Marked alterations of neutrophil functions during sepsis-induced immunosuppression

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RECEIVED APRIL 22, 2015; REVISED JULY 6, 2015; ACCEPTED JULY 11, 2015. DOI: 10.1189/jlb.4A0415-168RR

**ABSTRACT**

Severe septic syndromes deeply impair innate and adaptive immunity and are responsible for sepsis-induced immunosuppression. Although neutrophils represent the first line of defense against infection, little is known about their phenotype and functions a few days after sepsis, when the immunosuppressive phase is maximal (i.e., between d 3 and 8). The objective of the present study was to perform, for the first time, a global evaluation of neutrophil alterations in immunosuppressed septic patients (at d 3–4 and d 6–8) using phenotypic and functional studies. In addition, the potential association of these parameters and deleterious outcomes was assessed. Peripheral blood was collected from 43 septic shock patients and compared with that of 23 healthy controls. In the septic patients, our results highlight a markedly altered neutrophil chemotaxis (functional and chemokine receptor expressions), oxidative burst, and lactoferrin content and an increased number of circulating immature granulocytes (i.e., CD10dimCD16dim). These aspects were associated with an increased risk of death after septic shock. In contrast, phagocytosis and activation capacities were conserved. To conclude, circulating neutrophils present with phenotypic, functional, and morphologic alterations a few days after sepsis onset. These dysfunctions might participate in the deleterious role of sepsis-induced immunosuppression. The present results open new perspectives in the mechanisms favoring nosocomial infections after sepsis shock. They deserve to be further investigated in a larger clinical study and in animal models recapitulating these alterations. J. Leukoc. Biol. 98: 000-000; 2015.

**Introduction**

Septic syndrome represents the major cause of death among critically ill patients worldwide, constituting an important public health problem. Despite an overall modest decline in the proportional mortality, the total number of patients dying of sepsis is greater than in the past [1]. The incidence of sepsis is still increasing, reaching 10–30% of ICU admissions. A recent epidemiologic study also highlighted that mortality remains >30% for septic shock patients in Europe and the United States [2].

Septic syndromes deeply perturb immune homeostasis and impair innate and adaptive immunity. Both pro- and anti-inflammatory responses are initially induced in septic shock patients, with the secondary occurrence of sepsis-induced immunosuppression. The intensity and duration of this immunosuppressive phase are associated with increased mortality and nosocomial infections [3].

Among leukocytes, neutrophils are crucial components of the innate immune response and the first line of defense against infection. They also play a central role in the initiation of adaptive immune responses by secreting cytokines [4]. Thus, for the past decades, the role of neutrophils in the early phase after the onset of injury has been widely studied. An overwhelming activation of granulocytes and a reduced motility is thought to participate in the development of organ damage after sepsis, notably because of the massive production by neutrophils of ROS and proinflammatory cytokines at sites distant from the initial infection [5, 6]. Little is known about their phenotype and functions during the phase of sepsis-induced immunosuppression. However, an immunosuppressive neutrophil subset has recently been reported in the blood of volunteers challenged with LPS [7]. Fillay et al. [7] highlighted that suppression of T-cell function can be accomplished by a subset of human neutrophils that can be systemically induced in response to acute inflammation. In addition, they showed that these cells can...
be detected in the peripheral blood of few patients after trauma [7].

Considering the lack of information available on neutrophil status during sepsis-induced immunosuppression and given these new elements on neutrophil suppressive functions, our aim was to investigate neutrophil alterations during the sepsis-induced immunosuppressive phase. The objective of the present study was to perform, for the first time, a global evaluation of the neutrophil alterations in immunosuppressed septic patients using phenotypic and functional studies. In addition, we assessed the potential association between these parameters and deleterious outcomes.

