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## EARLY ASSESSMENT OF LEUKOCYTE ALTERATIONS AT DIAGNOSIS OF SEPTIC SHOCK

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**ABSTRACT**—A dramatic decrease in circulating lymphocyte number is regularly described after septic shock. However, it is unknown how early this alteration develops after diagnosis of shock and if it remains stable over time. Twenty-one septic shock patients with no comorbidities were included within 2 h after the beginning of vasopressive treatment. Flow cytometry phenotyping of circulating leukocyte subpopulations and quantitative real-time polymerase chain reaction of T-bet, GATA-3, FOXP3, and ROR $\gamma$  mRNA were performed in patients from the diagnosis of shock and every 6 h during the subsequent 48 h. From their admission in the intensive care unit, patients present with major alterations of circulating leukocyte count (leukocytosis, neutrophilia, and major lymphopenia). The numbers of every lymphocyte subpopulations (T, B, and natural killer cells) were diminished. Gene expression analysis of transcription factors specific for T<sub>H</sub>1, T<sub>H</sub>2, CD4<sup>+</sup>CD25<sup>+</sup> regulatory, and T<sub>H</sub>17 lymphocytes showed a severe decrease in comparison with healthy individuals' values. These alterations remain stable during the first 48 h after inclusion in the protocol despite early and aggressive resuscitation and antibiotherapy administered in patients. At the time of diagnosis of shock and admission in the intensive care unit, septic patients already present with severe lymphopenia involving every lymphocyte subsets including CD4<sup>+</sup> T-cell subpopulations. No significant variation could be detected within the first 48 h. This should be taken into account in the forthcoming clinical trials testing immunomodulating therapies in septic shock patients.

**KEYWORDS**—Septic shock, lymphocyte, FOXP3, T-bet, GATA-3, ROR $\gamma$

### INTRODUCTION

Despite marked improvement in critical care medicine and almost 20 years of anti-inflammatory clinical trials, septic syndromes (association of a systemic inflammatory reaction to an infection) are the leading cause of death in noncoronary intensive care units (ICUs) and a major health care problem worldwide (1–3). During the last 20 years, septic syndrome mortality has remained constant (from 30% to 50% for septic shock), and incidence has been continuously rising because of the constant aging of the population and the better care of comorbidities (diabetes, cancer, etc.). Severe sepsis, for example, is projected to represent one million cases per year before the year 2020 in the United States alone (1, 4).

Sepsis initiates a complex immunologic response that varies over time, with the concomitant occurrence of both proinflammatory and anti-inflammatory mechanisms alternatively predominating (2, 5). After a short proinflammatory phase, septic patients enter a stage of protracted immunosuppression illustrated by reactivation of dormant viruses (cytomegalovirus or herpes simplex virus) or infections due to germs normally pathogenic solely in immunocompromised host

(2, 5–7). Importantly, the intensity and duration of this phase of immunosuppression seem to be closely correlated with mortality and the development of nosocomial infections (2, 8, 9).

So far, studies have principally investigated dysfunctions of the innate side of the immune response after severe sepsis. However, dramatic alterations of the lymphocytic part of the immune response are also regularly described in patients (2, 5). These dysfunctions have been known for long and are mainly characterized by a decrease in total lymphocyte number (10, 11) and by a state of anergy illustrated both by a decreased proliferation after stimulation *ex vivo* (12) and by decreased response to standard skin tests (10, 13–16). The major importance of this lymphopenia in sepsis pathophysiology is illustrated by animal studies showing that prevention of lymphocyte apoptosis improves the likelihood of survival after sepsis (17–20).

Whereas lymphopenia has been shown to persist during the first week after admission of septic patients to the ICU (21), it is unknown when (i.e., how early after diagnosis of shock/admission in the ICU) these alterations develop, if they vary over time during the so-called golden hours after diagnosis of shock, and if the different lymphocyte subpopulations are equally diminished. This study was therefore designed to perform a systematic monitoring of the different circulating leukocyte subpopulations measured in septic shock patients from the time of diagnosis of shock and every 6 h during the first 48 h.

