RESEARCH ARTICLE

Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock

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Accepted for publication May 2, 2011

To cite this article: Tamayo E, Fernández A, Almansa R, Carrasco E, Heredia M, Lajo C, Goncalves L, Gómez-Herreras JI, Ortiz de Lejarazu R, Bermejo-Martin JF. Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. *Eur. Cytokine Netw.* 2011; 22(2): 82-7 doi:10.1684/ecn.2011.0281

ABSTRACT. The relationships between cytokine responses in septic shock are currently poorly understood. Some studies have pointed to a biphasic model, with an initial proinflammatory phase, followed by a reactive, anti-inflammatory response to explain the pathogenesis of the most severe form of sepsis. However, evidence for the coexistence of both responses has been found. In this study, the plasma levels of 17 cytokines and chemokines, in 20 patients with septic shock, 11 patients with systemic inflammatory response syndrome (SIRS), during the first 24 hours following diagnosis, and 10 healthy controls, were analyzed and compared. Patients with septic shock showed increased levels of IL-6, IL-8, MCP-1, MIP-1β, IFN-γ, GM-CSF and IL-10 compared to healthy controls. Patients with SIRS showed higher levels of IL-6, IL-8, MCP-1, MIP-1β, G-CSF and IL-10 than controls. Patients with septic shock showed higher levels of IL-8, GM-CSF, MIP-1β than those with SIRS. The Spearman test demonstrated a positive association between the pro-inflammatory mediators IL-6, IL-8, MCP-1, MIP-1β, IFN-γ, GM-CSF and the immunomodulatory cytokine IL-10 in septic shock. Consequently, correlation studies supported the notion that secretion of pro- and anti-inflammatory mediators in septic shock occurs as a simultaneous immune response program initiated early in the course of the disease, revealing that both types of cytokine play a role from the very beginning of this life-threatening condition.

Key words: pro-inflammatory, anti-inflammatory, septic, shock, simultaneous

Sepsis is an uncontrolled, systemic, inflammatory response which is a consequence of infection that, in its most severe form (septic shock), causes organ dysfunction and hypotension. Even today, septic shock remains associated with high mortality rates, presenting a challenge for both physicians and researchers [1]. Involvement of cytokine responses in the pathogenesis of sepsis tends to be explained using a bi-phasic model, with an initial state characterized by systemic production of inflammatory cytokines (IL-6, TNF-α, IFN-γ, IL-1), followed by the secretion of anti-inflammatory mediators such as IL-10 [2]. The early, pro-inflammatory state would contribute to tissue damage and mortality, while the secondary, anti-inflammatory phase (termed compensatory anti-inflammatory response syndrome, CARS), would induce an increased risk of secondary bacterial infection [3]. However, evidence contradicting the existence of two phases in the immune response in sepsis as a generalized phenomenon, also exists [4].

A better understanding of the regulation and overall balance between pro- and anti-inflammatory cytokine responses in sepsis is essential in order to explain the pathogenesis of this disease.

The emergence of several different approaches offers an opportunity to profile simultaneously a wide range of immune mediators, thus helping to obtain a clearer picture of the ongoing host immune response [5]. An analysis of systemic chemokine and cytokine levels in serum from patients with septic shock or systemic inflammatory response syndrome (SIRS) was performed using a 17-plex quantification kit. This allowed identification of the existence of simultaneous, antagonistic, immune response signatures, early in the onset of septic shock.

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doi: 10.1684/ecn.2011.0281

DONORS AND METHODS

Patients and controls

Patients and controls were prospectively recruited from the Intensive Care Unit (ICU) of the Hospital Clínico Universitario de Valladolid in Spain. Approval for the study protocol for both the scientific and the ethical aspects was obtained from the Scientific Committee for Clinical Research of our Hospital. Recommendations of the the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference were followed in order to define SIRS and septic shock [6]. Severity of illness was assessed by two different scores: the acute physiology and chronic health evaluation (APACHE) II score for the first 24 hours following diagnosis [7], and the sequential organ failure assessment (SOFA) score [8]. Twenty patients with septic shock and eleven patients fulfilling the criteria for SIRS were recruited for the study. Ten healthy volunteers were recruited from the staff of the University of Valladolid, and had an age range similar to patients included in the study.

