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Increased percentage of CD4+CD25+ regulatory T cells during septic shock is due to the decrease of CD4+CD25- lymphocytes

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Objective: The elevation of the percentage of regulatory CD4+CD25+ T lymphocytes (Treg) has been recently described during septic shock. The objective of the present study was to investigate whether this increased percentage was due to Treg proliferation.

Design: Observational study.

Setting: Adult intensive care units in a university hospital.

Subjects: Patients with septic shock (n = 54) and healthy individuals (n = 30).

Interventions: None.

Measurements and Main Results: In patients, we first confirmed the increased percentage of Treg among CD4+ lymphocytes in comparison with healthy individuals. Surprisingly, regarding absolute counting, we demonstrated that both T

CD4+ lineages (CD25+ and CD25-) were diminished immediately after the onset of shock. Then, whereas Treg returned rapidly to healthy donors values, CD4+CD25- T lymphocytes remained dramatically reduced. Finally, Foxp3 (Treg-related gene) messenger RNA quantification enabled us to definitively rule out a lack of proliferation since it was not increased during shock.

Conclusion: The increased percentage of Treg after shock is due not to their proliferation but to a decrease in CD4+CD25- T lymphocyte number. We hypothesize that it might be due to a resistance of Treg to apoptosis processes occurring during septic shock. (Crit Care Med 2004; 32:2329-2331)

KEY WORDS: septic shock; regulatory T cells; CD4+CD25+; Foxp3; immunosuppression; sepsis

Sepsis is still the leading cause of death in critically ill patients (1). It is now believed that those patients have features consistent with immunosuppression (1-2). The failure of the immune system may be defined by the impairment of monocytic functions, the dramatic apoptosis-induced loss of lymphocytes (B and T cells) and dendritic cells, and the anergy of T lymphocytes (1-2). We described an additional characteristic of sepsis-induced immunosuppression as we demonstrated an increased percentage of CD25+ T cells among CD4+ T lymphocytes in patients with septic shock (3). Although recently described in humans, CD4+CD25+ T lymphocytes (Treg) are already well defined as suppressor T cells. There is a growing body of evidence demonstrating that they are key cells in controlling different immune functions (4). In various animal models, bacterial infec-

tions induce elevation of Treg, which play a role in avoiding a systemic inflammation (5). These findings support the preliminary observation of an elevated percentage of Treg in patients with septic shock in whom it may contribute to sepsis-induced immunosuppression.

The goal of the present study was to investigate whether the elevation of the percentage of Treg during shock was due to their differentiation. To this end, we determined the absolute count of those cells and we measured Foxp3 messenger RNA (mRNA) by quantitative real-time polymerase chain reaction (qRTPCR) since, at present, Foxp3 appears to be the gene best correlated with Treg (6).

METHODS

Patients and Study Design. The study group consisted of 54 patients with septic shock according to the diagnostic criteria of the American College of Chest Physicians/Society of Critical Care Medicine (7). Septic shock was defined by an identifiable site of infection, hypotension persisting despite fluid resuscitation and requiring vasopressor therapy, and evidence of a systemic inflammatory response manifested by at least two of the following criteria: a) temperature >38°C or <36°C; b) heart rate >90 beats/min; c) respi-

ratory rate >20 breaths/min; d) white blood cell count >12,000/mm³ or <4000/mm³. Severity was assessed by the Simplified Acute Physiology Score II (8). Mortality was defined as death occurring within 28 days after the onset of shock. To provide a panel of values from healthy donors, we also included 30 individuals from laboratory staff of our hospital (aged 19-58 yrs, 17 females, 13 males, without known comorbidity). Cell phenotyping was performed at days 1-2 and days 3-6 after the onset of shock on residual blood after completing routine follow-up performed in our intensive care unit (ICU), in accordance with the human experimentation guidelines for clinical research of our institute. For mRNA measurements, blood samples (2.5 mL) were drawn directly into PAXGene Blood RNA tubes (PreAnalytix, Hilden, Germany). All patients or relatives gave informed consent. Hence, missing values are due to patients who died (nonsurvivors) and to patients (survivors) who recovered and left the ICU before 6 days.

