Twenty-eight–day survival (Fig. 1) was plotted using the Kaplan–Meier curve. Figs. 1–5 (and Supplemental Fig. 2) were based on the data generated in this study only. For data presented in Table I and Figs. 6 and 7 (and in Supplemental Tables I–II), cytokine values (i.e., IL-1β, IL-6, TNF-α, MIP-2, MCP-1, Eotaxin and IL-1ra, IL-10, TNF srI, TNF srII) obtained in this and two previous (25, 32) studies were combined for analysis. Specifically, the current dataset (n = 97) was supplemented by additional
measurements from the previous chronic (42 values total) and acute (70 values total) sepsis datasets. In all three studies, the CLP protocols were identical (i.e., severity, antibiotic treatment, and fluid resuscitation treatment). Detailed n distribution and the source of data plotted in all individual tables and figures are present in their respective legends.

Data in Figs. 2 and 6 were analyzed by either Student t test (Gaussian distribution) or Mann–Whitney U test (skewed distribution). Cytokine ratios (see Fig. 6) were computed for each individual animal, averaged across respective groups, and log-transformed (Y = Ln(Y)) for comparison. In Figs. 2 and 4, each time point was analyzed separately because the differences between SUR and DIE groups were the primary end points.

To generate the average inflammatory scores (see Figs. 5, 7), we normalized all cytokine values (each mediator individually) to the median DIE value of that specific cytokine. The scores for the same chronic-DIE and SUR groups are different in Figs. 5 and 7 because an effective graphic depiction required two different median DIE values to compute them; median was selected from the chronic dataset in Fig. 5 (henceforth referred to as the “composite cytokine score” [CCS]) and from combined acute and chronic sepsis datasets in Fig. 7 (henceforth referred to as the “normalized cytokine score” [NCS]). The means of all normalized cytokine values (now scores) were allocated to different groups based on outcome (see Fig. 5) and/or phase of sepsis (see Fig. 7, Table I), averaged, and presented as average response scores of each group. In Fig. 5, the outcome-dependent CCSs of chronically septic mice were additionally divided into the proinflammatory and anti-inflammatory panels. For comparison of global outcome-dependent responses between acute and chronic sepsis in Fig. 7, proinflammatory and anti-inflammatory cytokines were pooled together in each respective group. CCSs were then log-transformed and analyzed by one-way ANOVA followed by Tukey’s test.

Cytokine levels below the limit of detection were assigned a value that was equal to half of the lower limit of detection in the standard curve. Significance was assigned where p < 0.05, and all tests were two-tailed. All statistical analyses were performed using either Prism 5 (GraphPad Software, San Diego, CA) and SAS software 9.1.2 on Windows (SAS Institute, Cary, NC).

Results
Mortality in the chronic phase of CLP sepsis

To experimentally replicate the level of mortality typical for septic patients in the modern ICU, we subjected mice to CLP resulting in moderate severity and followed their survival for 28 d (Fig. 1). CLP-induced sepsis resulted in an acute mortality rate of 29% (or 26/97 of total deaths by day 5 post-CLP) followed by the additional chronic mortality rate of 30% (or 21/71 by day 28). Only those animals that survived/died within days 6–28 post-CLP were subjected to daily blood sampling. A total of 15 (of 21) late-death mice (designated by dotted lines) were subsequently included in the analysis.

Comparison of leukocyte trajectories in SUR versus DIE mice in chronic sepsis

To characterize protracted changes preceding chronic mortality for all parameters included in this study, we used the day of death during the chronic phase (any day between 6 and 28 d) as the reference point. We then clustered all parameter values recorded at 72, 48, and 24 h before the death of each mouse and compared them with the matching values recorded in SUR mice (see Study Design section and Supplemental Fig. 1 schematic). An identical analytical approach was used for data in Figs. 2–4 (and Supplemental Fig. 2).

We previously reported pronounced lymphopenia and neutropenia in acutely ill CLP mice (44, 45). To establish whether similar shifts

FIGURE 1. Twenty-eight–day survival curve after CLP-induced sepsis. CLP was performed (total n = 97) with an 18-gauge needle to produce ~30% mortality during the end of chronic phase of sepsis. Using a subjective cutoff, we separated 28-d follow-up into acute (days 1–5) and chronic (days 6–28) phases. A total of 21 deaths (21/71) occurred in chronic sepsis. Animals included in the analysis (15 mice) underwent daily 20-μL blood sampling between days 6 and 28. Solid line indicates the end of the acute sepsis phase, whereas dotted lines indicate the day of death of mice included in the analysis.

