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Decreased T-Cell Repertoire Diversity in Sepsis: A Preliminary Study*

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Objective: Septic syndromes are the leading causes of mortality in intensive care units. In patients, the occurrence of sepsis-induced immune suppression is associated with delayed mortality, although the exact role of lymphocyte dysfunctions is not well established. The objective of this study was to investigate T-cell receptor diversity, an important feature of T-cell response, in patients with septic shock.

Design: Preliminary prospective observational study.

Setting: Adult intensive care units in a university hospital.

Subjects: Patients with septic shock ($n = 41$) sampled twice after the onset of shock (early after inclusion [day 1] and at the end of the first week [day 7]).

Measurements and Main Results: Using a novel molecular biology technique, the combinatorial diversity of human T-cell receptor β -chain (*TRB* locus) was measured in peripheral blood. Patients with septic shock presented with a marked decreased T-cell receptor diversity after the onset of shock in comparison with nor-

mal values. Importantly, in paired samples, a very steep recovery slope of T-cell receptor diversity, never described in other clinical situations, was observed between day 1 and day 7 ($p < 0.0001$, Wilcoxon's paired test). Decreased T-cell receptor diversity was associated with mortality (log-rank test, $p = 0.0058$; hazard ratio = 4.48; 95% confidence interval 1.96–53.32), and the development of nosocomial infections ($p < 0.05$, Mann-Whitney *U* test).

Conclusion: Our results show for the first time that septic patients present with a marked decreased T-cell receptor diversity that returned rapidly toward normal values over time. This opens novel cognitive research perspectives that deserve to be investigated in experimental models of sepsis. After confirmation in larger cohorts of these preliminary results, T-cell receptor diversity measurements may become a crucial tool to monitor immune functions in ICU patients. (*Crit Care Med* 2013; 41:111–119)

Key Words: divpenia; lymphocyte; septic shock; T-cell receptor diversity

*See also p. 348.

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Severe sepsis and septic shock are common indications for admission to ICUs and affect millions of individuals around the world each year (1). They constitute the leading cause of morbidity and mortality for critically ill patients worldwide, although an enormous effort has been expended during the last 20 yrs to find new therapies that decrease the mortality resulting from this syndrome (1, 2).

It is generally accepted that septic patients develop immune alterations affecting both innate and adaptive immunity (3). So far, most work has been devoted to the study of the innate part of these sepsis-induced immune dysfunctions. Importantly, it has been shown that the intensity and duration of these alterations are associated with increased mortality and susceptibility to secondary/nosocomial infections (3). In contrast, far less work has been dedicated to the study of lymphocyte alterations, although a link between a reduced delayed-type hypersensitivity response (predominantly mediated by T-cells and a hallmark of immune suppression) in ICU patients and increased risk of nosocomial infections and death has been described >30 yrs ago (4).

In this study, we investigated a novel aspect of sepsis-induced lymphocyte alterations. Using a novel molecular biology tech-

nique covering the whole T-cell receptor (TCR) repertoire (*TRB* locus), we studied in patients with septic shock the combinatorial diversity of the TCR β -chain directly at the genomic level (5–7). Considering the importance of having a large and diverse TCR repertoire for the quality of the immune response to infection, we reasoned that if TCR diversity was decreased after septic shock, this might participate in increased mortality and susceptibility to secondary nosocomial infections in patients.

PATIENTS AND METHODS

Patients

The study group consisted of 41 consecutive patients with septic shock, according to the diagnostic criteria of the American College of Chest Physician/Society of Critical Care Medicine (8). The exclusion criteria were patients <18 yrs of age and subjects with aplasia or immunosuppressive disease (e.g., human immunodeficiency virus). Septic shock was defined by an identifiable site of infection, persisting hypotension despite fluid resuscitation requiring vasopressor therapy, and evidence of a systemic inflammatory response manifested by at least two of the following criteria: 1) temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$; 2) heart rate >90 beats/min; 3) respiratory rate >20 breaths/min; and 4) white blood cell count $>12,000/\text{mm}^3$ or $<4000/\text{mm}^3$. Severity was assessed by the Simplified Acute Physiologic Score II calculated at inclusion in the protocol (9). The development of organ dysfunction was assessed by the Sequential Organ Failure Assessment score (SOFA; range, 0–24), measured after 24 hrs of ICU stay (10). Mortality was defined as death occurring within 28 days after the onset of shock. The onset of septic shock was defined as the beginning of vasopressor therapy. None of these patients was infected by superantigen-producing bacteria. Secondary ICU-acquired infections were defined according to European definitions of the European Centre for Disease Prevention and Control (11).

Biological analyses were performed on residual blood after completing routine follow-up performed in the ICU. EDTA-anticoagulated blood was collected from patients at two time points: days 1–3 (D1) and days 5–8 (D7) after diagnosis of septic shock. Because the laboratory did not operate on Saturdays and Sundays, patients were included on weekdays during working hours. In addition to patients who died or left the ICU before the second sampling time, this accounted for the majority of missing samples.

This work belongs to a global study on ICU-induced immune dysfunctions. It has been approved by our Institutional Review Board for ethics (Comité de Protection des Personnes) and registered at French Ministry of Research and Enseignement (#DC-2008–509). It is also recorded at the Commission Nationale de l'Informatique et des Libertés.

