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Guillaume Monneret, Anne-Lise Debard, Fabienne Venet, Julien Bohe, Olivier Hequet, et al.. Marked elevation of human circulating CD4+CD25+ regulatory T cells in sepsis-induced immunoparalysis. *Critical Care Medicine*, 2003, 31 (7), pp.2068-2071. <10.1097/01.CCM.0000069345.78884.0F>. <hal-04066384>

HAL Id: hal-04066384

<https://hcl.hal.science/hal-04066384v1>

Submitted on 12 Apr 2023

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Marked elevation of human circulating CD4+CD25+ regulatory T cells in sepsis-induced immunoparalysis

Guillaume Monneret, PharmD, PhD; Anne-Lise Debard, PharmD; Fabienne Venet; Julien Bohe, MD; Olivier Hequet, MD; Jacques Bienvenu, PharmD, PhD; Alain Lepape, MD

Objective: Immunoparalysis has recently emerged as a possible cause explaining the failure of clinical trials in septic shock. Because human peripheral blood CD4+CD25+ T cells have been characterized as suppressor T cells, we hypothesized they might be increased in sepsis-induced immunoparalysis.

Design: Prospective, observational, clinical study.

Setting: Adult intensive care units in a university hospital.

Subjects: Patients with septic shock (n = 16) and healthy individuals (n = 36).

Interventions: None.

Measurements and Main Results: In patients with septic shock (mortality rate at 28 days, 56%; mean admission Simplified Acute Physiology Score II, 47), we first illustrated immunoparalysis by showing a severe diminished monocytic human leukocyte antigen

(HLA)-DR expression. Afterward, compared with control values, we found in these patients a marked elevation of circulating CD4+CD25+ T cells that were also CD45RO+ and CD69- and overexpressed CTLA-4. Importantly, nonsurvivors (n = 9) presented prolonged lower monocytic HLA-DR expression and higher percentage of CD4+CD25+ T-suppressor T cells.

Conclusions: These data support the concept that the persistence of a pronounced immunoparalysis after septic shock is associated with a poor outcome. Whether CD4+CD25+ T cells directly participate in sepsis-induced immunoparalysis remains to be investigated. (Crit Care Med 2003; 31:2068–2071)

KEY WORDS: human leukocyte antigen-DR; immunoparalysis; T lymphocytes; CD4+CD25+ T cells; septic shock

The concept of immunoparalysis recently has been proposed to explain the failure of 20 yrs of clinical trials using anti-inflammatory drugs in septic shock. In fact, the initial inflammatory stage of sepsis is rapidly counterbalanced by an anti-inflammatory response, which may adversely affect immune functions (1, 2). This secondary immunoparalysis state is first characterized by a monocytic deactivation: Phagocytic function, proinflammatory cytokine release, and antigen-presenting capacity (likely due to a decreased expression of human leukocyte antigen [HLA]-DR) are severely depressed (1, 2). On the other hand, T cells become hyporesponsive in terms of proliferation and turn toward a type 2 profile: Interleukin (IL)-4, tumor growth factor-β, and

IL-10 productions are increased, whereas those of IL-12 and interferon-γ are suppressed (1–2). Importantly, the persistence of immunoparalysis, as monitored by measuring monocytic HLA-DR expression, correlates with an increased risk for fatal outcome (3).

Recently, human peripheral blood CD4+CD25+ T cells have been characterized as suppressor T cells (4–5). The mechanism by which these cells mediate their effects remains under debate as well as the definition of their cellular targets. It is reported they act on T cells by inducing their anergy through a cell-contact mechanism in which CTLA-4 would be involved (6). On the other hand, CD4+CD25+ T cells also are thought to produce IL-10 and tumor growth factor-β and to suppress interferon-γ production (6). In line with these data, Suto et al. (7) suggested that regulatory T cells act by inhibiting the activation of Th1 T cells. Accordingly, Zelenika et al. (8) showed that CD4+CD25+ T cells express a subset of Th2 gene transcripts, whereas Iellem et al. (9) described a specific expression of type 2 chemokines receptor on their surface.

Given these properties, we hypothesized that CD4+CD25+ might have a

kind of type 2 role to play in sepsis induced-immunoparalysis. The present study was designed to determine whether CD4+CD25+ T cells are increased during the stage of immunoparalysis in patients with septic shock.