### PATIENTS AND METHODS

#### Patients

The study group consisted in 21 patients diagnosed with septic shock according to the diagnostic criteria of the American College of Chest Physician/Society

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of Critical Care Medicine (22). To minimize heterogeneity, patients were included only if they present with no comorbidity. Septic shock was defined, according to classic definition, 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 greater than 38°C or less than 36°C, (b) heart rate greater than 90 beats/min, (c) respiratory rate greater than 20 breaths/min, and (d) white blood cell count of more than 12,000/ $\mu$ L or less than 4,000/ $\mu$ L. Severity was assessed by the Simplified Acute Physiology Scale II (23). Mortality was defined as death occurring within 28 days after the onset of shock. Cell phenotyping was performed within 2 h after the onset of vasopressor therapy, arbitrary chosen as the inclusion time, and every 6 h during the following 48 h. This prospective study was reviewed by the institutional ethics committee. Informed consent was obtained for each patient or relatives before the first blood drawing.

### Flow cytometry

Apart from white blood cell and differential leukocyte count (obtained from the routine hematology laboratory), the following lymphocyte subsets were analyzed: total T lymphocytes (CD45<sup>+</sup>CD3<sup>+</sup>), CD4<sup>+</sup> T lymphocytes (CD45<sup>+</sup>CD4<sup>+</sup>CD3<sup>+</sup>), CD8<sup>+</sup> T lymphocytes (CD45<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup>), total B cells (CD45<sup>+</sup>CD19<sup>+</sup>), and natural killer cells (NK cells, CD45<sup>+</sup>CD3<sup>-</sup>CD56<sup>+</sup>). The flow cytometry gating strategy is shown in Figure 1.

Flow cytometric (FC500; Beckman-Coulter, Hialeah, Fla) expression of cell-surface markers was assessed on peripheral whole blood collected on EDTA anticoagulant tubes. Monoclonal antibodies were used according to the manufacturer's recommendations: fluorescein isothiocyanate (FITC)-labeled anti-CD45, PC5-labeled anti-CD3, PE-labeled anti-CD4, ECD-labeled anti-CD8, ECD-labeled anti-CD19, and PE-labeled anti-CD56 (Beckman-Coulter). The red cells were lysed on the automated TQ Prep system (Beckman-Coulter). Absolute count was performed using Flow-Count Fluorospheres according to manufacturer's recommendations (Beckman-Coulter). Results are expressed as numbers of cells per microliter of blood. Age-matched reference values were provided by the routine flow cytometry laboratory of our institution.

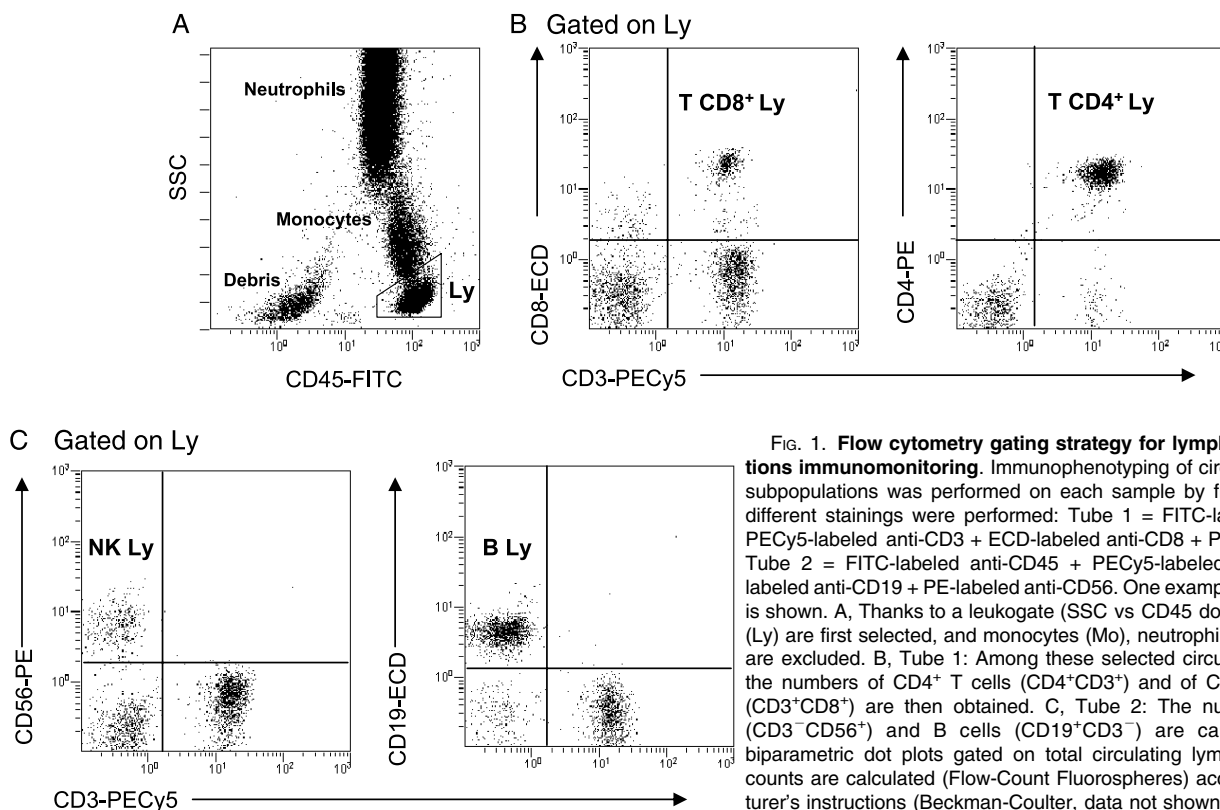
### Quantitative real-time polymerase chain reaction analysis of FOXP3, T-bet, GATA-3, and ROR $\gamma$