Normal values for TCR β -chain combinatorial diversity were defined based on a cohort of 27 healthy volunteers extracted from a larger study (SuSa Study, AFSSAPS clinical trial number 2010-A00428-31, ImmunID; age range, 39–70

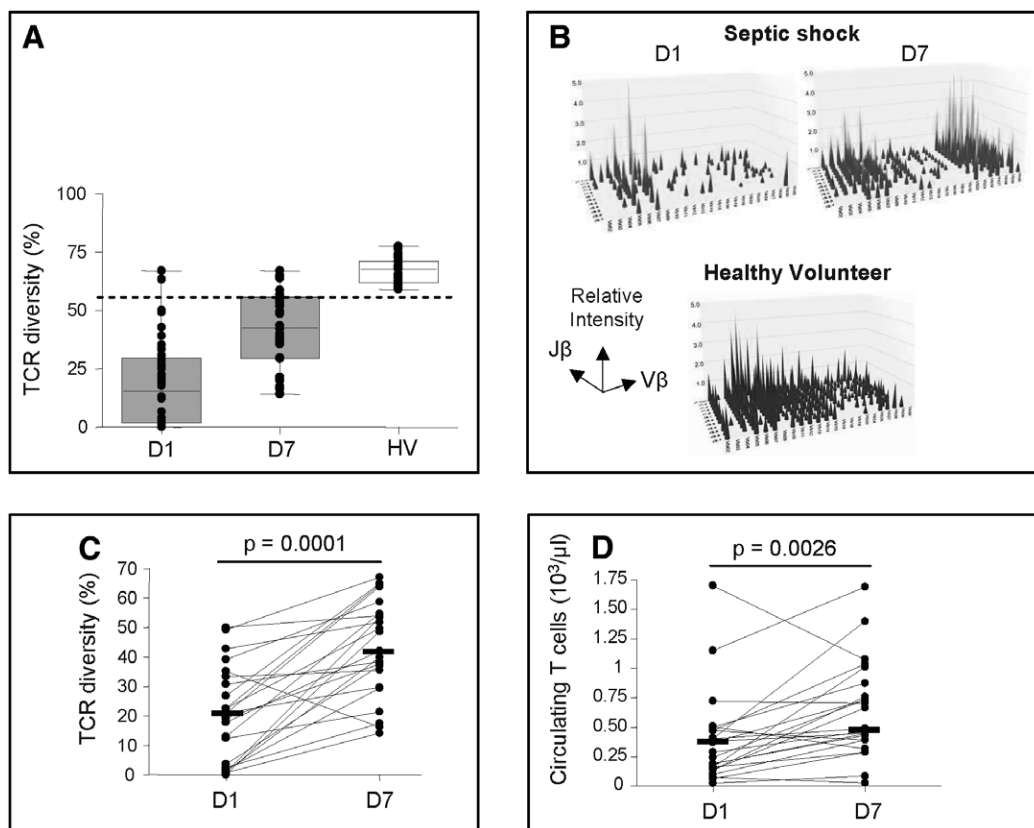


Figure 1. Reduced T-cell receptor (TCR) diversity in patients with septic shock. **A**, T-cell receptor β -chain diversity (TCR diversity) was measured by molecular biology in 41 patients with septic shock between day 1 and day 3 (D1, $n = 38$) and day 5 and day 8 (D7, $n = 26$) after the onset of shock (gray boxes) and in 27 healthy volunteers (HV, open box). Results are presented as box plots and individual values for the percentage of TCR diversity. Dotted line represents inferior limit of normal TCR diversity value (56%) defined based on values measured in HV. **B**, Representative examples of TCR diversity measurements are shown for one septic patient at D1 and D7 and one HV. x-axis represents 23 T-cell receptor β V (TRBV) genes ($V\beta$); y-axis represents 12 T-cell receptor β J (TRBJ) genes ($J\beta$); and z-axis represents the relative intensity of each $V\beta$ – $J\beta$ combinatorial TCR chain amplified. **C**, TCR diversity was monitored in 23 septic patients with paired samples at D1 and D7. Individual values and medians are shown. **D**, The number of circulating T-cells (10^3 cells/ μL) in 23 septic patients with paired samples at D1 and D7 are shown. Individual values and medians are presented. Nonparametric Wilcoxon's paired test was used to compare overtime evolution of TCR diversity and number of T-cells in patients.

ys; 11 females and 16 males). Normal median TCR diversity was 68% (95% confidence interval [CI] 65–69). For the purpose of this preliminary study, we defined 56% (mean – 2 SD) as the lower limit of normal TCR diversity (Fig. 1A).