**MATERIALS AND METHODS**

**Study population**

Septic shock was defined according to the diagnostic criteria of the American College of Chest Physicians/Society of Critical Care Medicine [8] by an identifiable site of infection, persisting hypotension despite fluid resuscitation requiring vasopressor therapy, and evidence of a systemic inflammatory response manifested by ≥2 of the following criteria: (a) temperature >38°C or <36°C; (b) heart rate >90 beats/min; (c) respiratory rate >20 breaths/min; and (d) white blood cell count >12,000/mm3 or <4000/mm3. The exclusion criteria were age <18 yr and the presence of aplasia or immunosuppressive disease (e.g., HIV infection). During the follow-up period, clinical and biologic data were collected. The data collection included demographic characteristics (age, gender); admission category (elective or emergency surgery, medicine); referral pattern (community, hospital, or ICU-acquired septic shock); microbiological findings (infection characteristics [e.g., source and identified microorganisms]), and comorbidities (e.g., chronic obstructive pulmonary disease, chronic heart failure, malignant diseases, diabetes). Two clinical scores were recorded: the initial severity assessed by the SAPS II (range 0–194) and the SOFA score (range 0–24) at admission. Mortality was defined as death occurring within 28 d after the onset of shock. Secondary ICU-acquired infections were defined according to European definitions of the European Centre for Disease Prevention and Control.

This work was a part of a global study on ICU-induced immune dysfunctions. Our institutional review board for ethics (Comité de Protection des Personnes) approved the study and waived the need for patient informed consent, because the study was observational and biomarker expression was measured using residual blood after completion of the routine follow-up period. The present study is also registered at the French Ministry of Research and Teaching (DC:2008-509) and recorded at the Commission Nationale de l'Informatique et des Libertés.

Forty-three septic shock patients admitted to the ICUs of the Lyon-Sud University Hospitals (France) were prospectively enrolled in our study. Peripheral blood was collected in sampling tubes containing EDTA or heparin at d 3 or 4 (d3–4) and d 6–8 (d6–8) after the onset of shock. Only patients alive at d 3 or 4 after the onset of shock were considered. The onset of septic shock was defined as the beginning of vasopressor therapy. Considering the complexity of the functional, morphologic, and transcriptomic studies and the limited available amount of blood, it was not possible to perform all experiments on every patient; thus, the sample size for each experiment has been provided in the text or figures. Reference values and control samples were obtained from a cohort of 23 healthy volunteers who had provided informed consent (median age 36 yr; 15 women, 8 men).

To confirm that the patients had presented with immunosuppression, mHLA-DR was assessed by flow cytometry (FC500; Beckman-Coulter, Miami, FL, USA) at d3–4, as previously described [9]. An mHLA-DR nadir is known to occur at this time point [10]. The results are expressed as the number of antibodies bound per cell. The absolute lymphocyte counts and percentages of regulatory Treg at d3–4 are also reported [11].

**Phenotypic study**

The PerFix-No Centrifuge Assay Kit (Beckman-Coulter, Hialeah, FL, USA) was used with 7-color flow cytometry immunophenotyping. Staining of fresh EDTA whole blood was performed using FITC-labeled anti-myeloperoxidase, PE-labeled anti-lactoferrin, ECD-labeled anti-CD162L, PE-cyanine7-labeled anti-CD10, AA750-labeled anti-CD11b, PB-labeled anti-CD16, and Krome Orange-labeled anti-CD14. All reagents were purchased from Beckman-Coulter. According to the manufacturer’s instructions, the samples were first fixed with the fixative reagent and incubated for 15 min. Next, the aliquots were simultaneously permeabilized using the permeabilizing reagent and stained with fluorochrome-conjugated antibodies. After 35 min of incubation, the samples were fixed using a solution containing formaldehyde. Cytometry analyses were performed on a Navios flow cytometer using the Navios software (Beckman-Coulter). Neutrophils were identified on a forward light scatter/SSC histogram and a CD14/SSC plot was used to exclude monocytes. Neutrophil subsets were eventually sorted using a FacsAria II (BD Biosciences, San Jose, CA, USA), and cytopsins were made based on CD10, CD16, and CD62L staining. At the end of the cell sorting, microscope slides were observed using the May-Gr¨unwald-Giema staining protocol. Fluorescence thresholds to assess percentages and sorting of granulocyte subsets were set up by comparison with healthy controls such that >95% of the control samples exhibited CD10high CD162Lhigh, or CD62Lhigh neutrophils.