Peripheral blood mRNA expression was measured using samples collected directly in PAXGene Blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland). Total RNA was extracted using the PAXGene Blood RNA kit

(PreAnalytix). The residual genomic DNA was digested using the RNase-free DNase set (Qiagen, Germantown, Md). The integrity and quality of the total RNA were assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, Calif) by means of the RNA 6000 Nano assay (Agilent). Total RNAs were reverse transcribed into cDNA using SuperScript real-time polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, Calif) according to manufacturer's instructions. mRNA expression was quantified using quantitative RT-PCR (qRT-PCR) as previously described (24). Briefly, PCR reactions were performed in a LightCycler 2.0 instrument using the Fast-Start DNA Master SYBR Green I RT-PCR kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, Ind). Thermocycling was performed in a final volume of 20  $\mu$ L containing 3 mM MgCl<sub>2</sub> and 0.5  $\mu$ M each of the required primers. Polymerase chain reaction was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of a touch-down PCR protocol (10 s at 95°C, 10-s annealing at 68°C to 58°C, and 16-s extension at 72°C). mRNA expression of the housekeeping gene peptidylprolyl isomerase B (PIIB), encoding for cyclophilin B, and the four transcription factors (FOXP3, T-bet, GATA-3, and ROR $\gamma$ ) were investigated using specific cDNA standards and ready-to-use primer mixes obtained from Search-LC (Heidelberg, Germany). The efficiency of PIIB mRNA levels has been already demonstrated as reference for target mRNA quantification in human peripheral blood (25). Serial dilutions of the cDNA standard were prepared in quadruplicate to generate standard curves. Relative standard curves, describing the PCR efficiency of each target genes and PIIB, were created and used to perform efficiency-corrected quantification with the LightCycler software version 4.05. An internal calibrator (pooled mRNA extracted from 10 healthy volunteers' whole blood sampled using PAXGene tubes) was used to compare each reverse transcription. The results were expressed as normalized ratio of FOXP3, T-bet, GATA-3, or ROR $\gamma$  mRNA/PIIB mRNA and compared with results from a cohort of age-matched healthy volunteers.