STUDY DESIGN

Patients. The study group consisted of 16 patients (mean age, 58 yrs; male/female ratio, 14/2) with septic shock according to the diagnostic criteria for septic shock of the American College of Chest Physicians/Society of Critical Care Medicine (10). Severity was assessed by the Simplified Acute Physiology Score II (11); mean admission score was 47 (range, 24–70). Mortality was defined as death occurring within 28 days after diagnosis. Because we focused our interest on the secondary stage (immunoparalysis) of septic shock, we excluded patients who did not survive >48 hrs after being admitted to intensive care units. None of the patients received corticosteroids as therapy for septic shock. Most patients presented with no preexisting underlying disease, and only two patients (one survivor, one nonsurvivor) presented with metastatic cancer. To provide a panel of

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DOI: 10.1097/01.CCM.0000069345.78884.0F

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control values, we also included 36 healthy individuals from our laboratory (age 22–48). Cell phenotyping in whole blood was performed as regularly as possible during 10 days after the onset of shock when residual blood was available after completing routine follow-up performed in our intensive care units (details in sampling are given in Table 1), in accordance with the human experimentation guidelines for clinical research of our institute.

Flow Cytometry and Cell Isolation. Flow cytometric (EPICS XL, Beckman-Coulter, Hialeah, FL) expression of cell-surface markers was assessed on peripheral whole blood collected in EDTA anticoagulant tubes. Monocytes were characterized by expression of CD14 (12) and regulatory T cells by coexpression of CD3, CD4, and CD25 (4–5). A number of monoclonal antibodies and their respective isotype controls were used according to the manufacturer's recommendations: PC5-labeled anti-CD45, fluorescein isothiocyanate-labeled anti-CD14, ECD-labeled anti-CD3, PE-labeled anti-CD25, ECD-labeled anti-CD45RO, PC5-labeled

anti CD69 (Immunotech, Marseille, France), PE-labeled anti-HLA-DR, Cy-Chrome-labeled anti-CTLA-4, PE-labeled anti-CD25 (Becton Dickinson-Pharmingen, San Jose, CA). Results are expressed as mean \pm SEM as percentages of HLA-DR-positive monocytes out of total monocyte population, as percentages of CD25-positive cells out of total CD4-T cell population (CD3+CD4+), and as percentages of CD69-, CD45RO-, or CTLA-4-positive cells out of total T regulatory population (CD3+CD4+CD25+). The different thresholds for positivity were defined with the respective isotype controls. Plate-bound anti-CD3 and soluble anti-CD28 (Becton Dickinson-Pharmingen) were used for polyclonal activation of T cells. For CD4+CD25+ isolation, CD4+ T cells were first isolated from peripheral blood mononuclear cells with a negative CD4+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Then, regulatory T lymphocytes were obtained after an additional purification by using CD25 microbeads (Miltenyi Biotec) as previously described (4). Purity was assessed by flow

cytometry (>90%) by using the anti-CD25 monoclonal from Becton Dickinson.

Statistical Study. Due to the sample size and some missing data at certain time points in the follow-up, the comparison between groups was made with a nonparametric Mann Whitney test, whereas the Wilcoxon paired test was used to assess daily individual variation in CD4+CD25+ percentages. We considered $p < .05$ to be significant, without correction of the number of tests performed.

RESULTS AND DISCUSSION

In the first days following septic shock, the expression of HLA-DR on monocytes was severely reduced in septic patients (Table 1). This illustrated the rapid onset of immunoparalysis. Mortality defined as death occurring within 28 days after diagnosis was 56%. When survivors and nonsurvivors were compared, there was no difference in HLA-DR monocytic expression until day 5. However, at days 7–10, we found higher values in survivors, suggesting an ongoing recovery of their immunologic functions (Table 1). These data agree with several studies which have shown that lower expression of monocytic HLA-DR in the first days after diagnosis is associated with a poor prognosis (1–3). We extended these results by illustrating that the recovery observed at days 7–10 in survivors was not found in nonsurvivors.

Rapidly following the onset of septic shock, we observed a progressive and significant elevation of CD4+CD25+ T cells above control values (Table 1 and Fig. 1). The highest values were found at day 5 (30 ± 2 ; median, 27; range, 23–42), which was significantly increased compared with control values ($p < .001$). Because CD25 is the IL-2 receptor α chain, we verified that this increase in CD4+CD25+ cells was not due to a sepsis-induced up-regulation of CD25 (Fig. 2). As expected, the majority of CD4+CD25+ cells did not express a phenotype of recently activated T cells since they express low levels of CD69 ($11 \pm 0.6\%$) and were CD45RO positive ($86 \pm 3\%$), which is similar to the human CD4+CD25+ regulatory T-cell phenotype described by others (4, 5). Moreover, after activation with plate-bound anti-CD3 plus soluble anti-CD28, most CD4+ cells became strongly CD25-CD69 double