The following antibodies were also used to study chemokine receptors: PE-labeled anti-CXCR2, allophycocyanin-labeled anti-CXCR1, and PB-labeled anti-CD16 (Beckman-Coulter). After 30 min of incubation with the antibodies, RBCs were lysed using FACS Lysing Solution (BD Biosciences). Samples were run on a Navios flow cytometer (Beckman-Coulter). The results are expressed as the MFI for each of the parameters studied.

**Activation capacity**

Fresh EDTA whole blood was stimulated with increasing IL-8 (R&D Systems, Minneapolis, MN, USA) or IMLP (Sigma-Aldrich, St. Louis, MO, USA) doses at 37°C in a water bath for 15 min. Staining with ECD-labeled anti-CD62L and AA750-labeled anti-CD11b (Beckman-Coulter) was performed during 30 min after RBC lysis (FACS Lysing Solution, BD Biosciences). The samples were analyzed on a Navios (Beckman-Coulter). The MFI of CD62L and CD11b was examined for each condition. The PerFix EXPOSE reagents from Beckman-Coulter were used to study STAT5 phosphorylation. Staining of fresh EDTA whole blood was performed using PE-labeled antiphosphorylated STAT5 and allophycocyanin-labeled anti-CD14 (Beckman-Coulter) antibodies. The blood samples were first stimulated with increasing recombinant human GM-CSF (ImmuNoTools, Friesoythe, Germany) concentrations at 37°C in a water bath for 15 min. The samples were fixed for 10 min using a fixative reagent. Aliquots were then permeabilized using the permeabilizing reagent (which also lyses RBCs) and incubated for 5 min at 37°C in a water bath. The samples were centrifuged, and intracellular staining was performed with a mixture of staining reagent, including fluorochrome-conjugated antibodies for 30 min. After a last washing step, the samples were fixed using a solution containing formaldehyde. Samples were run on a Navios flow cytometer (Beckman-Coulter). The MFI of phosphorylated STAT5 is reported in neutrophils.

**Chemotaxis**

The Migratext Kit (Glycotope Biotechnology, Berlin, Germany) allows the determination of the number of neutrophils that have migrated through cell culture inserts toward a concentration gradient of the chemotactant, similar to the Boyden chamber technique. The chemotactants used are fMLP (10⁻⁸ M; Sigma-Aldrich), IL-8 (10 ng/ml, R&D Systems), and GRO-α (10 ng/ml; R&D Systems), all diluted in incubation buffer. The neutrophils were isolated from heparinized whole blood by spontaneous sedimentation over leukocyte separation medium containing dextran and placed into cell culture inserts. Chemotaxis was conducted at 37°C (heat chamber) toward a gradient of chemotactant compared with a control of incubation buffer. The cells were then stained with FITC-labeled anti-CD62L and counting beads. Just before flow cytometry analysis on a Navios flow cytometer.
Phagocytosis

Quantitative determination of neutrophil phagocytosis (ingestion of bacteria) was assessed using the Phagotest Kit (Glycotope Biotechnology). It measures the percentage of phagocytes that have ingested bacteria and their activity (number of bacteria per cell). Heparinized whole blood was incubated with the FITC-labeled Escherichia coli at 37°C (water bath); a negative control sample remained on ice. Phagocytosis was stopped by placing the samples on ice and adding a quenching solution. This solution allows the discrimination between attachment and internalization of bacteria by quenching the FITC fluorescence of surface bound bacteria, leaving the fluorescence of internalized particles unaltered. After 2 washing steps, the erythrocytes were lysed. The DNA staining solution, which was added just before flow cytometry analysis, excludes aggregation artifacts of bacteria or cells. The MFI (number of ingested bacteria) in neutrophils was then analyzed on a Navios flow cytometer (Beckman-Coulter).