### Statistics

Comparisons between groups were made using the nonparametric Mann-Whitney *U* test (analysis of transcription factor mRNA expressions), whereas comparisons versus normal/test values (medians) were performed using the signed rank sum test with *P* < 0.05 considered as statistically significant with correction by the number of tests performed. The nonparametric Wilcoxon paired test was used to assess variations between hour 0 (H0) and hour 48 (H48) for each lymphocyte subpopulation. Finally, variability over time for each marker was evaluated using linear mixed-effects models. In these



**FIG. 1. Flow cytometry gating strategy for lymphocyte subpopulations immunomonitoring.** Immunophenotyping of circulating lymphocyte subpopulations was performed on each sample by flow cytometry. Two different stainings were performed: Tube 1 = FITC-labeled anti-CD45 + PECy5-labeled anti-CD3 + ECD-labeled anti-CD8 + PE-labeled anti-CD4. Tube 2 = FITC-labeled anti-CD45 + PECy5-labeled anti-CD3 + ECD-labeled anti-CD19 + PE-labeled anti-CD56. One example in a septic patient is shown. A, Thanks to a leukogate (SSC vs CD45 dot plot), lymphocytes (Ly) are first selected, and monocytes (Mo), neutrophils (Neu), and debris are excluded. B, Tube 1: Among these selected circulating lymphocytes, the numbers of CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD3<sup>+</sup>) and of CD8<sup>+</sup> T lymphocytes (CD3<sup>+</sup>CD8<sup>+</sup>) are then obtained. C, Tube 2: The numbers of NK cells (CD3<sup>-</sup>CD56<sup>+</sup>) and B cells (CD19<sup>+</sup>CD3<sup>-</sup>) are calculated based on biparametric dot plots gated on total circulating lymphocytes. Absolute counts are calculated (Flow-Count Fluorospheres) according to manufacturer's instructions (Beckman-Coulter, data not shown).

models, the null hypothesis was that, for each marker, the slope calculated over 48 h was equal to zero. The analyses were performed using Sigma Stat software (version 3.5; Systat Software, Chicago, Ill).

## RESULTS

Twenty-one septic shock patients with no comorbidities, as shown by a low Charlson score, were prospectively included in the study. Clinical data are presented in Table 1. Four patients died (28-day mortality: 19%), among whom 2 died during the first 48 h. There was no delay between diagnosis of shock and admission in the ICU (Table 1).

Serial blood samples were obtained from patients at the time of diagnosis of shock (i.e., within 2 h after the beginning of vasoactive treatment) and every 6 h during the first 48 h afterward. Flow cytometry phenotyping of circulating cells was performed, and results were compared with normal, age-matched values from our routine immunology laboratory.

We first observed that septic shock patients present with a marked and significant leukocytosis that was present from the inclusion time and remained constant during the whole monitoring (linear mixed-effects model, Table 2). This increase in white blood cell count was due to an increase in the number of circulating neutrophils, whereas the number of circulating monocytes was overall not modified in patients in comparison with normal values (Table 2). In marked contrast, total lymphocyte absolute number was markedly and significantly diminished from the inclusion time (Table 2) and remained extremely low during the whole monitoring (i.e., during the first 48 h after inclusion, linear mixed-effects model).

Regarding circulating lymphocyte subpopulations, a marked decrease in T, B, and NK cells was observed (Table 2; Fig. 2). This diminution was present from the time of admission in the ICU and stable during the first 48 h (linear mixed-effects model; Table 2; Fig. 2A). No significant variation could be observed between values measured at H0 and H48 (Fig. 2A).

Within T lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> T-cell numbers were equally decreased during the whole monitoring (Table 2; Fig. 2, A and B). No significant variations (neither increase nor decrease, linear mixed-effects model) were observed during the first 48 h (Fig. 2, A and B), although for T cells and CD4<sup>+</sup> T lymphocytes, the nadir was measured at inclusion in the protocol. As an example, box plots as well as individual values for CD4<sup>+</sup> T cells are presented in Figure 2B.

Finally, to evaluate the potential variations of CD4<sup>+</sup> T-cell subpopulations, transcription factors specific for T<sub>H</sub>2 (GATA-3), T<sub>H</sub>1 (T-bet), regulatory T cell (Treg; FOXP3), and newly described T<sub>H</sub>17 (ROR $\gamma$ ) cells were measured by qRT-PCR in patients and compared with a cohort of age-matched healthy individuals (n = 11, 4 women and 7 men; mean age, 51 [SD, 6] years). Not surprisingly, a significantly reduced expression of each transcription factors was observed. This decrease was measured at inclusion and at each time point during the whole monitoring (Fig. 3). Interestingly, when normalized based on the number of circulating CD4<sup>+</sup> T lymphocytes for each patient at each time point, every transcription factors returned within the range of normal values (data not shown).