Human ImmunTraCkeR β Test/Combinatorial Diversity of TCR β -Chain

Human T-lymphocyte repertoire diversity was measured using Human ImmunTraCkeR β test (ImmunID Technologies, Grenoble, France), a novel molecular biology technique on genomic DNA. Genomic DNA was extracted using standard techniques from total peripheral mononuclear blood cells of patients and control subjects after informed consent. Multi-N-plex polymerase chain reaction (PCR) was performed using an up stream primer specific of all functional members of a given T-cell receptor β V (TRBV) family and a downstream primer specific of a given T-cell receptor β J (TRBJ) segment. This assay allows for the simultaneous detection of several V–J rearrangements in the same reaction. Using this technique, it is possible to detect 276 different TRBV–TRBJ rearrangements covering 100% of the possible combinatorial rearrangements. All V–J1, J2, J3, J4, and Jn products were separated as a function of their size with a maximum amplicon size of ~5 kb. PCR signals were detected and analyzed using the Constel'ID software developed by ImmunID Technologies. PCR products were generated using iProof GC rich Master Mix (Bio-Rad, Hercules, CA). The cycling conditions were as follows: Touchdown PCR cycle protocol was performed beginning with 98°C for 3 mins, 98°C for 20 secs, 72°C for 20 secs, and 72°C for 3 mins 30 secs; the annealing temperature was reduced by 1° for every cycle until it reached 68°C, which was then repeated 23 times. Finally, one cycle of 10 mins at 72°C was performed. To perform semi-quantitative analysis, PCR reactions were stopped at the exponential step of the PCR. To normalize DNA quantity in each reaction, the actin gene was amplified in the same PCR run. DNA isolated from kidney and/or human embryonic kidney cell lines, in which no rearrangement takes place, was used as a negative PCR control. PCR products were separated on a 0.8% agarose gel, directly stained with SYBR Green I, and quantified using a charge-coupled device camera equipped with BIO-1D (Vilbert Lourmart, France). Constel'ID software was used for further analytical studies including the generation of three-dimensional repertoire illustration. Results are expressed as percentages of detected rearrangements among the total 276 possible combinatorial rearrangements. A decreased percentage (i.e., a decreased TCR diversity) defines a state of divpenia.

Flow Cytometry

The following lymphocyte subsets were analyzed: total T lymphocytes (CD45⁺CD3⁺), CD4⁺ T lymphocytes (CD45⁺CD4⁺CD3⁺), CD8⁺ T lymphocytes (CD45⁺CD8⁺CD3⁺), total B-cells (CD45⁺CD19⁺), natural killer cells (CD45⁺CD3⁺CD56⁺), and regulatory T-cells (CD4⁺CD25^{high}CD127^{low}), as previously described (12). Human leukocyte antigen-DR (HLA-DR) expression was monitored on circulating monocytes (mHLA-DR), as previously described (13). Results are expressed as

number of cells per microliter of blood for lymphocyte subsets, as percentages of regulatory T-cells among total CD4⁺ lymphocyte population, and as percentages of positive cells among total monocyte subset for mHLA-DR measurements. Age-matched reference values were provided by the routine flow cytometry laboratory of our institution.

Statistics

Results are presented as box plots with individual values. Lower limit for the reference values is shown as a dot line.

TABLE 1. Demographic, Clinical, and Immunological Data for Patients With Septic Shock

| Clinical Data | |
|--|------------|
| Age at admission (yrs) ^a | 71 [57–77] |
| Male gender | 29 (71) |
| Simplified Acute Physiologic Score II at diagnosis of shock ^a | 57 [44–67] |
| Sequential Organ Failure Assessment score ^a | 9.5 [8–12] |
| Main diagnosis category | |
| Medical | 17 (41) |
| Surgery | 24 (58) |
| Comorbidities | |
| 0 | 21 (51) |
| ≥1 | 20 (49) |
| Infection | |
| Bacilli Gram-negative | 14 (34) |
| Cocci Gram-positive | 10 (24) |
| Fungi | 3 (7) |
| Others | 10 (24) |
| Type of infection | |
| Community-acquired | 22 (54) |
| Hospital-acquired | 15 (37) |
| ICU-acquired | 4 (10) |
| Site of infection | |
| Pulmonary | 12 (29) |
| Abdominal | 13 (32) |
| Other | 16 (39) |
| Mortality | |
| Nonsurvivors | 16 (39) |
| Nosocomial infections | |
| Occurrence | 4 (10) |

(Continued)

TABLE 1 (Continued).

| Immunological Data | |
|--|---------------------------------|
| Total lymphocytes ^a | |
| D1 | 0.6 × 10 ³ [0.2–0.9] |
| D7 | 0.8 × 10 ³ [0.6–1.3] |
| B lymphocytes ^a | |
| D1 | 80 [40–200] |
| D7 | 110 [70–260] |
| Natural killer cells ^a | |
| D1 | 60 [30–100] |
| D7 | 80 [50–130] |
| CD3 ⁺ T lymphocytes ^a | |
| D1 | 400 [100–500] |
| D7 | 500 [300–900] |
| CD3 ⁺ CD4 ⁺ T lymphocytes ^a | |
| D1 | 200 [100–400] |
| D7 | 400 [200–600] |
| CD3 ⁺ CD8 ⁺ T lymphocytes ^a | |
| D1 | 70 [40–200] |
| D7 | 160 [70–260] |
| Regulatory T-cells (%) ^a | |
| D1 | 8.5 [6–12.5] |
| D7 | 11 [8–13] |
| mHLA-DR (%) ^a | |
| D1 | 31 [18–53] |
| D7 | 42 [26–60] |

D = day.

^aContinuous data as well as biological parameters are presented as medians and interquartile ranges [Q1–Q3]. Simplified Acute Physiologic Score II calculated at inclusion in the protocol; Sequential Organ Failure Assessment score measured after 24 hrs of intensive care unit stay. Blood samples were obtained from 41 patients with septic shock at two time points after the onset of shock. Patients were sampled within the first 3 days (D1) and between day 5 and day 8 (D7) after the onset of shock. For clinical parameters, categorical data are presented as number of cases and percentages respective to the total patient's population, within brackets.

