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CD24-Trigged Caspase-Dependent Apoptosis via Mitochondrial Membrane Depolarization and Reactive Oxygen Species Production of Human Neutrophils Is Impaired in Sepsis

Marianna Parlato,* Fernando Souza-Fonseca-Guimaraes,* François Philippart,† Benoît Misset,‡,‡ Captain Study Group,1 Minou Adib-Conquy,* and Jean-Marc Cavaillon*

Apoptosis is the most common pathway of neutrophil death under both physiological and inflammatory conditions. In this study, we describe an apoptotic pathway in human neutrophils that is triggered via the surface molecule CD24. In normal neutrophils, CD24 ligation induces death through depolarization of the mitochondrial membrane in a manner dependent on caspase-3 and caspase-9 and reactive oxygen species. Proinflammatory cytokines such as TNF-α, IFN-γ, and GM-CSF upregulated the expression of CD24 in vitro, favoring the emergence of a new CD16<sup>high</sup>/CD24<sup>high</sup> subset of cultured neutrophils. We observed that CD24 expression (at both mRNA and protein levels) was significantly downregulated in neutrophils from sepsis patients but not from patients with systemic inflammatory response syndrome. This downregulation was reproduced by incubation of neutrophils from healthy controls with corticosteroids or with plasma collected from sepsis patients, but not with IL-10 or TGF-β. Decreased CD24 expression observed on sepsis neutrophils was associated with lack of functionality of the molecule, because cross-ligation of CD24 failed to trigger apoptosis in neutrophils from sepsis patients. Our results suggest a novel aspect of CD24-mediated immunoregulation and represent, to our knowledge, the first report showing the role of CD24 in the delayed/defective cell death in sepsis. *The Journal of Immunology, 2014, 192: 000–000.

Neutrophils are the most abundant leukocyte population in human peripheral blood and play an essential role in the inflammatory response and in the clearance of microbes. Neutrophils engulf pathogens and, through generation of reactive oxygen metabolites, degranulation, enzymatic activation, and exocytosis of DNA, are able to kill them (1). However, these substances can cause “collateral damage,” as they are toxic not only to invading bacteria but also to the host. The presence of such highly toxic intracellular content requires timely and tight regulation of neutrophil death both to avoid broad spillover into the surrounding tissue and, finally, to achieve inflammation resolution (2). Under normal conditions in the circulation, neutrophil turnover is very rapid, with estimates of neutrophil half-life ranging from 6 to 10 hours up to 5 days (3), after which they undergo apoptosis spontaneously. In the presence of proinflammatory mediators and microbe-derived molecules such as endotoxin (LPS), and in particular environmental conditions such as hypoxia, the neutrophil life span becomes significantly extended (4–6). This delayed cell death is associated with the accumulation of activated neutrophils at the site of inflammation, induction of host tissue damage, and consecutive organ failure in critically ill patients. Indeed, in sepsis, one of the leading causes of mortality in intensive care units (7), a profound inhibition of neutrophil programmed cell death has been reported (8–10). These observations suggest that the deregulation of neutrophil programmed cell death may contribute to the exacerbation of the inflammatory response.

CD24 is a small heavily glycosylated cell-surface protein that is linked to the membrane by a GPI anchor and localized in a lipid raft (11). In hematopoietic cells, CD24 is expressed on B cells, T cells, eosinophils, dendritic cells, and, most prominently, neutrophils (12). It has been stated that CD24 expression on T cells is required for optimal homeostatic proliferation (13), whereas several other reports showed that cross-linking of CD24 transduces apoptotic signals in other cell types (14–16). Therefore, both enhanced expression and lack of CD24 can affect cellular functions. CD24 also plays a role in inflammation because it was recently shown to bind a variety of danger-associated molecular patterns, such as high mobility group box protein 1, members of the heat shock protein (HSP) family, and nucleolins (17), and to repress the host response to tissue injury through its interaction with Siglec-10 in dendritic cells (18).

In this report, we investigated whether CD24 was involved in the modulation of neutrophil functions. We show that pro- and anti-inflammatory mediators alter the expression of CD24 and, most importantly, that CD24 cross-ligation can significantly accelerate neutrophil death in a caspase-dependent fashion. We also report that the expression of CD24 is downregulated in neutrophils from sepsis patients, associated with an impaired CD24-mediated cy-
toxic response and neutrophil death. Our results suggest a novel aspect of CD24-mediated immunoregulation, which may play a role in the pathophysiology of sepsis.