Of note, significantly lower numbers of circulating neutrophils and CD4<sup>+</sup> T lymphocytes were measured at H0 in

TABLE 1. Demographic, clinical, and biological data for septic shock patients

Parameters	Patients (n = 21), n (%)
Age at admission,* y	58 (58–79)
Sex	
Male	12 (57)
Simplified Acute Physiology Scale II at diagnosis of shock*	45 (34–57)
McCabe score	
0	15 (71)
1	5 (25)
2	1 (5)
Charlson score*	2 (1–3)
Delay shock-ICU,* d	0 (0–1)
Main diagnosis category	
Medical	12 (57)
Surgery	9 (45)
Infection	
Diagnosis	
Only radiologically diagnosed	2 (10)
Only surgically diagnosed	2 (10)
Microbiologically documented	17 (81)
Bacilli gram-negative	13 (62)
Cocci gram-positive	7 (33)
Fungi	2 (10)
Others	0 (0)
Type of infection	
Community acquired	13 (65)
Hospital acquired	7 (33)
ICU acquired	1 (5)
Site of infection	
Pulmonary	4 (20)
Abdominal	10 (48)
Urinary	3 (14)
Catheter	2 (9)
Others	2 (9)
Mortality	
Survivors	17 (81)

For categorical data, results are presented as number of cases and percentages respective to the total patient's population between brackets. For continuous data, results are presented as medians and interquartile ranges (Q1–Q3).

\*Continuous data.

patients with pulmonary infections in comparison with patients with abdominal infections. No significant differences were observed between patients with gram-negative bacilli (n = 9) and gram-positive cocci (n = 3) infections (we did not consider mixed-germ infections), or between survivors (n = 17) and nonsurvivors (n = 4, data not shown).

## DISCUSSION

As a result of their ability to interact with cells of the innate immune system (i.e., macrophages, dendritic cells, neutrophils, NK cells) and with other cells of the adaptive immune

TABLE 2. Circulating white blood cell (WBC) phenotyping in septic shock patients

	H0	H6	H12	H18	H24	H30	H36	H42	H48	Normal values
WBC, × 10 <sup>3</sup>	12 (7–16)	16* (11–20)	15* (9–21)	13* (10–20)	13* (10–17)	13* (10–17)	13* (9–15)	12* (10–16)	13* (8–15)	4–10
Neutrophils, × 10 <sup>3</sup>	10* (5–13)	15* (10–18)	15† (8–19)	11* (9–16)	11† (8–15)	10† (8–15)	11* (8–13)	11† (9–15)	11* (6–13)	1.7–7
Monocytes, × 10 <sup>3</sup>	0.5 (0.4–0.7)	0.8 (0.5–1)	0.7 (0.6–1.1)	0.7 (0.4–0.9)	0.8 (0.6–1)	0.7* (0.5–0.9)	0.7 (0.5–0.9)	0.7* (0.5–0.8)	0.7 (0.5–0.8)	0.1–1
Lymphocytes, × 10 <sup>3</sup>	0.5† (0.3–0.8)	0.6† (0.5–1.1)	0.5† (0.4–0.9)	0.7† (0.5–0.9)	0.7† (0.5–1)	0.7† (0.5–1)	0.6† (0.5–0.9)	0.7† (0.4–1)	0.5† (0.3–0.9)	1.3–3.2
B cells	87* (51–132)	112* (69–201)	104* (79–171)	105* (86–174)	115* (83–211)	111* (83–162)	102* (82–161)	95* (81–182)	86* (69–156)	150–450
NK cells	49† (29–67)	66† (26–91)	51† (25–74)	37† (28–66)	42† (22–64)	47† (21–68)	36† (19–63)	32† (19–48)	29† (13–42)	50–600
T cells	313* (172–640)	419* (293–883)	349† (290–704)	580† (289–700)	585† (381–722)	517† (396–696)	516† (307–666)	524† (289–724)	463† (225–728)	900–1,900
CD4 <sup>+</sup> T	168* (101–376)	219* (182–544)	202† (155–362)	262† (170–457)	326† (222–514)	330† (162–480)	321† (168–428)	353† (144–448)	230† (139–464)	500–1,250
CD8 <sup>+</sup> T	146* (57–268)	176* (87–359)	128† (80–216)	193† (83–323)	209† (111–286)	173† (116–277)	156† (82–274)	162† (81–262)	149† (82–303)	350–900