Table 1. Immunomonitoring of patients with septic shock

	Day 1	Day 3	Day 5	Days 7–10	Control Values
HLA-DR+ (% of monocytes)					
Survivors	29 \pm 5 27 [16–45] n = 6	30 \pm 5 30 [11–54] n = 7	42 \pm 6 38 [27–70] n = 7	67 \pm 2 ^a 70 [51–71] n = 7	89 \pm 1 90 (from Ref. 12)
Nonsurvivors	23 \pm 4 24 [8–38] n = 5	17 \pm 3 18 [4–33] n = 8	27 \pm 4 27 [10–43] n = 7	29 \pm 3 25 [21–48] n = 9	
CD4+CD25+ (% of CD4+)					
Survivors	23 \pm 3 21 [15–32] n = 6	24 \pm 3 24 [16–35] n = 7	30 \pm 3 30 [24–41] n = 6	24 \pm 3 ^a 24 [12–32] n = 7	20 \pm 1 18 [12–26] n = 36
Nonsurvivors	23 \pm 4 18 [15–39] n = 6	25 \pm 5 22 [10–57] n = 8	29 \pm 3 26 [23–42] n = 5	38 \pm 5 37 [19–55] n = 9	
CTLA-4 (% of CD4+CD25+)					
Survivors	—	—	—	8 \pm 1 8 [6–10] n = 4	5 \pm 1 6 [2–8] n = 10
Nonsurvivors	—	—	—	19 \pm 7 11 [5–50] n = 6	

Changes of human lymphocyte antigen-reaction of degeneration (HLA-DR) expression on monocytes (percent of monocytes expressing HLA-DR), changes of CD4+CD25+ suppressor T cells (percent of total CD4 positive T cell population), and expression of CTLA-4 on CD4+CD25+ T (percent of CD4+CD25+ cells expressing CTLA-4) are expressed as mean \pm SE, median, [range of values], and number of samples, in patients who survived (n = 7) or died (n = 9).

^a $p < .05$ (Mann Whitney test) between survivors and nonsurvivors.

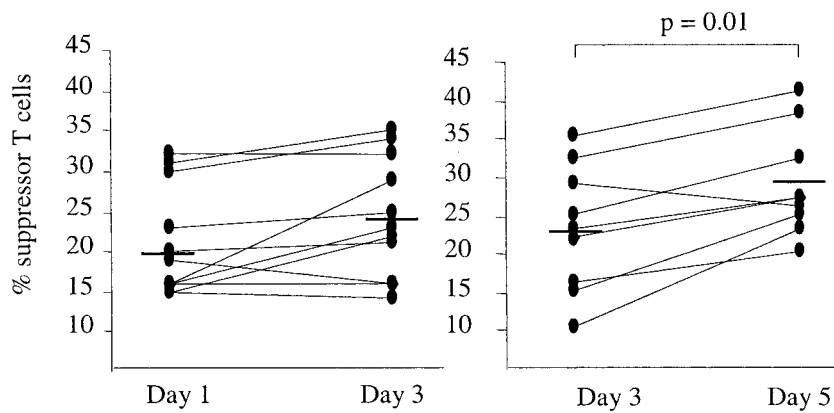


Figure 1. Elevation of CD4+CD25+ T cells in patients after septic shock. Individual values were taken into account when paired samples were available at the two time points considered: day 1 to day 3 (n = 11) and day 3 to day 5 (n = 9). Horizontal lines represent medians. The Wilcoxon paired test was used to assess significance between days.

These data support the concept that the persistence of a pronounced immunoparalysis after septic shock is associated with a poor outcome.

Interestingly, the elevation of CD4+CD25+ T cells was similar until day 5 in both survivors and nonsurvivors, suggesting a normal homeostatic mechanism. Afterward, however, a difference was observed (Table 1). At days 7–10, in survivors, the percentage of CD4+CD25+ T cells was 24%, whereas in sharp contrast, these cells still represented 38% of total CD4+ T lymphocytes in nonsurvivors.

Considering that regulatory T cell effects are believed to be mediated, for the most part, by CTLA-4, we also looked at its expression in ten donors and ten patients at days 7–10. We found that CTLA-4 levels were increased in cells from septic patients (15 ± 4; median, 10; range, 5–50) compared with control values (5 ± 1; median, 6; range, 2–8; *p* < .005). Due to the small number of patients, we could not demonstrate a difference between survivors and nonsurvivors regarding CTLA-4 expression. Even if we found higher values in nonsurvivors than in survivors (Table 1), this point remains to be investigated in a larger study to confirm the idea that elevated CTLA-4 expression is associated with patients who are not going to survive.