Samples and cytokine profiling

A single blood sample was obtained from each patient or control in tubes containing ethylenediaminetetraacetic acid (EDTA) within the first 24 hours following diagnosis of septic shock or SIRS. Plasma samples were obtained after proper centrifugation and these were stored at -80°C until cytokine profiling. Plasma chemokine and cytokine levels were evaluated using the multiplex Biorad© 17 plex assay, following manufacturer's instructions. This system allows for quantitative measurement of 17 different chemokines, cytokines, growth-factors and immune mediators, while consuming only a small amount of biological material. Furthermore, this system has good representation of analytes for inflammatory cytokines, anti-inflammatory cytokines, Th1 cytokines, Th2 cytokines, Th17 cytokines and chemokines, allowing for testing of different levels of regulatory cytokines in patients' plasma. Limits of detection were as follows (pg/mL): IL-1b (2,4); IL-6 (2,1); IL-8 (1,8); IL-7 (2,4); IL-17 (2.1); G-CSF (1,5); MCP-1 (1,8); MIP-1b (1,4); IL-2 (1,3); IL-4 (0,2); IL-5 (2,5); IL-10 (1,8); IL-12(p70) (2,6); IL-13 (2,6); GM-CSF (0,7); IFN-γ (2,4); TNF-α (6,4).

Statistical analysis

The Mann-Whitney U test was employed for cytokine comparison purposes, since the Saphiro-Wilk test demonstrated the absence of normal distribution of the data, and the Levene test demonstrated the absence of homogeneity of variance in the compared groups. Correlation studies between cytokine levels and clinical parameters were performed by using the Spearman test. All statistical tests were two-sided, and $p<0.05$ was considered significant. Spearman correlation coefficients were also represented as heat maps using the JColorGrid software (University of California San Francisco and University of California Berkeley) [9].

RESULTS

Clinical and demographic characteristics (table 1)

Patients were elderly individuals in both groups. The most common co-morbidity was cardiovascular disease. The most common immediate antecedent in the septic shock group was abdominal surgery. Procalcitonin and C-reactive protein levels were higher in the shocked patients compared to those with SIRS ($p<0.05$), as were the leucocytes counts ($p<0.05$).

Comparisons of immune mediator levels (figure 1)

IL-1β, IL-7, IL-5, and IL-13 levels were below the limit of detection of the method employed for each mediator in the vast majority of the patients studied. As a consequence, they were not considered in the analysis. Patients with septic shock demonstrated increased levels of IL-6, IL-8, MCP-1, MIP-1β, IFN-γ, GM-CSF and IL-10 compared to healthy controls. Patients with SIRS showed significantly higher levels of IL-6, IL-8, MCP-1, MIP-1β, G-CSF and IL-10 compared to the control group. Seven patients with septic shock showed detectable levels of IL-17; the other seven showed increased levels of TNF-α. Six patients from this group showed detectable levels of IL-2 and IL-4, while three patients showed detectable levels of IL-12p70. Only one patient with SIRS showed detectable levels of IL-17 (13.9 pg/mL), while another showed detectable levels of IL-2, IL-4, IL-12p70 and TNF-α (data not shown). None of the controls had detectable levels of IL-17, IL-2, IL-4, IL-12p70 or TNF-α. When the levels of immune mediators were compared between the septic shock group and the SIRS group, the shocked group showed higher levels of IL-8, GM-CSF and MIP-1β ($p<0.05$).

Correlation of immune mediator levels (figure 2)

Correlation studies demonstrate a positive association between levels of IL-6, IL-8, MCP-1, MIP-1β, IFN-γ, GM-CSF and IL-10 in those patients with septic shock, with Spearman correlation coefficients (r) >0.4. This positive correlation demonstrates that these mediators are secreted simultaneously in response to the insult leading to septic shock. In patients with SIRS, the following positive correlations were found: [IL-6, IL-8), [IL-6, G-CSF], [IL-8, IFN-γ], [G-CSF, MCP-1], [MCP-1, MIP-1β], with r coefficients >0.5 (data not shown).

DISCUSSION

The presence of increased plasma levels of IL-6, IL-8, MCP-1, MIP-1β, IFN-γ and GM-CSF found in our patients are in agreement with a previous study [10], which reports the existence of a strong, systemic, pro-inflammatory cytokine and chemokine response in the most severe form of sepsis, septic shock. Most have already been studied as biomarkers in sepsis [11]. These mediators are chemotactic molecules that attract monocytes, neutrophils, T cells and macrophages to the injured tissues,

Table 1
Clinical and demographic data.