Blood samples were obtained from 21 septic shock patients within 2 h after the beginning of vasopressive treatment and every 6 h during 48 h afterward. The following leukocyte subsets were analyzed: total WBC count, number of neutrophils, monocytes, lymphocytes, T lymphocytes (T cells) CD4<sup>+</sup> T lymphocytes (CD4<sup>+</sup> T), CD8<sup>+</sup> T lymphocytes (CD8<sup>+</sup> T), total B cells (B cells), and NK cells. Results are expressed as number of cells/μL [median (95% confidence intervals for the median)]. Normal values from our laboratory for age-matched individuals are shown. Comparisons versus normal/test values (medians) were performed using the signed rank sum test with correction for the number of tests performed. \*P < 0.0056, †P < 0.0001.

system, lymphocytes play a central role in the anti-infectious immune response not only as effectors but also as regulators of this response. This has been illustrated by the observation of a dysregulated, proinflammatory immune response after polymicrobial septic challenge in mice lacking T and B cells (19, 26).

Alterations of the lymphocytic response after septic shock have been known for long. They comprise (a) phenotypic alterations including an increase in inhibitory coreceptor expression associated with a decreased expression of activating coreceptors and CD3, (b) functional alterations mainly characterized by a decreased *ex vivo* lymphocyte proliferation associated with decreased T<sub>H</sub>1 and increased T<sub>H</sub>2 immune responses, (c) induction of regulatory T-cell populations, and (d) an increased apoptosis leading to a marked lymphopenia (2, 5).

This marked decrease in circulating lymphocyte number has been regularly reported in patients after sepsis and severe trauma (12, 25, 27–31). Most importantly, the extent of this lymphopenia has been associated with the development of nosocomial infections in ICU patients. For example, Hotchkiss et al. (28) observed a marked lymphoid depletion in adults dying of sepsis. These patients had reduced CD4<sup>+</sup> T- and B-cell numbers and the degree of reduction in cell numbers related

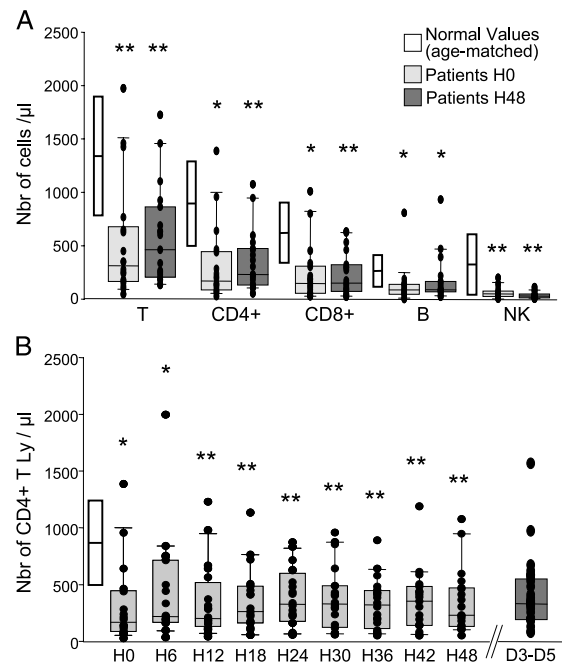
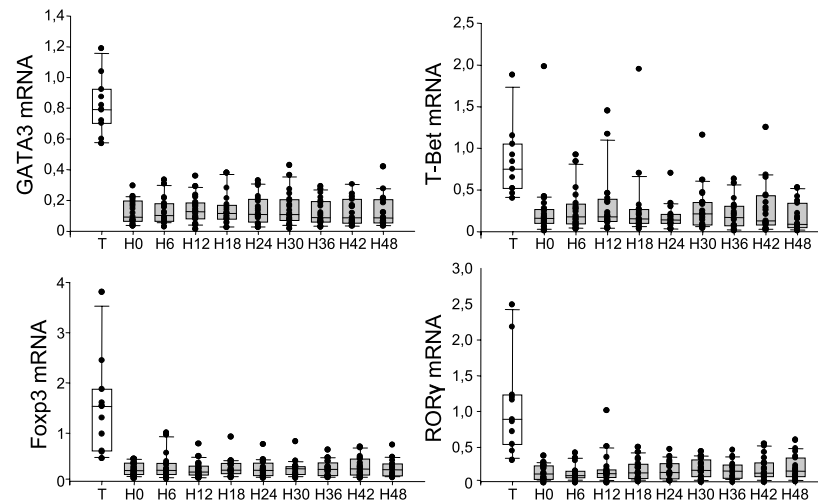