The following leukocyte subsets were analyzed: total lymphocyte number, CD3⁺ T lymphocytes, CD3⁺CD4⁺ T lymphocytes, CD3⁺CD8⁺ T lymphocytes, B lymphocytes, and natural killer cells, CD4⁺CD25^{high}CD127^{low} regulatory T-cells (Treg). Results are expressed as number of cells/μL and as percentages of Treg among total CD4⁺ lymphocyte population. Expression of human leukocyte antigen-DR (HLA-DR) was monitored on circulating monocytes (mHLA-DR). Results are presented as percentages of monocytes expressing HLA-DR. Normal values from our laboratory for age-matched individuals are 1.3 × 10³–3.2 × 10³ total lymphocytes/μL, 150–400 B-cells/μL, 50–600 natural killer cells/μL, 900–1900 T-cells/μL, 500–1250 CD4⁺ T-cells/μL, 350–900 CD8⁺ T-cells/μL, 5%–7% Treg, and >90% mHLA-DR.

Comparisons between groups were made using the nonparametric Mann-Whitney *U* test (survivors vs. nonsurvivors). The nonparametric Wilcoxon's paired test was used to assess variations between time points in septic patients. Spearman's correlation test was used to assess correlations between TCR diversity values and clinical and biological variables. Fisher's exact test was used to compare proportions. A receiver operat-

ing characteristic curve was performed to determine the cutoff values for TCR diversity and number of T lymphocytes with regard to prediction of mortality. The best cutoff value was selected based on likelihood ratio and Youden's index. Using these thresholds, Kaplan-Meier survival curves were obtained, and differences in survival between groups were evaluated using log-rank test. Univariate analysis through the Cox model was used for the estimation of the hazard ratio and 95% CI. Statistical analyses were performed using Prism software (version 4.03; GraphPad Software, La Jolla, CA). A *p* value < 0.05 was considered statistically significant.

RESULTS

Cohort Description

Forty-one patients with septic shock were included. Patients were sampled twice after the onset of shock: first between day 1 and day 3 (D1, *n* = 38 samples) and then between day 5 and day 8 (D7, *n* = 26 samples). Twenty-three patients were sampled at the two time points.

Clinical and immunological data are presented in **Table 1**. Twenty-eight-day mortality was 39% (*n* = 16 nonsurvivors), and four patients developed a nosocomial infection (10%).

As shown in Table 1, patients presented with usual immune alterations as described in the course of septic shock: a major lymphopenia associated with an increased percentage of circulating regulatory T-cells as well as a decreased percentage of monocytes expressing HLA-DR (12–14).

TCR Diversity Is Decreased in Septic Patients

TCR β-chain combinatorial diversity (TCR diversity) was measured on peripheral blood mononuclear cells in patients using a novel molecular biology technique. According to this indicator, the patients can or cannot be qualified as divpenic (low TCR diversity).

As shown in Figure 1A, TCR diversity was markedly decreased in most septic patients (95% values of individual TCR diversity below control values) at D1 and remained low at D7 in the majority of the cohort (77% below control values). Indeed, in patients, median TCR diversity was 15% at D1 (95% CI, 12–25) and 43% at D7 (95% CI, 36–49). Therefore, most septic patients in this cohort were divpenic. Representative examples of TCR diversity data for one septic patient at D1 and D7 and for one healthy volunteer are given in Figure 1B.

Interestingly, in the subgroup of 23 patients who were sampled twice, there was a significant increase in TCR diversity over time (*p* < 0.0001, Wilcoxon's paired test) (Fig. 1C). In fact, TCR diversity decreased in only one patient between D1 and D7 and the patient died 9 days after the onset of shock (of note, the number of circulating T-cells increased in this patient between D1 and D7). Meanwhile, in these patients, the number of circulating T-cells increased as well between D1 and D7 (Fig. 1D; *p* = 0.0026; Wilcoxon's paired test). However, in contrast to TCR diversity, this increase was not systematic in every septic patient.

Correlations Between TCR Diversity and Clinical and Immunological Parameters

No significant correlations were found between TCR diversity and SOFA score or Simplified Acute Physiologic Score II either at D1 or at D7 (Spearman's correlation test). No significant differences were observed between patients with or without comorbidities (the Mann-Whitney *U* test, data not shown).

Interestingly, a significant correlation was observed between TCR diversity and percentage of HLA-DR expressing monocytes when measured at D1 ($r = 0.3388$, $p = 0.0066$, Spearman's correlation test). However, no significant correlations were found between TCR diversity and percentage of regulatory T-cells either at D1 or at D7.

A correlation was found between TCR diversity and the number of circulating T-cells at D1 ($r = 0.5095$, $p = 0.0011$, Spearman's correlation test), but this correlation was lost at D7 showing that TCR diversity evolves independently of T-cell count after septic shock. No correlation was observed in healthy volunteers.

Low TCR Diversity May Possess a Predictive Value on Mortality

Sixteen patients died within 28 days after the onset of shock in this cohort. Five patients died before D8 and 11 patients died after D10. Mean time of death was 13 days postshock.