	Septic shock (n=20)	SIRS (n=11)
Description		
Gender (M/F)	12/8	9/2
Age (years)	68.00 (14.6)	76.6 (8.6)
Arterial hypertension (n)	7	9
Cardiopathy (n)	7	6
Diabetes mellitus (n)	1	1
Obesity (n)	0	1
Respiratory disease (n)	3	1
Cancer (n)	3	4
Renal disease (n)	3	0
Surgery (abdominal/cardiac/vascular)	15/5/0	5/5/1
Days at the CCU	12.00 (13.5)	1.00 (0.5)
Sampling day following admission to the CCU	6.00 (7.0)	1.00 (0.7)
Clinical status at diagnosis		
APACHE score	22.24 (6.7)	9.00 (2.4)
SOFA score	7.70 (3.7)	3.36 (2.6)
Mechanical ventilation	18/20	9/11
Heart rate	112.00 (20.4)	86.00 (16.2)
Respiratory rate	14.53 (4.5)	13.90 (3.6)
Temperature	37.46 (1.4)	36.7 (1.1)
Average blood pressure (BP)	67.54 (11.3)	82.2 (10.1)
Noradrenaline	20/20	2/11
Analytical status at diagnosis		
Hematocrit (%)	32.71 (6.6)	32.31 (5.0)
Leucocytes (number/mL)	17487.47 (10867.2)	11582 (4962.6)
Neutrophils (number/mL)	15431.2 (11120.8)	8475.1 (2768.9)
Procalcitonin	26.69 (38.7)	1.45 (3.4)
C Reactive Protein	184.2 (106.5)	45.9 (68.8)
Creatinine (mg/dL)	1.63 (1.1)	1.09 (0.4)
Patients with positive/negative microbiology cultures	18/2	0/11
Fatal outcome/survivors	11/9	1/10

playing not only a main role in fighting infection, but also in the pathogenesis of acute and chronic diseases with a strong inflammatory component [12]. At the same time, our results provide new evidence of the coexistence of the pro- and anti-inflammatory responses, as revealed by the increased levels of IL-10 in plasma. IL-10 is a molecule with immune-regulatory properties. Secretion of IL-10 in septic shock could represent an attempt to modulate the pro-inflammatory response observed in this condition. On the other hand, high levels of IL-10 could induce a state of functional immunodeficiency, compromising the host response to pathogens and the management of infection [13].

Previous works using the multiplex approach (Bozza *et al*. [5] and Mera *et al*. [14]) have revealed the relationship between severe septic disease and increases in IL-6, IL-8, MCP-1, IFN-γ, IL-10. These studies also reported increased levels of IL-4, IL-12p70 or TNF-α in those patients with the worst outcomes. In spite of the fact that we failed to find increased levels of these three cytokines in all of our patients, they were found in some of them. The small size of the cohort analyzed perhaps precluded obtaining more robust results regarding these cytokines. On the other hand, our results for GM-CSF and MIP-1β contradict, to a certain extent, those of Mera *et al*. [14], since they found lower levels of these molecules in