FIGURE 2. Protracted profiles of WBC (A), lymphocytes (LYM) (B), and neutrophils (NEU) (C) counts based on outcome during chronic sepsis. Daily blood samples were collected between days 6 and 28 post-CLP. The day of death (any day between 6 and 28 d) served as the reference point. DIE values were retrospectively plotted in the 72-, 48-, and 24-h predeath trajectory and were time matched with SUR values from the same post-CLP day (see Study Design). A 2:1 SUR/DIE ratio at each time point for each parameter: n = 20/10 (SUR/DIE) at 72 h; n = 28/14 at 48 and 24 h. Data (box and whiskers) presented as mean + SD. Dotted lines indicate normal range. *p < 0.05.
also occur during chronic sepsis, we compared circulating WBC and their subsets in DIE and SUR mice. Regardless of outcome, chronically septic mice displayed slight leukocytosis primarily because of pronounced neutrophilia (Fig. 2A, 2C). Lymphocyte counts were within the range of normal values at all time points. Compared with SUR, total WBC counts in DIE mice were similar at 72 and 48 h, but a 42% reduction was observed at 24 h before death (Fig. 2A). A similar decrease for DIE mice was observed in lymphocyte counts: a 30% decline at 48 h and 50% at 24 h compared with the respective SUR groups (Fig. 2B). Conversely, no significant differences in the numbers of neutrophils were observed between the SUR and DIE groups at any time point (Fig. 2C). These results indicate that the decrease in WBC counts recorded immediately before death was due to the reduction in lymphocyte but not neutrophil counts. In addition, this effect was accompanied by pronounced prelethal thrombocytopenia without overt signs of anemia (Supplemental Fig. 1).

Comparison of proinflammatory cytokine trajectories in SUR versus DIE mice in chronic sepsis

We have previously demonstrated that acute septic deaths after CLP were preceded by a robust increase of several inflammatory cytokines (8). To characterize the protracted evolution of inflammatory responses preceding late deaths in individual mice, we clustered their cytokine values to display profiles for the last 3 d before death (i.e., 72, 48, and 24 h). These values were then compared with time-matched SUR values.
A strikingly consistent pattern emerged when the data are analyzed relative to the day of death. This analysis is possible because the study design sampled all 15 mice on a daily basis. Compared with SUR, mean plasma levels of TNF-α (Fig. 3A), IL-6 (Fig. 3B), MIP-2 (Fig. 3C), and MCP-1 (Fig. 3D) recorded over the 3 d before death were significantly increased at 24 h.

A similar outcome-dependent difference (i.e., at 24 h) was also observed in virtually all other cytokines/chemokines we measured (Supplemental Table I). Conversely, the mean DIE versus SUR difference was not significant at 48 and 72 h (Fig. 3 and data not shown) in most of the recorded proinflammatory biomarkers, with the exception of MCP-1 (Fig. 3D, ∼6-fold difference at 48 h). SUR values of TNF-α, IL-6, MIP-2, and MCP-1 (Fig. 3), and remaining proinflammatory biomarkers (data not shown) were either below detection level or marginally low at the 48- and 72-h pre-death time points.

Comparison of anti-inflammatory cytokine trajectories in SUR versus DIE mice in chronic sepsis

It has been suggested that CARS predominates in the chronic phase of sepsis, and that late septic mortality is due to immunosuppression associated with a strong release of anti-inflammatory mediators (46). Using the analytical approach described earlier, we analyzed fluctuations of key anti-inflammatory cytokines known to contribute to suppressed immune responses.

Compared with the late proinflammatory response, the prelethal trajectory of anti-inflammatory biomarkers in chronic sepsis was virtually identical. At 24 h, mean DIE values of circulating IL-1ra (Fig. 4A), IL-10 (Fig. 4B), TNF srl (Fig. 4C), and TNF srlf (Fig. 4D) were 60-, 24-, 8-, and 4-fold higher compared with SUR mice, respectively. A similar outcome-dependent effect was also observed in five (of six) other anti-inflammatory cytokines recorded at 24 h before death (Supplemental Table II). In contrast, in none of the anti-inflammatory cytokines except TNF srl (Fig. 4C, ∼3-fold difference at 48 h) was the mean DIE concentration elevated (versus SUR) at 48 and 72 h (Fig. 4 and data not shown). Combined data from Figs. 3 and 4 show that the prelethal increase of both proinflammatory and anti-inflammatory cytokines during chronic sepsis is very transient and typically occurs only immediately before death.