Materials and Methods

Patients
All the intensive care unit patients belonged to the CAPTAIN study (Combined Approach for the Early Diagnosis of Sepsis; Clinical Trial, no. NCT01378169) and were from all seven intensive care units involved in this protocol. Study approval was obtained from the regional ethics committee (Session of November 19, 2010, no. 2010-A00908-31). A written informed consent was obtained from each patient or his or her relatives. Patients undergoing chemotherapy or immunosuppressive therapy, or those using steroids (>10 mg/d longer than 15 d) were excluded. All sepsis patients met the inclusion criteria according to the guidelines of the American College of Chest Physicians/Society of Critical Care Medicine (19). The diagnosis of sepsis was confirmed by two independent reviewers (F.P. and B.M.). A total of 46 patients were included in the study. Individuals with a confirmed infection were classified as sepsis patients (27) or, alternatively, as patients with systemic inflammatory response syndrome (SIRS) of noninfectious origin (19). Control values were obtained from a cohort of 21 healthy individuals (Etablissement Français du Sang, Groupe Hospitalier Pitié-Salpêtrière).

Reagents
The following human recombinant cytokines were used: TNF-α, IL-1β, IFN-γ, IL-6 (all from R&D Systems), IL-8 (PeproTech), and GM-CSF (Miltenyi Biotech). Diphenyleniodonium (DPI) was purchased from Calbiochem, and hydrocortisone, apocynin, and RU486 from Sigma-Aldrich.

Cell isolation and culture
Heparinized blood was collected from healthy individuals and daily from sepsis patients over a 3-d period starting on the first day sepsis was suspected. Neutrophils were isolated by centrifugation on a Ficoll-Hypaque density gradient. Non-monocytic cells were removed by Ficoll-Hypaque density gradient. Monocytes were allowed to adhere by washing. The ratio of monocytes to neutrophils was 1:20. Neutrophils were cultured for 2 h at 37˚C in RPMI 1640, and non-monocytic cells were removed by Ficoll-Hypaque. Monocytes were used to extract RNA, as described below. In a set of experiments, heparinized blood collected from healthy controls (diluted 1:2 in RPMI 1640) was directly stimulated with E. coli (M92%, as assessed by flow cytometry. Freshly isolated neutrophils were resuspended in RPMI 1640 supplemented with 100 U/ml penicillin, 100 g/ml streptomycin (Lonza), and 10% human serum (Bio-Whittaker) to a final concentration of 1 × 10^6 cells per milliliter. For some experiments, cells were used to extract RNA, as described below. In a set of experiments, heparinized blood collected from healthy controls (diluted 1:2 in RPMI 1640) was directly stimulated with Escherichia coli LPS (Sigma-Aldrich), heat-killed E. coli, and heat-killed Staphylococcus aureus (Calbiochem). For other experiments, the blood was centrifuged and the plasma removed and replaced with 100 μl plasma collected from sepsis patients. For cross-linking experiments, neutrophils were cultured in the presence of the isotype control. Nevertheless, the proportion of dead cells was significantly increased by CD24 ligation. Anti-CD24 mAb stimulation occurred with increased redistribution of phosphatidylserine (Annexin V positive) from the inner leaflet of the plasma membrane to the outer leaflet (Fig. 1B), a well-known feature of apoptotic neutrophils that also Ligation of CD24 resulted in the induction of significant neutrophil death in a time-dependent fashion (Fig. 1A). Neutrophils are short-lived and fragile cells in culture, as evidenced by the spontaneous death in the presence of the isotype control. Nevertheless, the proportion of dead cells was significantly increased by CD24 ligation. Anti-CD24 mAb stimulation occurred with increased redistribution of phosphatidylserine (Annexin V positive) from the inner leaflet of the plasma membrane to the outer leaflet (Fig. 1B), a well-known feature of apoptotic neutrophils (22).

RNA extraction
Total RNA was extracted from purified neutrophils in a single-step isolation system (TRizol Reagent; Invitrogen) and quantified using a Thermo Scientific NanoDrop 2000 spectrophotometer. DNA was generated by reverse transcription. Quantitative PCR was performed on a Stratagene MX3005P, using Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies). Primer sequences for CD24 are the following: hCD24 F1: 5'-GAC AGC AGA GTA ATG GTG GC-3'; hCD24 R1: 5'-GAG ACC ACG AGA AGA CTG GC-3'. The PCR consisted of 40 cycles at 94°C for 40 s, 58°C for 30 s, and 72°C for 40 s. Dissociation curve analysis confirmed the specificity of the SYBR Green–amplified product. Transcript levels for the CD24 gene were normalized against those of the housekeeping gene actin. The following primers were used: HsACTB F1: 5'-GGC AGA GCA ATG GTG GC-3; HsACTB R1: 5'-GGC AGA GCA ATG GTG GC-3.