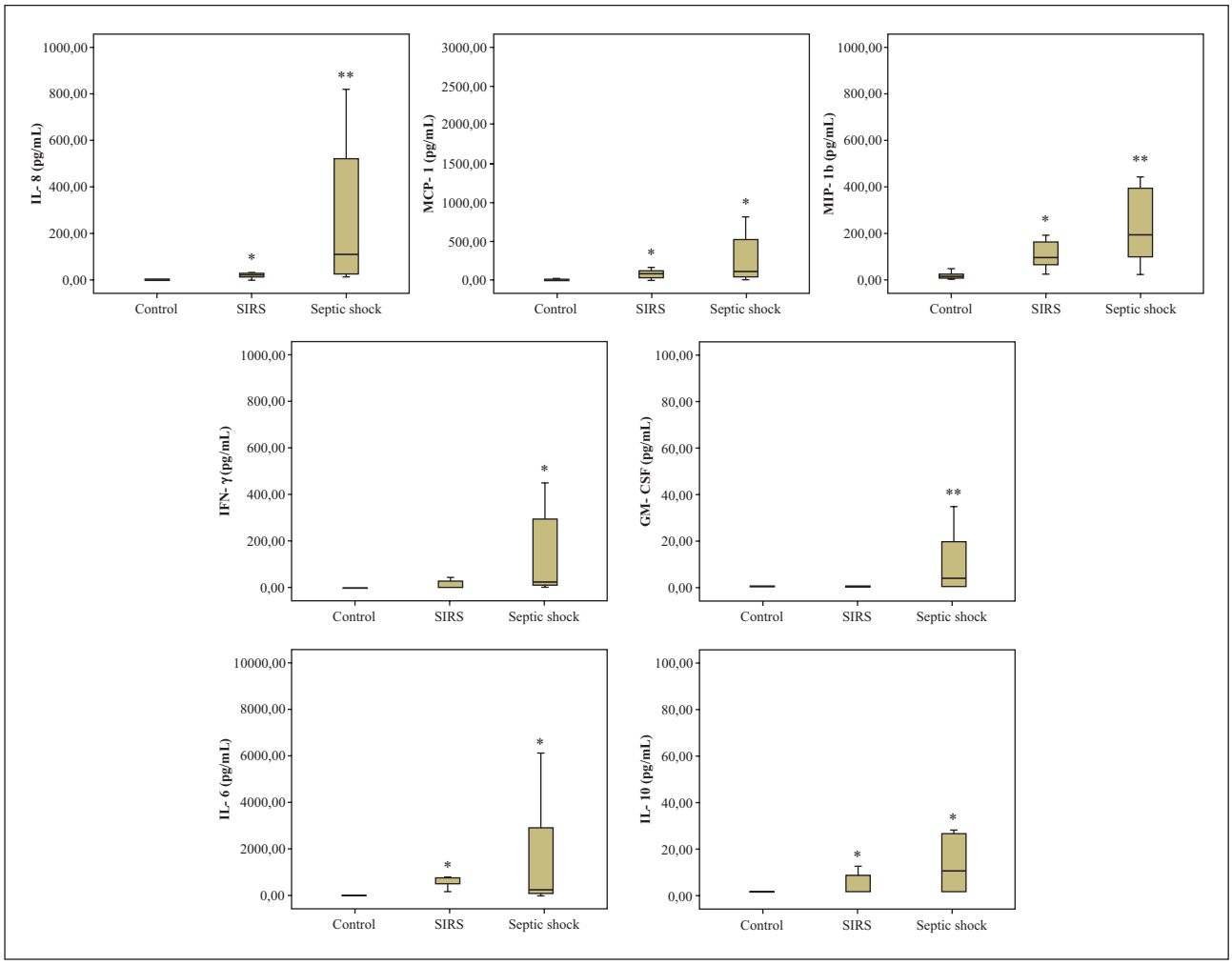

Figure 1

Immune mediator levels in healthy controls, SIRS and septic shock.
* Significantly different from the control. ** Significantly different from the the control and also the SIRS group ($p<0.05$).

patients with septic shock than in patients without shock. The different location of the initial infection site (respiratory and urinary in the work of Mera *et al*. abdominal in our work), could explain some differences in the profile of the cytokines found. According to the result of this study, increased levels of the Th17 cytokine, IL-17 in 35% of the patients with septic shock might indicate a potential role of this mediator in autoimmunity and antibacterial defense [12], in some cases of severe septic disease.

Most importantly, association observations of immune mediator levels in this study, confirm that both pro and anti-inflammatory responses occur in a simultaneous manner. In this sense, Cavaillon *et al*. had already found positive correlations between IL-8, MCP-1, MIP-b, RANTES and IL-1ra, IL-10 in sepsis [15]. In agreement with the results of Cavaillon *et al*., this study demonstrates that the simultaneous pro-inflammatory and anti-inflammatory responses occur from the very first moments of onset of the disease. The potential influence of this early, combined response on further pathogenic events, such as the development of a late phase of immunoparalysis [16], and also on mortality [17, 18] remains to be elucidated, since the design of this study did not include a follow-up of the patients.

Similarly to the results reported by Rodríguez-Gaspar *et al*. [19], patients with SIRS also demonstrated significant increases in pro-inflammatory (IL-6, IL-8, MCP-1, MIP-1β, G-CSF) and anti-inflammatory mediators (IL-10), compared to controls, although the increase in IL-10 levels was slight in these patients. The absence of positive correlations between pro-inflammatory mediators and IL-10 in patients with SIRS could be a consequence of the limited number of individuals included in this group, or alternatively, to a less severe pro-inflammatory state in SIRS compared to septic shock, with a limited compensatory secretion of IL-10. The absence of a suspected infection in SIRS could also explain the differences in the cytokine profiles compared with shock [20].