In total, the present study describes that circulating CD4+CD25+ T cells are increased during sepsis-induced immunoparalysis. Furthermore, the persistence of elevated percentages of these cells 10 days after the onset of septic shock was found in nonsurviving patients with low monocytic HLA-DR. To our knowledge, this is the first description of such an elevation of circulating CD4+CD25+ T cells in a human disease context. It provides an additional argument for rethinking the pathophysiology of septic shock. It seems likely that homeostatic modulatory mechanisms rap-

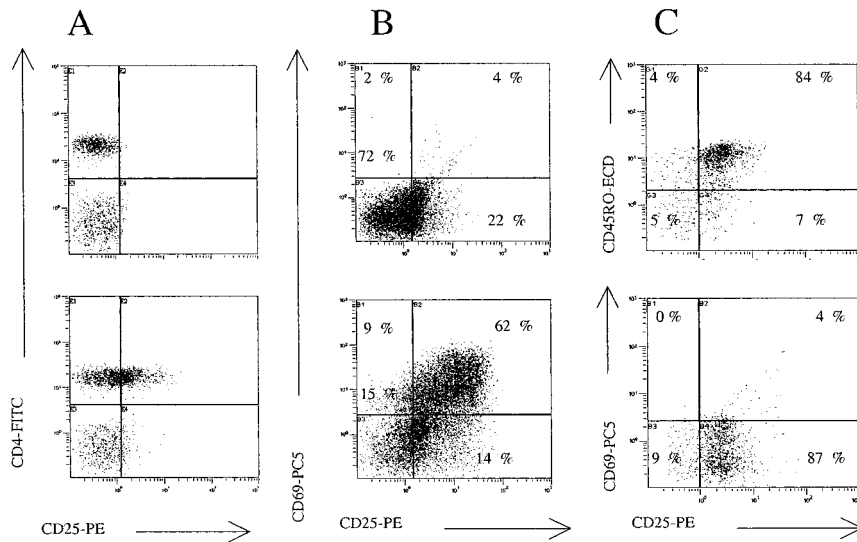


Figure 2. Human circulating CD4+CD25+ T cells in sepsis-induced immunoparalysis express low levels of CD25 and do not express CD69. *A*, identification of CD4+CD25+ T cells in whole blood in a representative patient during immunoparalysis (upper panel, patient + PE-isotype control; lower panel, patient + PE-anti-CD25). Cells were previously gated on their scatter characteristics and CD3 positive expression. *B*, CD4 T cells before (upper panel) and after (lower panel) activation with plate-bound anti-CD3 and anti-CD28 during 24 hrs (one representative experiment of four). Cells were previously gated on their CD3-CD4 characteristics. *C*, after purification, CD4+CD25+ T cells (ungated) express CD45RO (upper panel) and do not express CD69 (lower panel), one representative experiment of three.

positive (>60%), and CD25 expression levels after activation were higher compared with those measured on CD4+CD25+ cells isolated in patients (Fig. 2B). When expressed as means of fluorescence, CD25 levels were at 11 ± 1 and 43 ± 4 in patients' cells and CD3-activated cells, respectively. Together, these data strongly indicated that we were identifying regulatory T cells (CD25^{low}, CD69⁻) rather than recently activated T cells (CD25^{high}, CD69⁺). Besides, the hypothesis of an activated T population during immuno-

paralysis as illustrated by the low level of monocytic HLA-DR expression seems to be very unlikely. That would suggest the coexistence at the same time of a two-cell population with phenotypes corresponding to totally opposite stages of immune responses. We confirmed this whole blood phenotyping after purifying CD4+CD25+ T cells by using a magnetic cell separation system. In three additional donors, we obtained purified cells that were mainly positive for CD45RO (>94%) and negative for CD69 (<10%) (Fig. 2C).

idly occur after the onset of shock to counterbalance the initial inflammatory response. Nevertheless, in some patients, this may induce a prolonged and severe immunoparalysis that is associated with poor prognosis. A larger study is now needed to fully support this first description of elevated CD4+CD25+ in sepsis-induced paralysis and to investigate whether CD4+CD25+ may have a direct role in the different immunologic processes inducing immunoparalysis after a septic shock.

ACKNOWLEDGMENTS

This study was conducted thanks to the logistic and methodological support from the Centre d'Investigation Clinique (Clinical Research Centre) of INSERM and Hospices Civils de Lyon. We also thank J. Kingsley for his great help in preparing the manuscript and C. Fernandez, M. A. Guinand, and M. C. Gutowski for technical assistance in flow cytometric measurement.

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