No significant differences were observed in TCR diversity between survivors and nonsurvivors either at D1 or at D7. The percentage of monocytes expressing HLA-DR was significantly lower in nonsurvivors than in survivors at D7 but not at D1 ($p = 0.0109$ and 0.2442 , respectively, Mann-Whitney *U* test).

In both groups and in patients who were sampled twice, TCR diversity increased significantly between the two time points ($p = 0.0002$ in survivors, $p = 0.0195$ in nonsurvivors, Wilcoxon's paired test). Importantly, although most patients in the survivors' group (91%) and every patient in the nonsurvivors' group (100%) had a decreased TCR diversity at D1, only 67% of survivors remained with a decreased TCR diversity, whereas most of the nonsurvivors still presented with a decreased value at D7 (91%; **Table 2**).

Subsequently, the best thresholds for prediction of mortality were calculated for TCR diversity at D1, then at D7 using receiver operating characteristic curve analyses. At D1, the area under the curve was close to 0.5, illustrating the absence of predictive value for this parameter at this time point. However, at D7, the area under the curve was 0.7 (95% CI 0.5–0.9). The best cutoff value based on Youden's index and likelihood ratio was found to be 33% (Youden's index = 0.32; likelihood ratio = 3.4). Using this value, the Kaplan-Meier survival curves were obtained. Importantly, based on this threshold, a significantly different survival was observed between patients with or without low TCR diversity (**Fig. 2A**; log-rank test, $p = 0.0058$; hazard ratio = 4.48; 95% CI 1.96–53.32).

Of note, similar analyses were performed to determine the prognostic value for the number of circulating T-cells. The best cutoff value at D7 was found to be 730 cells/ μ L. However, based on this threshold, no significantly different survival was observed between the groups (data not shown). This illustrates that divpenia by itself possesses predictive value on mortality independently of the number of circulating lymphocytes.

TCR Diversity May Present With Predictive Value on Nosocomial Infections

Only four patients developed a nosocomial episode in our cohort. Every nosocomial infection was diagnosed later than 14 days after the onset of shock. Average diagnosis time was 16 days after shock. Interestingly, and despite the less number of patients in our cohort, patients who will develop a nosocomial episode present with significantly reduced TCR diversity at D1 in comparison with the patients who remain free of secondary infection (**Fig. 2B**). Furthermore, at D7, every patient who developed a nosocomial episode remained with a reduced diversity, whereas only 73% of the patients free of any nosocomial infection present with a low TCR diversity (**Table 2**).

Of note, neither at D1 nor at D7 did we observe any difference regarding the number of circulating T-cells between patients who will or will not develop a nosocomial episode (data not shown).

TABLE 2. T-Cell Receptor Diversity in Patients With Septic Shock

| | Survivors | | Nonsurvivors | | Noninfected | | Infected | |
|---|-----------|----|--------------|----|-------------|----|----------|-----|
| | D1 | D7 | D1 | D7 | D1 | D7 | D1 | D7 |
| Number of samples | 23 | 15 | 15 | 11 | 34 | 22 | 4 | 4 |
| Median | 13 | 49 | 18 | 40 | 18 | 41 | 1 | 49 |
| Quartile 1 | 2 | 37 | 2 | 20 | 2 | 29 | 1 | 31 |
| Quartile 3 | 29 | 64 | 34 | 52 | 32 | 58 | 12 | 51 |
| Divpenia (proportion <56%) ^a | 91 | 67 | 100 | 91 | 94 | 73 | 100 | 100 |

D = day.

^aLower limit of normal T-cell receptor diversity based on the evaluation of 27 healthy volunteers. T-cell receptor diversity (%) was evaluated on peripheral blood mononuclear cells extracted from 41 patients with septic shock at day (D) 1 (between D1 and D3 after the onset of shock, $n = 38$ samples) and D7 (between D5 and D8 after the onset of shock, $n = 26$ samples).