Flow Cytometry. Flow cytometric (EPICS XL, Beckman-Coulter, Hialeah, FL) expression of cell-surface markers was assessed on peripheral whole blood collected on EDTA anticoagulant tubes. Monocytes were characterized as previously described (3), and regulatory T cells were characterized by expression of CD3, CD4, CD25, and CD45RO and by the lack of expression of CD69 (3). A number of monoclonal antibodies and their respective isotype controls were used according to

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the manufacturer's recommendations: ECD-labeled anti-CD3, PE-labeled anti-CD25, PC5-labeled anti-CD69, fluorescein isothiocyanate-labeled anti-CD4, ECD-labeled anti-CD45RO (Immunotech, Marseille, France). The red cells were lysed on the automated TQ Prep system (Beckman-Coulter). Absolute count was performed using Flow-Count Fluorosphere according to the manufacturer's recommendation (Beckman-Coulter). Results are expressed as percentages of CD25 positive cells out of CD4+ lymphocytes (CD3+ CD4+) and as number of lymphocytes per microliter of whole blood.

Foxp3 mRNA Expression by qRT-PCR. Total RNA was extracted using PAXGene Blood RNA kit (PreAnalytix) and treated by DNase (RNase-Free DNase set, Qiagen). Total RNA was reverse transcribed into complementary DNA using ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Foxp3 mRNA level was quantified using qRT-PCR and the FastStart DNA Master SYBR Green I real-time PCR kit (Roche Molecular Biochemicals). The complementary DNA calibrator for Foxp3 was prepared with the following primer combination: forward primer, 5'-TCACCTACGCCACGCTCAT-3'; reverse primer, 5'-ACTCAGGTGTGCGCGATG-3' as previously described (9). Primer mixes and complementary DNA standard for the housekeeping gene PPIB (encoding for cyclophilin B) were obtained from Search-LC (Heidelberg, Germany). Relative quantification ratio between Foxp3 and the housekeeping gene PPIB was obtained with the LightCycler Relative Quantification Software.

Statistical Study. Due to the sample size, comparisons between groups were made using the nonparametric Mann-Whitney test. We considered $p < .05$ as significant without correction of the number of tests performed. Results are expressed as mean \pm SD.

RESULTS AND DISCUSSION

We included 54 patients exhibiting symptoms of septic shock. The demographic and clinical characteristics of the population are presented in Table 1. Mortality of the overall population was 24%. Thirty patients had no comorbidity. Main comorbidities were chronic obstructive pulmonary disease (17%), diabetes (9%), chronic cardiac failure (7%), and hemopathy (7%). Immunoparalysis was characterized by diminished expression of HLA-DR on monocytes (data not shown). Treg were distinguished from the recently activated T cells by the expression of CD45RO (marker of memory T cells, $86 \pm 15\%$) and by the lack of expression of CD69 (marker of recently activated lymphocytes, $11 \pm 7\%$). We observed a significant increase in the percentage of

Table 1. Demographic and clinical characteristics

| | No. (%) |
|--|------------|
| Male | 32 (59) |
| Female | 22 (41) |
| Age, yrs, mean (95% CI) | 63 (46–82) |
| Main diagnosis category | |
| Medical | 26 (48) |
| Surgery | 28 (52) |
| Microbiologically documented diagnosis | 42 (78) |
| Bacilli Gram (–) | 21 (39) |
| Cocci Gram (+) | 26 (48) |
| Fungi | 15 (28) |
| Others | 8 (15) |
| Type of infection | |
| Community acquired | 29 (54) |
| Nosocomial | 25 (46) |
| Site of infection | |
| Pulmonary | 24 (44) |
| Abdominal | 22 (41) |
| Others | 8 (15) |
| SAPS II at admission, mean (95% CI) | 51 (35–68) |

CI, confidence interval; SAPS, Simplified Acute Physiology Score.