Comparison of CCSs in chronic sepsis

Lethal outcome in sepsis is not caused by a single key mediator but is likely driven by concurrent deregulation of numerous immunoinflammatory pathways. To examine general and outcome-dependent inflammatory responses in chronic sepsis, we combined all cytokines into the two panels (proinflammatory [Fig. 5A] and anti-inflammatory [Fig. 5B]) and generated outcome-dependent CCSs (by normalizing all individual cytokine values; see Statistical Analysis section). The CCSs recorded at 24 h before death in DIE mice were higher than the ones calculated in SUR mice (Fig. 5): the difference reached ∼80-fold in the proinflammatory panel and 50-fold in the anti-inflammatory panel (p > 0.1 between proinflammatory and anti-inflammatory CCSs). These data demonstrate that lethality in chronic sepsis is preceded not only by a simultaneous but also a similar-grade release of proinflammatory and anti-inflammatory cytokines.

Comparison of cytokine ratios based on outcome and phase of sepsis

The data in Figs. 3–5 (and Supplemental Tables I, II) demonstrated that during chronic sepsis, both non-and lethal responses were associated with a simultaneous and similar release of both proinflammatory and anti-inflammatory cytokines. To further examine the systemic, outcome-dependent balance between proinflammatory and anti-inflammatory responses across acute and chronic sepsis, ratios for key proinflammatory and anti-inflammatory mediators were computed.

Despite occasional statistical intergroup differences, cytokine ratios were similar both across phases and outcomes. The most consistent difference was noted in the IL-1ra/IL-1β ratio, which showed an identical increase in DIE versus SUR mice in both acute and chronic phases of sepsis (Fig. 6A, 6B). The opposite finding was true for the TNF srl + II/TNF-α ratio: SUR was slightly higher compared with DIE, but only in the acute sepsis phase (Fig. 6G).

In addition, both acute sepsis IL-10/IL-6 ratios (also TNF srl + II/TNF-α ratio in acute DIE versus chronic DIE; Fig. 6G, 6H) were statistically lower compared with the corresponding ratios in chronic mice (Fig. 6E, 6F), whereas all remaining ratios were virtually identical. These data underline that regardless of the phase and/or outcome, a typical humoral response in sepsis always features proinflammatory and anti-inflammatory components.

Comparison of NCSs in acute and chronic sepsis

The earlier data are indicative of the mixed anti-inflammatory response syndrome (MARS)-like release pattern but do not reveal differences in the magnitude of septic responses between acute and chronic sepsis. In the next analysis, we aimed to compare the relative inflammatory response levels across the acute and chronic sepsis phases. To accomplish this, we combined cytokine data from this study and two previous studies investigating both acute (8) and chronic (35) sepsis. Similar to Fig. 5, cytokine scores were generated (see Statistical Analysis section). Scores were computed only for cytokines that overlapped in all three studies, and the scores were divided into four groups depending on outcome (i.e., SUR versus

![FIGURE 5. CCSs for proinflammatory (A) and anti-inflammatory (B) compartments based on outcome in chronic sepsis. All cytokine values were normalized (each mediator individually) to the median DIE value of that specific cytokine. The individual activation scores for each tested cytokine were then combined into the proinflammatory (i.e., IL-1β, IL-2, IL-5, IL-6, IL-12, IL-17, TNF-α, IFN-γ, ICAM-1, MIP-1α, MIP-2, MCP-1, Eotaxin, Eotaxin-2) and anti-inflammatory (IL-1ra, IL-4, IL-10, IL-13, TNF srl, TNF srlf) panels, and the average response scores were compared based on outcome. DIE (any day 6–28 d post-CLP): n = 15; SUR (alive by 28 d post-CLP): n = 30. Chronic DIE bar represents pooled cytokine values collected within 24 h of death. Cytokine values collected from SUR animals were sampled on the same post-CLP day as the DIE mice (see Study Design). Data presented as mean + SEM. *p < 0.01.](http://www.jimmunol.org/)

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DIE) and the phase of sepsis (i.e., acute versus chronic). For simplicity, no additional separation into the proinflammatory and anti-inflammatory panels was performed. The group scores for each individual cytokine are listed in Table I.