Oxidative burst

The FagoFlowEx Kit (Exbio Antibodies, Vestec, Czech Republic) was used to measure the oxidative burst of the neutrophils after their stimulation with E. coli in heparinized whole blood. After the ingestion of bacteria, phagocytes activate NADPH oxidase, producing reactive oxidative intermediates that can oxidize dihydrodihydrodramine 123 into fluorescent rhodamine 123, which is detected by flow cytometry. PMA was used as a positive control. Heparinized whole blood was stimulated with either E. coli or PMA, or was nonstimulated (negative control), before adding the substrate. After an incubation of 20 min at 37°C (water bath), RBCs were lysed, and samples were analyzed on a Navios flow cytometer (Beckman-Coulter) quickly after the last washing step. The stimulation index was measured; it is the MFI ratio of positive neutrophils of a stimulated sample (E. coli) to negative neutrophils of a negative control. According to the manufacturer’s instructions, it can be used to compare the respiratory burst intensity between blood samples.

Statistical analysis

The nonparametric Mann-Whitney U test was used to compare results between the septic patients and healthy volunteers. The nonparametric Wilcoxon paired test was performed to evaluate the evolution over time within a group. The receiver operating characteristic curves were established, and the best cutoff values were determined using the Youden index. Kaplan-Meier survival curves were obtained after patient stratification according to these values. Differences in survival between groups were evaluated using the log-rank test. To determine the variables associated with death, uni- and multivariate logistic regression analyses were performed, and odds ratios were estimated, with the previously described characteristics of injury-induced immunosuppression, with reduced mHLA-DR, low CD4+ lymphocyte count, and an increased percentage of circulating Treg cells compared with normal values.

Number of circulating neutrophils

As presented in Table 1, the number of circulating neutrophils was increased in the septic shock patients compared with the normal values (2.5–7.5 × 10⁷/L). A significant increase in the neutrophil count (P < 0.05) was noted in the nonsurvivors compared with the survivors at d6–8. However, this significance was lost on univariate analysis (detailed in the following sections).

Neutrophil immaturity—subset analysis

At d3–4 and d6–8, significantly decreased CD10 expression was measured on the neutrophils from septic patients compared with those from healthy volunteers. Furthermore, in paired samples, a slight increase over time was observed between d3–4 and d6–8 (Fig. 1A). The same trend was noted regarding CD16 expression, with decreased expression in septic patients compared with controls (Fig. 1B). Therefore, patients with septic shock exhibited increased proportions of immature CD10dimCD16dim granulocytes. For several patients, immature CD10dimCD16dim neutrophils were FACS sorted and stained with May-Grünwald-Giemsa. The morphology of this subset was compared with CD10brightCD16dim considered mature neutrophils (Fig. 1D). Immature CD10dimCD16dim granulocytes appeared as band cells (Fig. 1E).

In addition, as described by Pillay et al. [7], a potentially immunosuppressive CD16brightCD62Ldim neutrophil subset was studied. At d3–4 and d6–8, CD62L expression was lower in neutrophils from patients with sepsis than in those from healthy subjects (Fig. 1C). In addition, and as observed by Pillay et al. [7] and illustrated in Fig. 1F, CD16brightCD62Ldim neutrophils displayed a hypersegmented nuclear morphology. However, even if this subset was detected in some patients’ samples, it was not systematically present, and its frequency was low.

Activation capacity

Activation capacity in response to stimuli was measured by studying the differences in CD62L, and CD11b expression after stimulation. In response to increasing doses of fMLP or IL-8, we observed a similar decrease in CD62L expression for septic patients at d3–4 and healthy volunteers (Fig. 2A and B). We also observed increased CD11b expression in patients with septic shock (d3–4) and healthy controls when stimulated with increasing doses of fMLP or IL-8 (Fig. 2C and D). The same results were obtained when studying CD11b and CD62L expression in septic patients at d6–8 (data not shown).

STAT5 is a transcription factor for which phosphorylation is a key marker of cellular activation. We measured STAT5 phosphorylation to assess neutrophil activation in response to increasing concentrations of GM-CSF. We observed increased STAT5 phosphorylation in response to increasing doses of GM-CSF in both patients at d3–4 and healthy donors (Fig. 2E). The same results were obtained in septic patients at d6–8 (data not shown).