CD4+CD25+ T lymphocytes in patients at days 1–2 (32 ± 11) and days 3–6 (36 ± 12) in comparison with healthy donors (20 ± 6 , $p < .0001$, Fig. 1A). Otherwise, we did not find any correlation between the percentage of Treg and Simplified Acute Physiology Score II at admission or with percentages of HLA-DR expressing monocytes (data not shown). Regarding cell count, as reported elsewhere (1), we observed a lymphopenia at days 1–2 ($864 \pm 604/\mu\text{L}$) and days 3–6 ($1239 \pm 949/\mu\text{L}$) in comparison with healthy donors ($3113 \pm 1956/\mu\text{L}$, $p < .02$). We next investigated the absolute count of circulating Treg. In healthy donors, we obtained similar values to those published elsewhere (10). We found that early after the onset of shock, both T CD4+ lineages (CD25+ and CD25–) were diminished. However, their magnitudes of decrease were quite different: We observed a marked diminution of CD4CD25– lymphocytes (70% below normal values) whereas that of Treg was less pronounced (43%, Fig. 1, B and C). Then, whereas Treg returned rapidly to normal values, CD4+CD25– T lymphocytes remained dramatically reduced. This suggested that the increased percentage of Treg was not due to an elevation of those cells but rather to a decrease of CD4+CD25– lymphocytes. To definitely rule out a lack of proliferation, we quantified Foxp3 mRNA in peripheral blood. Although Treg represent a small number of cells in whole blood, the reliability of measuring spe-

cific mRNA from minority cell population has been recently validated for monocytes (11) and Th2 cells (12). Foxp3 is a transcriptional factor thought to be specifically linked to Treg. Its overexpression correlated with their differentiation (6). At the onset of shock, as expected, Foxp3 mRNA was not increased and was even found to be decreased in patients in comparison with healthy donors ($p = .015$) and it returned to normal values at days 3–6 (Fig. 1D). Hence, we did not observe any increase in Foxp3 mRNA after septic shock, which agrees with results from absolute counting. In total, it suggests that Treg elevation is the consequence of a mechanism that does not imply Treg by themselves.

The elevated percentage of Treg has now been observed in a total of 69 patients with septic shock (including those of our preliminary study) (3). We suggest that this increase, independently of disease severity, may belong to (or be a consequence of) the homeostatic modulatory mechanisms rapidly occurring after the onset of shock, also known as compensatory anti-inflammatory response syndrome (13). One important mechanism of action proposed for Treg is cell-contact dependent. Hence, the relative abundance of those cells at the onset of shock may have a major impact on a patient's immune response. Experiments are currently under process in our laboratory to define a role for those cells during sepsis-induced immunoparalysis.

The main result of the present study is to demonstrate that the increased proportion of the CD25+ subset among CD4+ T cells is not due to a proliferation of Treg but rather to a selective depletion of CD4+CD25– T cells. Indeed, we have shown that, despite a moderate initial decrease, Treg tended to remain in normal range during septic shock, whereas CD4+CD25– were found to be dramatically decreased until day 6. Consequently, it seems that Treg are weakly affected by cell death reported during septic shock. As proposed in gastrointestinal malignancies (10), one may hypothesize that these cells are less sensitive to the massive sepsis-induced apoptotic processes than other lymphocytes. Accordingly, Treg have been demonstrated to be resistant to dexamethasone-induced apoptosis in mice (14) even though glucocorticoids are believed to be involved in sepsis-induced apoptosis (1). It has been also