The highest NCS was recorded in mice that died during the acute phase of sepsis: NCS was ∼5-fold higher than in acute SUR mice and chronic DIE animals (Fig. 7). The difference was even more pronounced (∼35-fold) when compared with NCS in chronic SUR mice. In addition, the chronic SUR group NCS was, on average, 5-fold lower compared with both chronic DIE and acute SUR animals (all \( p < 0.01 \)).

**Discussion**

Measurement of circulating biomarkers constitutes the most practical method to rapidly describe the immunoinflammatory status of a septic emergency room/ICU patient. To characterize the chronic prelethal inflammatory response in the first part of the study (Figs. 1–5), we incorporated three vital experimental elements: 1) a model
Table 1. Average activation score for individual proinflammatory and anti-inflammatory cytokines separated based on the outcome and phase of CLP sepsis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>DIE(^a)</th>
<th>SUR(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Chronic</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4.78</td>
<td>0.65</td>
</tr>
<tr>
<td>IL-6 (Pro-I)</td>
<td>11.55</td>
<td>0.93</td>
</tr>
<tr>
<td>TNF-α (Pro-I)</td>
<td>4.96</td>
<td>0.33</td>
</tr>
<tr>
<td>MIP-2 (Pro-I)</td>
<td>8.72</td>
<td>1.26</td>
</tr>
<tr>
<td>MCP-1 (Pro-I)</td>
<td>1.11</td>
<td>0.96</td>
</tr>
<tr>
<td>Eotaxin (Pro-I)</td>
<td>3.76</td>
<td>2.09</td>
</tr>
<tr>
<td>IL-10 (Anti-I)</td>
<td>1.74</td>
<td>0.74</td>
</tr>
<tr>
<td>TNF srI (Anti-I)</td>
<td>1.88</td>
<td>1.70</td>
</tr>
<tr>
<td>TNF srII (Anti-I)</td>
<td>11.33</td>
<td>0.15</td>
</tr>
<tr>
<td>IL-1ra (Anti-I)</td>
<td>2.27</td>
<td>0.59</td>
</tr>
</tbody>
</table>

\(^a\)Prelethal cytokine values were collected within 24 h of death within the respective phase.
\(^b\)Cytokine values collected from SUR animals (alive at day 28 post-CLP) were sampled for comparison on the same post-CLP day as the DIE mice.

All cytokine values were normalized (see Statistical Analysis) to the median DIE value of that specific cytokine. Acute DIE/SUR: n = 35/group; chronic DIE: n = 29; chronic SUR: n = 58. Listed response scores were computed from combined cytokine values measured in this and two previous studies (25, 32).

Anti-I, Anti-inflammatory; Pro-I, proinflammatory.

of moderate severity sepsis (displaying frequent late mortality) by polymicrobial, CLP-induced peritonitis; 2) a study design where mice were never sacrificed to allow a natural progression of the disease to survival/death; and 3) small-volume daily sampling during the chronic phase of sepsis (days 6–28). The earlier design was dictated by the clinical reality, where a large fraction of septic patients die in the chronic disease stage, the onset of sepsis is typically unknown (compared with preclinical studies), and patients are monitored frequently to provide optimal management and avoid another and ultimate reference point: death.

Given that plethora of proinflammatory and anti-inflammatory cytokines were shown to correlate, both clinically (47–49) and experimentally (8, 50), with sepsis severity/poor prognosis, a clinical focus has been put on selecting patients with the greatest risk for death to produce relatively homogenous cohorts more suitable for personalized treatments. Comparison of the CCSs in the SUR and DIE groups at 24 h before death (Fig. 5) shows that similar to acute sepsis (8), late deaths can also be preceded by a marked release of circulating cytokines. In line with our initial findings (35), the current data reconfirm that individual cytokine/subject responses in chronic sepsis are highly mixed, regarding both the magnitude of the increase and the selection of released mediators. By longitudinally plotting the data from the chronic phase of sepsis in this study, we demonstrate an aspect of the chronic inflammatory response never shown before. Specifically, virtually no cytokine increases were observed earlier than 48 h before death, regardless of whether the mediator was in the proinflammatory or anti-inflammatory panel. This closely mirrors the cytokine response in acute CLP (8) where (SUR versus DIE) differences did not appear earlier than the 48 h before death. In the CLP setting, therefore, septic deaths (regardless whether acute or chronic) cannot be predicted earlier than ~48 h before the event. In this chronic sepsis study, none of the biomarkers exceeded area under the curve (>0.7 at 48 h before late deaths (except area under the curve = 0.77 for TNF srI; data not shown)). Although many seemingly promising clinical studies investigated the viability of cytokine prediction for long-term (typically 28 d) septic outcomes (51–53), none of the biomarkers/biomarker sets has ever translated into a routine and clinically usable tool (5, 41). Current observations in the chronic sepsis part of the study may, at least partially, explain this deficit. We show that compared with the relatively steady (hyperinflammatory) signature of acute sepsis (8), the feasibility of predicting outcome in chronic sepsis is much weaker. This weakness is primarily related to the transient and erratic appearance of cytokines in the bloodstream, limiting their use as predictors, regardless of whether as a single marker or in combination. Another aspect is the diverse strength of the signal, because cytokine values are much higher in the acute phase. In other words, although chronic-phase spikes in selected circulating cytokines (e.g., MIP-2) are predictive for late deaths, the magnitude of these increases is negligible when compared with their massive release in the acute phase. Interestingly, with the expanded range of biomarkers measured, we observed that the inflammatory response in mice dying of chronic sepsis displays an “all or nothing” character; that is, whenever an increase (or lack of response) in a given cytokine was recorded, a similar increasing (or no-responsiveness) pattern was also true for virtually all other (or majority of) remaining biomarkers.