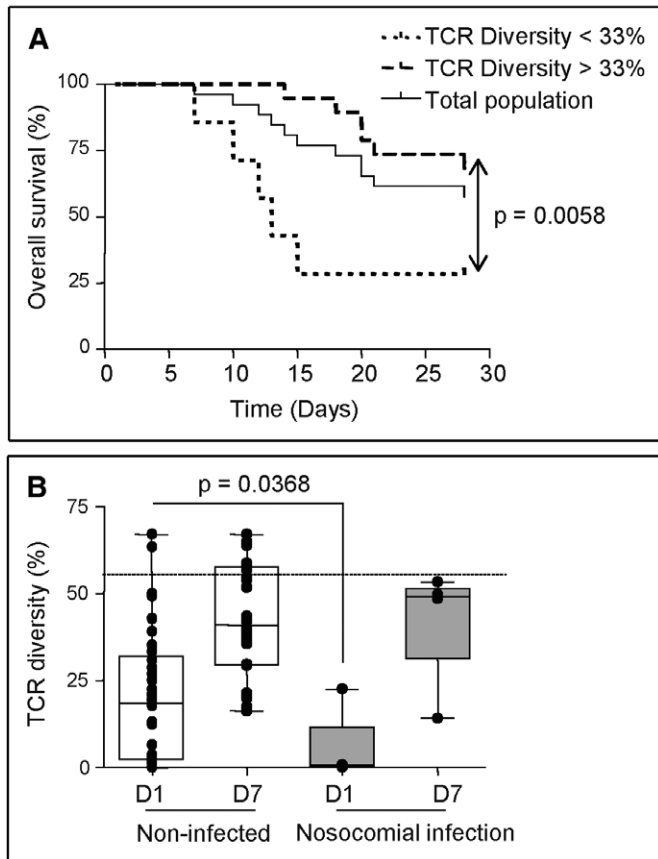


Figure 2. Reduced T-cell receptor (TCR) diversity in patients with septic shock is associated with increased risk of death and nosocomial infection. **A**, T-cell receptor β -chain diversity (TCR diversity) was measured by molecular biology in 41 patients with septic shock between day 1 and day 3 (D1, $n = 38$) and day 5 and day 8 (D7, $n = 26$) after the onset of shock. The Kaplan-Meier survival curves were obtained after patients with septic shock were stratified based on TCR diversity value at 33% at D7 determined by receiver operating characteristic curve analysis. Log-rank test was used to analyze the difference in 28-day survival between patient groups. **B**, TCR diversity was monitored in 41 septic patients sampled at D1 and/or D7. Results are presented as box plots and individual values for patients who remained free of any nosocomial episode (*open boxes*, $n = 34$ at D1, $n = 22$ at D7) and patients who developed a nosocomial infection (*gray boxes*, $n = 4$ at D1 and at D7). The nonparametric Mann-Whitney U test was used to compare TCR diversity between groups of patients.

Predictive Value of Numeration Diversity Lymphocyte Scoring

Finally, to test whether the combination of TCR diversity and number of circulating T-cells was more informative than each parameter considered alone, patients were stratified based on these parameters at D1 and at D7 using thresholds previously calculated through receiver operating characteristic curve analyses (TCR diversity < 33%, number of circulating T-cells < 730 cells/ μ L). Based on these two parameters, patients sampled at D1 were classified into four groups (**Fig. 3A**; $n = 38$). The numeration diversity lymphocyte (NDL)1 group (presenting with low TCR diversity and low number of T-cells, $n = 26$) was compared with NDL2 (higher TCR diversity and low number of T-cells, $n = 5$), NDL3 (low TCR diversity and higher number of T-cells,

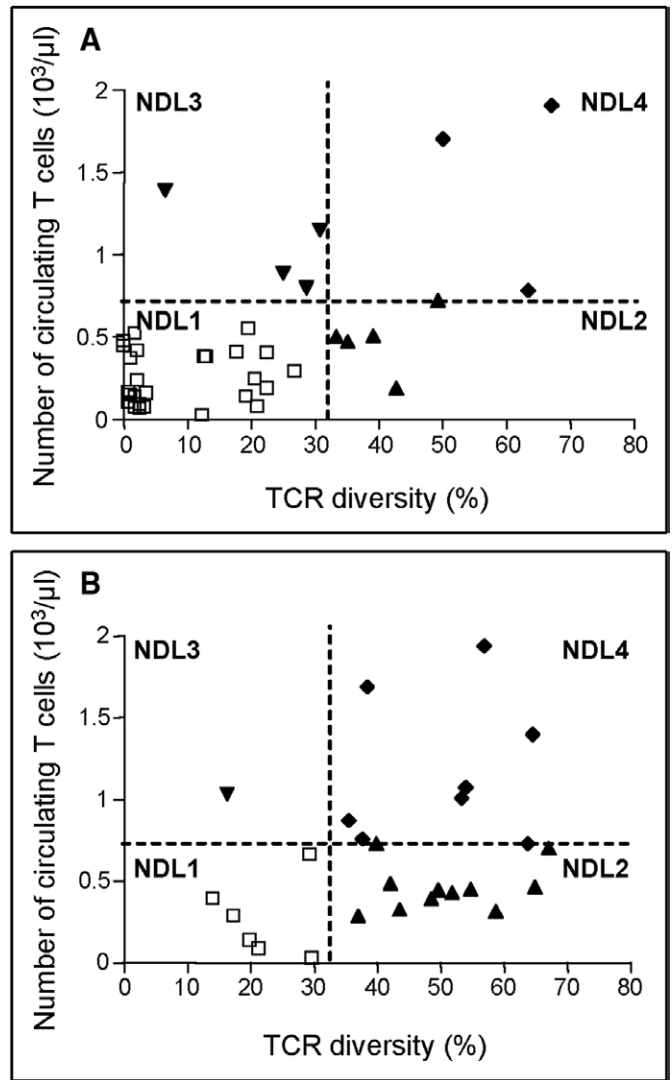


Figure 3. Numeration diversity scoring at day (D1) and day 7 (D7). T-cell receptor (TCR) diversity and number of circulating T-cells ($10^3/\mu$ L) were monitored in 38 septic patients at D1 (**A**) and 26 septic patients at D7 (**B**). Based on thresholds previously determined (33% TCR diversity, 730 T-cells/ μ L), four groups were created: numeration diversity lymphocyte (NDL)1 score corresponding to lymphopenic patients (low TCR diversity, low number of T-cells, $n = 26$), NDL2 score corresponding to lymphopenic patients (higher TCR diversity, low T-cells, $n = 5$), NDL3 score corresponding to dipenic patients (low TCR diversity, higher T-cells, $n = 4$), and NDL4 score corresponding to normality (higher TCR diversity, higher T-cells, $n = 3$). Results are presented as individual values.