FIG. 2. Septic shock patients present with severe lymphopenia from the time of admission and during the first 48 h. Blood samples were obtained from 21 septic shock patients within 2 h after the beginning of vasopressive treatment and every 6 h during 48 h afterward. The following lymphocyte subsets were analyzed by flow cytometry: total T lymphocytes (CD45<sup>+</sup>CD3<sup>+</sup>), CD4<sup>+</sup> T lymphocytes (CD45<sup>+</sup>CD4<sup>+</sup>CD3<sup>+</sup>), CD8<sup>+</sup> T lymphocytes (CD45<sup>+</sup>CD8<sup>+</sup>CD3<sup>+</sup>), total B cells (CD45<sup>+</sup>CD19<sup>+</sup>), and NK cells (CD45<sup>+</sup>CD3<sup>-</sup>CD56<sup>+</sup>). A, Box plots as well as individual values are presented for measurements at H0 (light gray boxes) and H48 (dark gray boxes) for each lymphocyte subpopulations. Box plots (median ± 2 SDs, open boxes) for age-matched normal values from our laboratory are shown for each lymphocyte subpopulations. B, Total phenotyping for circulating CD4<sup>+</sup> T lymphocytes is presented (light gray boxes) in comparison with normal values from our laboratory. In addition, values for circulating CD4<sup>+</sup> T cells previously published by our group (34) at days 3 to 5 in a similar cohort of septic shock patients are shown (dark gray box). Comparisons versus normal/test values (medians) were performed using the signed rank sum test with correction for the number of tests performed. \*P < 0.0056, \*\*P < 0.0001 versus normal values.



**Fig. 3. Gene expressions for CD4<sup>+</sup> T-cell-specific transcription factors are dramatically decreased after septic shock.** Blood samples were obtained from 21 septic shock patients within 2 h after the beginning of vasopressive treatment and every 6 h during 48 h afterwards. mRNA expressions for transcription factors specific for T<sub>H</sub>2 (GATA-3), T<sub>H</sub>1 (T-bet), CD4<sup>+</sup>CD25<sup>+</sup> Treg cells (FOXP3), and T<sub>H</sub>17 (ROR $\gamma$ ) cells were measured by qRT-PCR. As a control group, gene expressions were measured in a cohort of healthy individuals ( $n = 11$ , 4 women and 7 men; mean age, 51 [SD, 6] years). Results for each transcription factor measured at each time point in septic shock patients (gray boxes) and healthy individuals (open boxes) are presented as box plots and individual values. Results are expressed as normalized ratio of FOXP3, T-bet, GATA-3, or ROR $\gamma$  mRNA versus PPIB mRNA. The decrease was statistically significant ( $P < 0.0056$ ) at all time points for every transcription factor. Comparisons between groups were made using the nonparametric Mann Whitney  $U$  test with correction for the number of tests performed.

to the duration of sepsis before death. Similarly, Felmet et al. (29) observed that an absolute lymphocyte count of less than 1,000 cell/ $\mu$ L not only identified critically ill children at high risk for nosocomial infection, but also that an absolute lymphocyte count of less than 1,000/ $\mu$ L persisting for 7 days identified those at risk for death from uneradicated nosocomial infection.