The two most valuable findings, however, come from a combined, across-the-board comparison of outcome-dependent cytokine responses in the acute and chronic phases of sepsis. First, based on the comparison of proinflammatory/anti-inflammatory ratios from this (chronic sepsis) and our two previous (acute and chronic sepsis) experiments (8, 35), we prove that the proposed “Sepsis: Always in MARS” paradigm (54) can be uniformly applied regardless of outcome and the phase of disease. Specifically, whenever sepsis provokes a given systemic inflammatory response, the associated release of anti-inflammatory cytokines closely matches the speed and/or robustness of the proinflammatory mediator secretion (and vice versa). Consequently, neither acute nor chronic sepsis mortality appear to be associated with a distinct predominant proinflammatory and/or anti-inflammatory signature in the blood (despite differences in the relative response magnitude; see later). The most recent clinical studies corroborate the earlier observations (10, 11). The clinical translation of the earlier argued concept is simple: in the blood, the true cytokine makeup of SIRS/CARS is a one of MARS, and these definitions should be redefined accordingly.

Second, we demonstrate that because of the 5-fold difference in the magnitude of the response between acute DIE and chronic DIE, an effective identification of late CLP mortality in the milieu of an acute cytokine response is next to impossible. In other words, 5 ng/ml...
circuiting IL-6 measured in a CLP mouse may herald either its impending death (hence a need for an immediate therapeutic intervention) in the chronic phase of sepsis or, conversely, indicate that the host mounted a successful struggle against the disease in its early, acute stage (discouraging an aggressive anti-inflammatory treatment). The above can be also directly extrapolated to the hospital ICU. The cytokine response profiles represented by NCSs indicate that the current precept of selecting septic patients with the greatest risk for death (based on the secreted cytokines) is heavily biased toward the hyperinflammatory (MARS-like) signature; that is, it tends to include only those who display a relatively strong cytokine response, whereas leaving both “nonresponders” and “low-responders” behind. As a result, chronic septic patients with a typically weaker prelethal cytokine response are unable to rise above a given diagnostic threshold calibrated on the magnitude of the early inflammatory response. Thus, these patients will be neither identified nor treated (with the latter omission paradoxically bene-

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Disclosures
The authors have no financial conflicts of interest.

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puncture followed by methicillin-resistant *Staphylococcus aureus* pneumonia increases mortality in mice and blunts production of local and systemic cytokines. Shock 35: 87–94.


Supplementary Figure 1 - Legend:

Schematic of the chronic sepsis study design.

Daily blood samples were collected between days 6-28 post-CLP (chronic phase of sepsis). The day of death (any day between 6-28 days) served as the reference point for any DIE mouse. Regardless of the day of death, all DIE values were retrospectively tallied together and plotted in the 72h, 48h and 24h prior-to-death trajectory. Each data set (typically 72h, 48h and 24h time-points) collected from a DIE mouse (e.g. mouse 2) was time-matched with the same number of values collected from a random SUR mouse (e.g. mouse B) on the same post-CLP days. For simplicity, a 1:1 ratio is indicated in the schematic. The true SUR-to-DIE ratio in the study was 2:1 for each time-point/parameter.
Supplementary Figure 2 - Legend:

Protracted profiles of hemoglobin concentration, red blood cell and platelet counts based on outcome during chronic CLP sepsis.