In conclusion, correlation studies for cytokine levels in plasma, support the notion that secretion of pro- and anti-inflammatory mediators in septic shock occurs as a simultaneous, immune response program initiated early in the course of the disease. The exact role of this simultaneous response (detrimental or beneficial), in the evolution of patients with sepsis is worth further investigation.

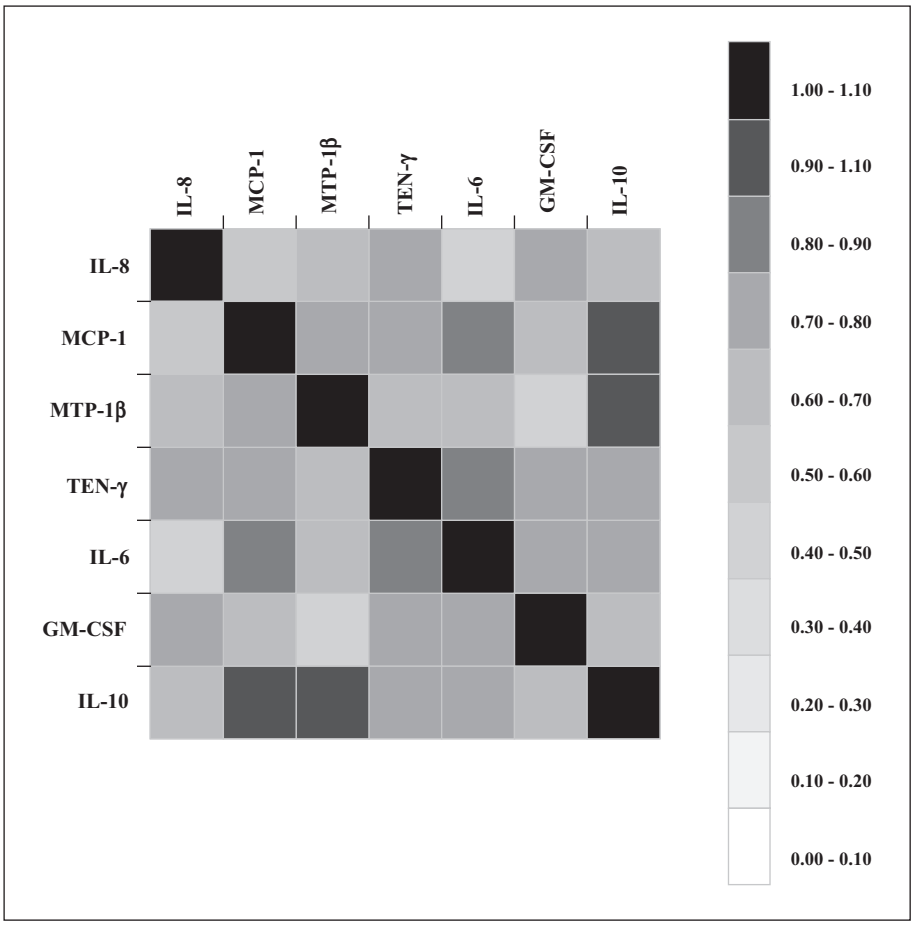

Figure 2
Heat map representing the Spearman coefficient (r) corresponding to the correlations found to be significant at the level $p<0.05$. Values of "r" coefficients were represented using a grey scale. The figure was prepared using the Jcolorgrid software.

Acknowledgments. *The authors wish to thank Lucia Rico and Veronica Iglesias for their technical support. Likewise, the authors are grateful to the nursing team of the Internal Medicine Unit of our Hospital, who kindly collected the samples. We also thank Alicia San José for her assistance with the English language aspects.*

Disclosure. *This study was possible because of a grant obtained from the Spanish Ministry of Science and Innovation and the "Consejería de Sanidad Junta de Castilla y León" ("Programa para favorecer la incorporación de grupos de investigación en las Instituciones del Sistema Nacional de Salud, EMER07/050" y "Proyectos de Investigación Sanitaria, PI 10/01362).*
None of the authors has any conflict of interest to disclose.

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