$n = 4$), and NDL4 (higher TCR diversity and higher number of T-cells, $n = 3$). At this time point, the majority of patients belonged to group NDL1 (68% patients), whereas group NDL2 represented 13%, group NDL3 11%, and group NDL4 8% of the total population.

The NDL1 group showed a significantly lower percentage of mHLA-DR expressing monocytes than the other three groups (median, 26%; 95% CI 21–39 for group NDL1; median, 55%; 95% CI 35–67 for the other groups; $p < 0.05$, Mann-Whitney U test). However, no differences between NDL1 and the other groups were observed for Simplified Acute Physiologic Score II and SOFA scores. Interestingly, every nosocomial infection

occurred in the NDL1 group, and mortality was 38% vs. 25% in the other pooled groups.

At D7 ($n = 26$; Fig. 3B), patients' distribution according to these NDL groups was NDL1 ($n = 6$), NDL2 ($n = 11$), NDL3 ($n = 1$), and NDL4 ($n = 8$). Therefore, the majority of patients now belonged to group NDL2 (42% patients), whereas group NDL1 represented 23%, group NDL3—4%, and group NDL4—31% of the total population. Interestingly, at this time point, initial SOFA score was significantly higher in group NDL1 as compared with the other three groups (median, 13; 95% CI 11–15 in NDL1; median, 9; 95% CI 9–11 in the other groups; $p < 0.01$, Mann-Whitney U test), whereas the percentage of mHLA-DR expressing monocyte was lower (median, 25%; 95% CI 12–41 in NDL1; median, 51; 95% CI 39–58 in the other groups; $p < 0.05$, Mann-Whitney U test). Furthermore, mortality in the NDL1 group was 67% (four nonsurvivors among six patients), whereas only seven patients among 20 died in the other groups (mortality = 35%).

DISCUSSION

As a result of their ability to interact with the cells of the innate immune system and with the other cells of the adaptive immune 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-cells and B-cells (15, 16). Without showing any clear association with the increased risk of death or nosocomial infections in patients, some alterations of the lymphocytic response have been previously reported after septic shock. They comprise the following: 1) phenotypic alterations including an increase in inhibitory coreceptor expression associated with a decreased expression of activating coreceptors and CD3; 2) functional alterations mainly characterized by a decreased *ex vivo* lymphocyte proliferation associated with decreased Th1 and increased Th2 immune responses; 3) induction of regulatory T-cell populations; and 4) increased apoptosis participating in the initial lymphopenia (1, 3). Such lymphocyte dysfunctions were recently observed in patients' lymphoid organs in a postmortem study (17). The authors observed that patients who died of sepsis present with features consistent with the development of immunosuppression after sepsis (reduced cytokine production after TCR stimulation of splenocytes, increased expression of inhibitory receptors and ligands as well as expansion of suppressor cell populations in both lung and spleen cells). These results illustrate that sepsis-induced immune suppression is a profound mechanism that can be observed not only in circulating blood cells, but also in lymphoid organs.

In the current study, we reinforce and expand these results by investigating at the molecular level a novel aspect of sepsis-induced lymphocyte alterations: the diversity of the TCR β -chain repertoire. To the best of our knowledge, we observe for the first time that septic patients present with a dramatic decrease of peripheral T-cell repertoire. This was associated with other usual immune dysfunctions described in such cohorts of

patients (lymphopenia, decreased percentage of HLA-DR expressing monocytes) and most importantly with increased risk of death and nosocomial infections. The diversity of T-cells plays a central role in the host's ability to mount protective anti-infectious immune responses (18, 19) and an optimal level of TCR diversity allows for efficient protection against pathogens (19). Indeed, because the organism cannot predict the precise pathogen-derived antigens that will be encountered, the immune system relies on the generation and maintenance of a diverse TCR repertoire (19). It follows that the size and diversity of the available T-cell repertoire are crucial in shaping the immune response to a given antigen.

One physiological (aging) and several pathological/therapeutic (HIV infection/highly active antiretroviral therapy, T-cell leukemia, and bone marrow transplantation) conditions have yielded reduced TCR diversity in humans (19, 20). Importantly, decreased TCR repertoire diversity has been associated with viral escape in chronic viral infections such as HCV (21), and changes in the TCR repertoire have been correlated with outcomes of renal transplantation (22, 23). In ICU patients, such reduced TCR repertoire, as observed in our study, might participate in increased susceptibility to nosocomial infections in the context of use of invasive devices (intubation, urinary catheter, central venous catheter, etc.). Furthermore, this might play a role in the reactivation of viral infections (cytomegalovirus, Epstein–Barr virus), normally solely occurring in immunocompromised hosts and also observed in ICU patients (24, 25).