Daily blood samples were collected between days 6-28 post-CLP. The day of death (any day between 6-28 days) served as the reference point. DIE values were retrospectively plotted in the 72h, 48h and 24h prior-to-death trajectory and were time-matched with SUR values from the same post-CLP day (see study design). 2:1 SUR to DIE ratio at each time-point for each parameter: n= 20/10 (SUR/DIE) at 72h; n=30/15 at 48h and 24h. Data (bars) presented as mean + SD. Dotted lines indicate normal range. * p < 0.05
**Supplementary Table I.** Comparison of circulating pro-inflammatory cytokine concentrations between mice dying and surviving during the chronic phase of sepsis

<table>
<thead>
<tr>
<th>Cytokine&lt;sup&gt;a&lt;/sup&gt; (ng/ml)</th>
<th>Dying&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Surviving&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin (IL)-1β</td>
<td>1.02 ± 2.4</td>
<td>0.17 ± 0.3</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.05 ± 1.3*</td>
<td>0.10 ± 0.3</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.24 ± 0.37</td>
<td>0.05 ± 0.6</td>
</tr>
<tr>
<td>IL-6</td>
<td>5.52 ± 15.6*</td>
<td>0.03 ± 0.1</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.58 ± 1.1*</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.27 ± 0.48*</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.15 ± 0.21*</td>
<td>0.01 ± 0.04</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.52 ± 0.90*</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>7.75 ± 4.2*</td>
<td>4.33 ± 2.4</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>11.1 ± 18.4*</td>
<td>0.45 ± 0.7</td>
</tr>
<tr>
<td>MIP-2</td>
<td>3.60 ± 6.7*</td>
<td>0.08 ± 0.4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>3.62 ± 4.1*</td>
<td>0.51 ± 0.9</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>6.72 ± 13.9*</td>
<td>2.63 ± 5.6</td>
</tr>
<tr>
<td>Eotaxin-2</td>
<td>4.21 ± 5.3</td>
<td>1.32 ± 1.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data were expressed as the mean ± standard deviation (SD). To increase statistical power, data from the current and previous (25) chronic sepsis study were combined for several cytokines: DIE n=29 (IL-1β, IL-6, TNFα, MIP-2, MCP-1, Eotaxin) and n=15 (IL-2, IL-5, IL-12, IL-17, IFNγ, ICAM-1, MIP-1α, Eotaxin-2); Surviving n=58 and 30 (cytokine distribution as in DIE).

<sup>b</sup> Values collected within 24h of chronic death (any 6-28 day post-CLP).

<sup>c</sup> Cytokine values collected from Surviving (two per one dying) animals (alive at day 28 post-CLP) were sampled for comparison on the same post-CLP day as the Dying mice.

* p<0.01;  † p<0.05 versus Surviving.
**Supplementary Table II.** Comparison of circulating anti-inflammatory cytokine concentrations between mice dying \(^b\) and surviving during the chronic phase of sepsis

<table>
<thead>
<tr>
<th>Cytokine(^a) (ng/ml)</th>
<th>Dying(^b)</th>
<th>Surviving(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukin (IL)-1ra</td>
<td>43.1 ± 48.9*</td>
<td>1.71 ± 4.71</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.96 ± 4.81*</td>
<td>0.10 ± 0.20</td>
</tr>
<tr>
<td>TNF srI</td>
<td>5.59 ± 11.8*</td>
<td>0.88 ± 0.41</td>
</tr>
<tr>
<td>TNF srII</td>
<td>0.29 ± 0.36*</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.29 ± 2.28#</td>
<td>0.13 ± 0.15</td>
</tr>
<tr>
<td>IL-13</td>
<td>6.28 ± 11.5</td>
<td>0.69 ± 1.09</td>
</tr>
</tbody>
</table>

\(^a\) Data were expressed as the mean ± standard deviation (SD). To increase statistical power, data from the current and previous (25) chronic sepsis study were combined for several cytokines: Dying n=29 (IL-1ra, IL-10, TNF srI, TNF srII) and n=15 (IL-4, IL-13); Surviving n=30 or 58 (distribution as in Dying, respectively);

\(^b\) Values collected within 24h of chronic death (any 6-28 day post-CLP).

\(^c\) Cytokine values collected from Surviving (two per one dying) animals (alive at day 28 post-CLP) were sampled for comparison on the same post-CLP day as the Dying mice.

\(* p<0.001; \ # p<0.05 \) versus Surviving.