Most importantly, no difference was noted between patients and healthy volunteers in both experiments (Fig. 2).
We also investigated whether the chemotactic response was impaired in patients with septic shock. At d3–4 and d6–8, CD11b expression was decreased in septic patients compared with healthy volunteers (Fig. 3A). Regarding the chemokine receptors, CXCR1 and CXCR2, a significant decrease in their expression was noted in patients at d6–8, although a decrease over time was observed between d3–4 and d6–8 (Fig. 3B and C). We measured neutrophil migration in response to fMLP, GRO-α, and IL-8. In agreement with the decreased chemokine receptor expression, septic patients presented with a significantly decreased number of emigrated neutrophils in the stimulated wells at d3–4. The same trend was noted regardless of the chemoattractant used (Table 2). Overall, these results showed that the chemotactic response is altered a few days after septic shock.

**Phagocytosis**
Phagocytosis was evaluated by measuring using flow cytometry the ingestion of fluorescent E. coli by neutrophils. No significant
differences were observed between patients and controls at either d3–4 or d6–8 (Supplemental Fig. 1).

**Bactericidal parameters**

The intracellular myeloperoxidase content was significantly decreased in neutrophils from patients with septic shock at d3–4 and d6–8 relative to those from healthy volunteers (Fig. 3D). Similarly, the expression of intracellular lactoferrin was significantly decreased in the septic patients compared with that in the healthy volunteers (Fig. 3E). Finally, we assessed oxidative burst by measuring, using flow cytometry, oxidation of a substrate by NAPDH oxidase after ingestion of bacteria. We observed that the stimulation index, as a measure of oxidative burst capacity, was significantly reduced in patients versus controls (Fig. 3F). In paired samples, a significant increase over time was observed between the d3–4 and d6–8 samples. Overall, these results suggest that the production of bactericidal agents is impaired after septic shock.

**Logistic regression analysis**

Despite the limited number of nonsurvivors (n = 8), multivariate analyses were finally performed to test the predictive value on 28-d mortality of nonstratified variables selected on the basis of the univariate analysis results (variables presenting with P ≤ 0.15; data not shown). Only myeloperoxidase, lactoferrin, CD62L, CD11b, CD10, and CD16 expression, the percentage of CD10brightCD16dim neutrophils measured at d6–8, and the SAPS II score were included in 2 parameter models for 34 patients (Table 3). Therefore, each multivariate model always included the SAPS II score (calculated from 12 routine physiologic measurements, including age, WBCs, and others) and a biologic parameter measured on neutrophils at d6–8. In these models, lower intracellular expression of myeloperoxidase, lactoferrin, or extracellular CD62L expression measured at d6–8 remained significantly associated with a higher risk of death after septic shock (myeloperoxidase: odds ratio 8.39, 95% CI 1.185–59.441, P = 0.033; lactoferrin: odds ratio 1.25, 95% CI 1.019–1.530, P = 0.032; CD62L: odds ratio 1.52, 95% CI 1.003–2.307, P = 0.048; Table 3).

Among these parameters, and even if the results could be considered underpowered, diminished myeloperoxidase expression seemed to appear as the best predictor to identify a group of septic shock patients at high risk of death. Kaplan-Meier survival curves were established after stratification using the calculated thresholds (Youden index − myeloperoxidase d6–8 expression = 3). The survival rates of patients were significantly different when stratified according to myeloperoxidase expression. Patients with higher expression had significantly better survival (P = 0.040, log-rank test; Fig. 4).

It is noteworthy that although not found on multivariate analysis, but in accordance with a recent study performed by Guerin et al. [12], the percentage of immature CD10brightCD16dim...
neutrophils appeared more important in nonsurvivors compared with survivors at d6–8 (survivors: 2.05%, IQR 0.92–6.03; nonsurvivors: 5.89%, IQR 4.95–25.1; P = 0.023; data not shown). We therefore established Kaplan-Meier survival curves after stratification by the Youden index for the percentage of immature granulocytes measured at d6–8 (i.e. 4%). Patients with a lower proportion of CD10dimCD16dim neutrophils had significantly better survival than did patients presenting with a higher percentage (P = 0.040, log-rank test; Supplemental Fig. 2).

**DISCUSSION**

It is now well established that, in parallel with a massive proinflammatory response leading to shock and organ failure, septic patients rapidly develop an immunosuppressive state associated with severe immune dysfunction [13]. In addition, the intensity and duration of these alterations have been linked to an increased risk of death and the development of ICU-acquired infections [14].