Only two studies previously investigated the TCR repertoire in sepsis by using flow cytometry: one in patients and one in the murine model of cecal ligation and puncture. The first study included nine patients with *Staphylococcus aureus* septic shock (26), and no specific T-cell repertoire modifications were observed. In the second study, Unsinger et al (27) showed that after cecal ligation and puncture, mice presented with lymphopenia but that the TCR repertoire measured 21 days after cecal ligation and puncture was not skewed toward any V β type but resembled the repertoire found in normal mice. The authors concluded that, after sepsis, T-cells were not primed to any antigen resulting from the infection. Importantly, in both studies, TCR repertoire was measured by flow cytometry. Using this technique, a maximum of 25 different V β clones could be detected (28). In contrast, 276 TCR V β –J β rearrangements could be identified by the present molecular biology technique. Furthermore, in both previously published studies, TCR repertoire was evaluated at late time points after the onset of shock (after 21 days in mice and within 10 days in patients). Considering the fast recovery slope observed in our cohort, this could explain the discrepancy with our results. However, a study comparing these two techniques of TCR repertoire measurement should be conducted. To our knowledge, such study has never been done yet.

This fast increase in TCR repertoire observed in patients between D1 and D7 is of the utmost importance in this study. Indeed, to our knowledge, such a rapid recovery of TCR diversity has not been described previously in any other clinical context. This argues against a novel thymus-based lymphocyte

generation but rather suggests a systemic liberation of lymphocytes trapped in peripheral compartments. This assumption is in agreement with the reduced percentage of CD45RA⁺ (naive) but not of CD45RO⁺ (memory) T-cells previously described in septic patients (14, 29, 30). This is further confirmed by our observation that lymphocyte reconstitution after septic shock occurs mainly through a switch from NDL1 to NDL2 group (i.e., through increased diversity) and not through a switch from NDL1 to NDL3 group (i.e., through an increase in cell number). This suggests a uniform reconstitution of TCR diversity through rapid mobilization from lymphocyte peripheral stores rather than immune reaction-based active oligoclonal expansion. These results open novel cognitive research perspectives that deserve to be further investigated in experimental models of sepsis. Similarly, the evaluation of TCR diversity of memory vs. naive T-cells and its overtime evolution in patients with septic shock should be evaluated in a specifically designed study. Indeed, the dynamic measurement of TRB diversity and the composition of T-cell subpopulations (i.e., naive/memory/regulatory) over time in peripheral tissues as well as in spleen and thymus would help in understanding the mechanisms of the rapid recovery of TCR diversity in patients with septic shock and of sepsis-induced immune suppression. More generally, this fast increase in TCR diversity after the onset of shock suggests that this temporal immune suppression belongs to the homeostatic response that takes place after septic shock. It is the lack of (or slow) recovery of immune functions that is associated with unfavorable outcome (as observed with decreased mHLA-DR). This is in favor of the initiation of immunotherapy in sepsis to facilitate this restoration in selected patients with persistent immune alterations.

Our study has some limitations. First, this preliminary study included a small cohort of patients. Therefore, no multivariate analysis could be performed to test the independent predictive value of TCR diversity on mortality vs. clinical variables (Simplified Acute Physiologic Score II and SOFA scores) or other immune parameters (mHLA-DR or number of circulating T-cells). Similarly, the low number of nosocomial infections clearly weakens the statistical analysis of our data because it is quite difficult to obtain significant results with such unbalanced groups of patients. This may be explained by the rather high mortality rate (i.e., 39%) in our cohort knowing that, by definition, patients who died cannot develop nosocomial infections. Second, the impact of therapeutic interventions on TCR diversity was not evaluated. Finally, the absence of an exact age-matched control group prevents us to definitively conclude on the sepsis-induced decrease in TCR diversity. With that said, such a low percentage of TCR variability observed at D1 in septic patients could not solely be explained by their increasing age, considering results for TCR repertoire diversity in elderly published in the literature (18) as well as the fact that no correlation with age was measured at D7 in patients or within the cohort of healthy volunteers (maximum age = 70 yrs, data not shown).

In total, in a cohort of patients with well-documented immune dysfunctions (lymphopenia, decreased HLA-DR expression on monocytes), we describe for the first time an

additional aspect of sepsis-induced immune suppression. Our results add to the current knowledge of lymphocyte dysfunctions in sepsis and of their association with increased mortality and risk of nosocomial infections (3, 31, 32). Furthermore, our results show that the measurement of TCR repertoire diversity, as a novel immune response indicator, may provide predictive information regarding risk of death and nosocomial infection independently of the number of circulating lymphocytes. As a consequence, immunostimulating therapies are now proposed in the treatment of sepsis. One potential candidate could be recombinant human IL-7 because, in addition to its capacity to increase circulating lymphocyte number and function (33), its *in vivo* injection in healthy volunteers was able to increase TCR diversity without inducing any major side effect (34). Furthermore, in mice, initial studies showed that recombinant human IL-7 was able to reduce mortality after cecal ligation and puncture (35, 36).

CONCLUSION

In this study, we describe for the first time at the molecular level a novel aspect of sepsis-induced lymphocyte dysfunctions. We observed that patients with septic shock present with a severely decreased TCR β -chain diversity after the onset of shock. Importantly, independent of the number of circulating T-cells, patients who did not restore rapidly their diversity presented with both increased risk of death and of nosocomial infections. On validation in a large multicentric clinical study, divpenia assessment may become an interesting tool for immunomonitoring. Furthermore, on a more general basis, the observation of a rapid recovery of TCR diversity in peripheral lymphocytes, never described in other clinical situations, opens a novel and interesting cognitive research avenue to understand the underlying mechanisms of immune homeostasis after septic shock.

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