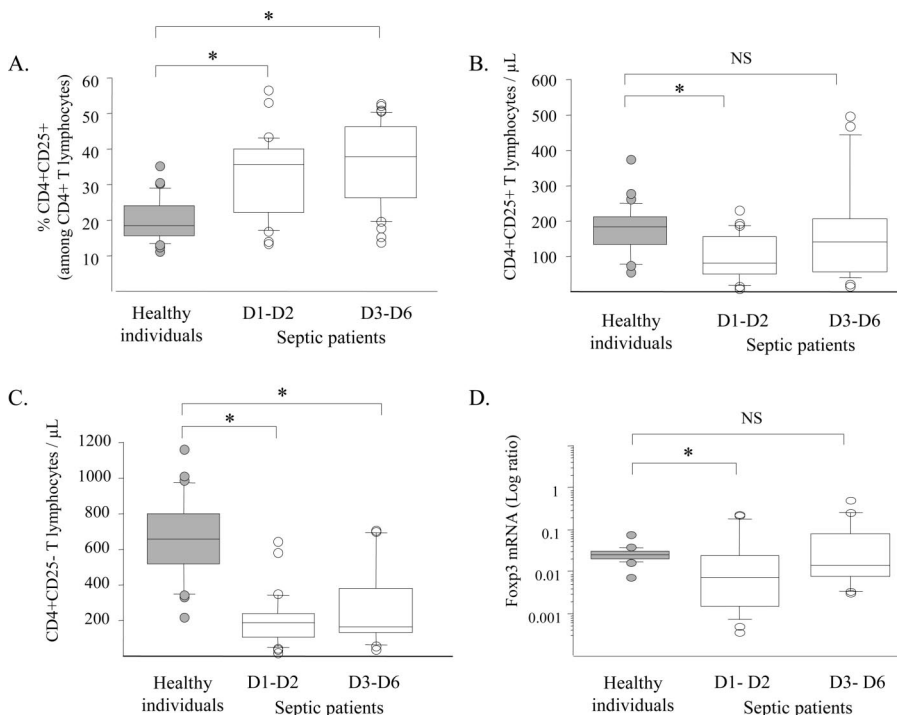


Figure 1. Increased proportion of regulatory CD4+CD25+ T lymphocytes (Treg) during septic shock is due to the decrease of CD4+CD25- lymphocytes. **A**, CD4+ T cells were examined for their percentage of regulatory T cells (CD4+CD25+CD45RO+CD69-). Circulating lymphocytes from 54 patients (open boxes) were phenotyped at days 1–2 (D1–D2, n = 31) and days 3–6 (D3–D6, n = 46) after the onset of shock. As control, values from 30 healthy donors (measured once) are represented (gray box). Data are shown as a box-plot representation. The nonparametric Mann-Whitney test was used to assess significance between groups (**p* < .0001 vs. control values). Including results from our previous study, the percentage of Treg has now been determined in a total of 66 healthy individuals; results are 20.0 ± 0.6 % (median, 18.6) on the overall normal population. **B**, absolute counting of CD4+CD25+ T lymphocytes was performed in whole blood from 30 patients with septic shock (open boxes) and 30 healthy individuals (gray box). Patients' lymphocytes were counted at days 1–2 (n = 26) and days 3–6 (n = 23) after the onset of shock; those of donors were measured once. Data are shown as a box-plot representation. The nonparametric Mann-Whitney test was used to assess significance between groups (**p* = .0001 vs. control values). **C**, absolute counting of CD4+CD25- T lymphocytes was performed in whole blood from 30 patients with septic shock (open boxes) and 30 healthy individuals (gray box). Patients' lymphocytes were counted at days 1–2 (n = 26) and days 3–6 (n = 23) after the onset of shock; those of donors were measured once. Data are shown as a box-plot representation. The nonparametric Mann-Whitney test was used to assess significance between groups (**p* < .0001 vs. control values). **D**, Foxp3 gene expression was quantified in peripheral blood of 27 patients with septic shock at days 1–2 (n = 22) and days 3–6 (n = 14) after the onset of shock using real-time polymerase chain reaction (open boxes). Results are expressed as relative quantification ratio between Foxp3 and the housekeeping gene PPIB encoding for cyclophilin B. Normal expression levels were measured once in 16 healthy donors (gray box). Data are shown as a box-plot representation. The nonparametric Mann-Whitney test was used to assess significance between groups (**p* < .02 vs. control values). mRNA, messenger RNA.

reported that Treg are resistant to Fas-dependent apoptosis *in vitro* (15). This hypothesis remains to be further investigated. There has been growing recognition that apoptotic processes play a major role in septic shock pathophysiology. A better understanding of the interaction between apoptosis and immunosuppression may provide new targets for therapy of this hitherto deadly disease.

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