Neutrophils are the most abundant leukocytes providing the first line of host defense against a wide range of infectious pathogens [4]. Therefore, it is no surprise that these cells have a pivotal role in the defense against bacterial infections, including sepsis. In line with this, the role of these cells has been studied at the onset of sepsis pathophysiology. In brief, the recruitment of neutrophils from bone marrow is augmented and their apoptosis is reduced. Moreover, neutrophil oxidative burst and phagocytosis were increased in patients with septic shock but chemotaxis was strongly inhibited. This reduced motility associated with acute activation of neutrophils is believed to play a role in the development of organ damage after sepsis. The extensive production of ROS and proinflammatory cytokines by
neutrophils at sites distant to the initial infection might be a part of the physiopathology of sepsis [5, 6]. However, these studies were conducted during the proinflammatory phase of sepsis, and no data are yet available during the delayed immunosuppressive phase. However, recent works, notably that by Pillay et al. [7], have suggested putative immunosuppressive properties for neutrophils, highlighting a possible involvement of these cells during sepsis-induced immunosuppression.

The aim of the present study was thus to perform an extensive investigation of neutrophil alterations during sepsis-induced immunosuppression through phenotypic and functional studies. Although most studies have reported overwhelming activation of neutrophils during the sepsis early proinflammatory phase [5, 6], our results showed, for the first time in patients, that circulating neutrophils present with phenotypic and functional alterations during sepsis-induced immunosuppression. Our main results included that septic patients present with (a) a persisting increased proportion of immature cells in circulating neutrophils, (b) major neutrophil functional alterations, and (c) both aspects are associated with an increased risk of death after septic shock.

We found an increased frequency of immature CD10^dimCD16^dim neutrophils in septic shock patients sampled at d3–4 and d6–8 and decreased expression of CD10 and CD16 on granulocytes, characteristic of immature myeloid cells [15]. This is important, because Drifte et al. [16] recently showed that circulating immature neutrophils from patients with severe sepsis and septic shock are less potent in supporting innate immune defenses compared with mature neutrophils. Therefore, this increased proportion of immature cells might participate in the

Figure 3. Chemotaxis and bactericidal parameters. Cell surface expression was measured as the MFI on neutrophils from septic shock patients at d3–4, white, n = 34) and d6–8 (striped, n = 30) serial samples after the onset of shock compared with healthy volunteers (HVs; gray, n = 23) for CD11b (A). Similarly, MFI on neutrophils from septic shock patients at d3–4 (n = 11), d6–8 (n = 10; serial samples), and HVs (n = 14) are reported for CXCR1 (B) and CXCR2 (C). MFI of intracellular myeloperoxidase (D) and intracellular lactoferrin (E) were also measured for patients at d3–4 (n = 34), d6–8 (n = 30; serial samples), and HVs (n = 25). The stimulation index (MFI ratio of positive neutrophils of stimulated sample [E. coli] to negative neutrophils of negative control) after E. coli stimulation in neutrophils are presented for patients at d3–4 (n = 11), d6–8 (n = 10; serial samples), and HVs (n = 14) (F). The nonparametric Mann-Whitney U test was used to compare the results between groups. The nonparametric Wilcoxon paired test was used to evaluate evolution over time within a group. **P < 0.01. *P < 0.05.
Alterations of neutrophil functions we observed in our patients. This is supported by our observation of a lower oxidative burst capacity (decreased stimulation index) in CD10<sup>dim</sup>CD16<sup>dim</sup> neutrophils compared with CD10<sup>bright</sup>CD16<sup>bright</sup> cells (data not shown).

In addition, we observed markedly decreased function of circulating neutrophils in the septic shock patients. In particular, neutrophil chemotaxis was decreased (reduced integrin, selectin, and chemokine receptor expression with altered ex vivo migration in response to different chemoattractants). Similar results have been found by Tavares-Murta et al. [17] in response to fMLP, GRO-α, and IL-8. Data presented as median and IQR (quartile 1 to quartile 3; nonparametric Mann-Whitney U test used to compare results between groups. *P < 0.05.