However, in the literature, circulating lymphocyte numbers have been mainly monitored in patients starting from day 1 after the onset of shock and usually only once within the first days. What happens before day 1 (i.e., between the time of diagnosis of shock and the end of the first day after shock) is unknown. Based on the biphasic model usually described for the immune response after severe sepsis (2, 5, 19) and on the fact that the adaptive/lymphocytic immune response is thought to be involved in a second time/late on after an infection, one may postulate that lymphocyte number may be within the normal range at diagnosis of shock but decreases later on. In this context, this study is the first to perform a systematic monitoring of every circulating leukocyte subset in septic shock patients, starting from the time of admission in the ICU and every 6 h during the first 48 h. This systematic monitoring has allowed us to obtain a precise view of the development of these alterations and of their variability over time during the first 48 h.

Importantly, we observed that leukocyte alterations described in the literature after septic shock are already present at the time of inclusion in the study: leukocytosis associated with marked neutrophilia and profound lymphopenia. Monocyte number, in contrary, was not modified, although it is well known that many of their functions are altered (2, 5). Among lymphocytes, the numbers of every subpopulation were decreased as well. Interestingly, when measuring CD4<sup>+</sup> T-cell-specific transcription factors (T-bet for T<sub>H</sub>1, GATA-3 for T<sub>H</sub>2, FOXP3 for Treg, and ROR $\gamma$  for T<sub>H</sub>17), they all appeared equally diminished. Because qRT-PCR was performed on mRNA

extracted from whole blood, these decreased expressions could be related with the marked diminution in CD4<sup>+</sup> T-cell number. Despite the apparent normalization of these mRNA levels when corrected by CD4<sup>+</sup> T-cell absolute count, we cannot definitively rule out a specific downregulation of one of these transcription factors in some CD4<sup>+</sup> cell subsets. This thus deserves to be confirmed in isolated cells.

These results are in line with previous studies by our group measuring either T-bet and GATA-3 or FOXP3 mRNA at days 1 and 2 after the onset of shock (25, 32). Regarding ROR $\gamma$ , very few studies have been published so far investigating the role of T<sub>H</sub>17 cells in sepsis pathophysiology. In a murine model of sepsis, Flierl et al. (33) observed that IL-17a circulating levels were increased after sepsis and that specific blockade of this cytokine was associated with decreased mortality. However, the present results tend to show that ROR $\gamma$  behaves after septic shock as other T-cell-specific transcription factors.

The second important point of this study is that these alterations remain stable during the first 48 h after diagnosis of shock despite early and aggressive resuscitation therapy and antibiotherapy administered in patients. When put into perspective with our previously published data, it seems that they persist even longer, as illustrated in Figure 2B. Indeed, values for total T cells and for CD4<sup>+</sup> T cells measured at H48 in this study are very similar to values measured at days 3 to 5 in previous studies by our group (25, 30, 34). This suggests that early and aggressive therapy in patients has no effect on lymphopenia and that it may be interesting to install a more specific treatment to restore normal lymphocyte numbers because lymphopenia has been shown to be related with increased mortality after sepsis.

Overall, our data suggest that the causes of this prolonged lymphopenia and lymphoid depletion, likely multifactorial, most probably occur before admission of patients to the ICU. This highlights the major difference between murine models

of sepsis, in which this onset of shock can be precisely timed, and clinical situations, in which when patients are admitted to the ICU several hours may have elapsed after the onset of shock. This is most likely the major limitation of our study: although patients were included immediately after the diagnosis of shock (illustrated by the requirement for catecholamines), the “real” start of the infection could never be determined precisely. It is probable that the infectious process has started well before and most likely is culminating with the septic shock clinical picture. Another limitation would be that our limited cohort has a low mortality probably in relation with the absence of comorbidities and also because of a very early management of septic shock in our ICUs. With that said, despite this putative lower severity, lymphopenia measured in these patients is still extremely marked.

Finally, our data, although apparently incremental to the already described lymphopenia after septic shock, show for the first time that these major lymphocytic alterations described are present at the time of diagnosis, remain stable during the first 48 h afterward, and concern every lymphocyte subset. We thus believe this study provides several important and complementary information to previous literature in the field and increases our understanding of the lymphocytic alterations of the immune response occurring in septic shock patients. The sepsis-induced loss of lymphocytes may be particularly important because of the extensive and very early depletion of critical immune effector cells. This should be taken into account in the forthcoming clinical trials testing immunomodulating therapies in septic shock patients.

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