Similarly, we found a clear reduction in oxidative burst for late samples measured using the ex vivo functional test. This is in line with a decrease in myeloperoxidase and lactoferrin intracellular expression. Many studies have noted an upregulation in ROS generation 48 h after sepsis onset that could participate in organ dysfunctions after sepsis [18, 19]. We found contrasting results that might illustrate the shift from an initial overwhelming proinflammatory response with exacerbated neutrophil function to an immunosuppressive phase with functional alterations after septic shock.

Although our study was purely observational and thus did not allow to draw conclusions regarding the effective role of neutrophil alterations in the development of immunosuppression in patients with septic shock, diminished myeloperoxidase expression appeared to be the best predictor to identify a group of septic shock patients at high risk of death (odds ratio 8.39, 95% CI 1.85–59.44, *P = 0.033). However, this result must be taken with caution, given the limited number of nonsurvivors in our cohort. Similarly, patients with a lower proportion of CD10<sup>dim</sup>CD16<sup>dim</sup> granulocytes had significantly better survival compared with patients presenting with a higher percentage (*P = 0.040, log-rank test). This last aspect is in accordance with data from Guérin et al. [12]. They associated the prediction of the early deterioration of patients with sepsis with the presence of the

### TABLE 2. Ratio of emigrated neutrophils in stimulated wells versus control wells

<table>
<thead>
<tr>
<th>Variable</th>
<th>fMLP</th>
<th>GRO-α</th>
<th>IL-8</th>
</tr>
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<tbody>
<tr>
<td>Healthy volunteers (<em>n</em> = 7)</td>
<td>4.9 (0.55–10.49)</td>
<td>2.97 (1.69–15.10)</td>
<td>6.01 (2.21–37.63)</td>
</tr>
<tr>
<td>Septic shock patients d3–4 (<em>n</em> = 9)</td>
<td>0.16&lt;sup&gt;a&lt;/sup&gt; (0.03–0.49)</td>
<td>0.92&lt;sup&gt;a&lt;/sup&gt; (0.29–1.65)</td>
<td>1.96&lt;sup&gt;a&lt;/sup&gt; (0.88–3.94)</td>
</tr>
<tr>
<td>Septic shock patients d3–4 (<em>n</em> = 6)</td>
<td>0.62 (0.15–1.05)</td>
<td>3 (1.11–9.04)</td>
<td>4.21 (2.51–6.61)</td>
</tr>
</tbody>
</table>

The ratio of emigrated neutrophils in stimulated wells to emigrated neutrophils in control wells (incubation buffer) are presented for neutrophils from healthy volunteers (*n* = 7) compared with that for septic shock patients at d3–4 (*n* = 9) and d6–8 (*n* = 6; different patients) in response to fMLP, GRO-α, and IL-8. Data presented as median and IQR (quartile 1 to quartile 3; nonparametric Mann-Whitney U test used to compare results between groups. *P < 0.05.

### TABLE 3. Multivariate analysis results

<table>
<thead>
<tr>
<th>Multivariate analysis parameter</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th><em>P value</em></th>
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<tr>
<td>Myeloperoxidase</td>
<td>8.39</td>
<td>1.185–59.441</td>
<td>0.033&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>SAPS II</td>
<td>0.96</td>
<td>0.874–1.043</td>
<td>0.305</td>
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<td>Lactoferrin</td>
<td>1.25</td>
<td>1.019–1.530</td>
<td>0.032&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>SAPS II</td>
<td>0.96</td>
<td>0.885–1.036</td>
<td>0.283</td>
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<tr>
<td>CD102L</td>
<td>1.52</td>
<td>1.003–2.307</td>
<td>0.048&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>SAPS II</td>
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<td>0.898–1.035</td>
<td>0.311</td>
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<tr>
<td>CD11b</td>
<td>8.40</td>
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<td>SAPS II</td>
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<td>0.812–1.028</td>
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<td>CD10</td>
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<td>SAPS II</td>
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<td>0.884–1.027</td>
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<td>CD16</td>
<td>1.31</td>
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<td>SAPS II</td>
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<tr>
<td>Neutrophil count</td>
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<td>SAPS II</td>
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<td>0.884–1.044</td>
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<td>% CD10&lt;sup&gt;dim&lt;/sup&gt;CD16&lt;sup&gt;dim&lt;/sup&gt;</td>
<td>0.86</td>
<td>0.730–1.007</td>
<td>0.061</td>
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<tr>
<td>SAPS II</td>
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<td>0.882–1.021</td>
<td>0.160</td>
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</tbody>
</table>

Multivariate logistic regression analysis used to identify variables associated with death (*n* = 35 survivors and 8 nonsurvivors). SAPS II score included models with myeloperoxidase, lactoferrin, CD102L, CD11b, CD10, and CD16 expression (MFI), neutrophil count, and percentage of CD10<sup>dim</sup>CD16<sup>dim</sup> neutrophils measured at d6–8. SAPS II score was calculated after admission. *Statistically significant (*P < 0.05).
CD10^dim^CD16^dim^ subset [12]. This suggests that these neutrophils might participate in the deleterious role of immunosuppression after septic shock. In particular, it is well known that primary innate immunodeficiencies affecting neutrophil function such as oxidative burst and chemotaxis lead to severe pathologic entities characterized by an increased susceptibility to infections. Thus, it is tempting to speculate that neutrophil alterations could participate in the increased risk of nosocomial infection observed in septic patients. This aspect needs to be further evaluated in experimental studies, recapitulating in mice the results we observed in patients and including additional microbiological experiments such as bacterial killing or clearance.

Finally, not all neutrophil functions were altered in our study. In particular, the phagocytosis capacity was preserved. Likewise, the activation capacity was also conserved in patients during sepsis-induced immunosuppression compared with controls. We observed a similar increased CD11b and decreased CD62L expression on neutrophils after either IL-8 or fMLP stimulation. Likewise, when measuring STAT5 phosphorylation in response to increasing concentrations of GM-CSF, no differences were observed a similar increased CD11b and decreased CD62L expression on neutrophils after either IL-8 or fMLP stimulation. Likewise, when measuring STAT5 phosphorylation in response to increasing concentrations of GM-CSF, no differences were observed.

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CONCLUSIONS

Taken together, in this extensive study of neutrophil phenotype and function during sepsis-induced immunosuppression, our results have highlighted a markedly altered neutrophil chemotaxis and oxidative burst and an increased number of immature circulating granulocytes in patients with septic shock. Both aspects were associated with an increased risk of death after septic shock. The association of selected parameters with subsequent mortality requires confirmation in a larger clinical study. In addition, the importance of neutrophil alterations during sepsis-induced immunosuppression could be evaluated in animal models.

AUTHORSHIP


ACKNOWLEDGMENTS

This study was mainly supported by funds from Hospices Civils de Lyon. The study was also supported by Beckman-Coulter and bioMérieux by donations of laboratory equipment and supplies. These private companies had no role in the study design or data collection or interpretation. Beckman-Coulter and bioMérieux had no role in manuscript preparation or the decision to submit it for publication. The authors thank Anne Portier, Immunology Laboratory, Hôpital E. Herriot, Lyon, and Elisabeth Cerrato, Hospices Civils de Lyon, bioMérieux Joint Research Unit, for their help in performing preanalytical handling of the samples; and Hélène Vallin, Nathalie Panel, and Marion Provent (Clinical Research Center, Lyon-Sud) for their work on patient inclusion and clinical data acquisition.

DISCLOSURES

The authors declare no competing financial interests.

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Figure 4. Survival curve. Kaplan-Meier survival curves were established after stratification by myeloperoxidase expression at 6–8 (MFI = 3; Youden index). A significant difference was measured between the 2 curves (P = 0.040, log-rank test).
KEY WORDS:  
septic shock · CD10 · CD16 · myeloperoxidase
Marked alterations of neutrophil functions during sepsis-induced immunosuppression

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J Leukoc Biol published online July 29, 2015
Access the most recent version at doi:10.1189/jlb.4A0415-168RR

Supplemental Material
http://www.jleukbio.org/content/suppl/2015/07/29/jlb.4A0415-168RR.DC1

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