Flow cytometry
Expression of cell surface markers was assessed directly on heparinized whole blood, following erythrocyte lysis by flow cytometry (MACSquant; Miltenyi Biotech). Anti-CD24 (ML5, allophycocyanin conjugated) and anti-CD66 (ASL-32, A488 conjugated) were obtained from BioLegend. Anti-CD16 (3G8, PE conjugated) and anti-CD42b (HIP1, PE conjugated) were purchased from BD Pharmingen. All the mAbs were used according to the manufacturer’s recommendations, and negative controls were isotype-matched mouse mAbs. Neutrophils were gated using side scatter dot plots versus CD66 expression. Data analysis was performed using MACSquant software (MACSquantify; Miltenyi Biotech).

Determination of cell death and apoptosis
Apoptosis was assessed by flow cytometry (MACSquant; Miltenyi Biotech) using Annexin V^FITC according to the manufacturer’s instructions with the addition of propidium iodide (BD Biosciences). Mitochondrial membrane potential was evaluated (ΔΨm) by flow cytometry using the cationic dye JC-1 (BD Biosciences). Caspase-3 and caspase-9 protease activity in protein extracts was measured by cleavage of fluorogenic substrate (respectively, Ac-DEVD-AMC and Ac-LEHD-AMC) (Alexis Biochemicals). Cell lysates were prepared in caspase assay buffer (0.2% [wt/vol] Triton X-100; 50 mM HEPES, pH 8; 20 mM EDTA; 1 mM PMSF; 2 mg/ml apotinin, 10 mg/ml leupeptin). Enzymatic reactions were monitored in a plate fluorometer (Tecan) at 37°C, and the enzymatic activity was assessed after 5 h as maximum fluorescence (in relative arbitrary units) generated by the release of AMC (excitation, 390 nm; emission, 460 nm).

Statistical analysis
Data are presented as means ± SEM. Statistical analyses were performed using the nonparametric Mann–Whitney U test. For multiple comparisons within a data set, one-way ANOVAs with a Tukey posttest were performed. A p < 0.05 was considered significant.

Results
CD24 ligation triggers caspase-dependent apoptosis in neutrophils
Several authors report that CD24 ligation triggers apoptosis in hematopoietic and nonhematopoietic cells (14–16, 21). Thus, we investigated whether cross-linking of CD24 mediated by a specific mAb triggered any physiological response in neutrophils. Ligation of CD24 resulted in the induction of significant neutrophil death in a time-dependent fashion (Fig. 1A). Neutrophils are short-lived and fragile cells in culture, as evidenced by the spontaneous death in the presence of the isotype control. Nevertheless, the proportion of dead cells was significantly increased by CD24 ligation. Anti-CD24 mAb stimulation occurred with increased redistribution of phosphatidylserine (Annexin V positive) from the inner leaflet of the plasma membrane to the outer leaflet (Fig. 1B), a well-known feature of apoptotic neutrophils (22).

We also measured the neutrophil cell surface expression of CD16 (FcyRIII receptor), which is known to be lost from the surface during apoptosis (23). During the culture, a population of neutrophils with markedly reduced expression of CD16 appeared (CD16low neutrophils) and increased significantly compared with the isotype control in a time-dependent manner following CD24 cross-linkage (Fig. 1C). By double staining with Annexin V, the CD16low population was confirmed to be apoptotic neutrophils, as it was also Annexin V positive, as shown in Fig. 1D. Because caspases play a critical role in the execution phase of neutrophil apoptosis (22), we investigated whether caspase activation was required to induce apoptosis via CD24. Lysates from CD24-treated cells and isotype control (IC)–treated cells were assessed for cleavage of caspase-3 and the caspase-9 substrate. As shown in Fig. 1E, ligation of CD24 resulted in increased DEVDDase and LEHDDase activity, indicating that CD24 transduces apoptotic signal through the caspase-3/caspase-9 pathway. To determine whether reactive oxygen species (ROS) were involved in CD24-mediated death, we used a pharmacological approach. In neutrophils, ROS are mostly generated by the mitochondrial flavoprotein inhibitor DPI (24). Of note, we found that the inhibition of basal ROS generation efficiently blocked CD24-mediated cell death (Fig. 1F). CD24-mediated cell death inhibition by NADPH oxidase–derived oxidants was also confirmed using apocynin (25).
FIGURE 1. Cross-linking of CD24 induces apoptosis in human neutrophils. (A) Purified blood neutrophils were cultured in the presence of anti-human CD24 (500 ng/ml) and secondary Abs (500 ng/ml) and then stained with Annexin V and propidium iodide. (B) Percentage of Annexin V–positive neutrophils was quantified. A representative experiment shows the increase of neutrophils undergoing death following CD24 cross-linking after 4 h (upper panels) and 16 h (lower panels), compared with IC-treated cells. (C) Percentages of CD16low and CD16high neutrophils were quantified following CD24 cross-ligation. (D) Representative dot plots of surface expression of Annexin V and CD16 on neutrophils after 4 h (upper panels) and 16 h (lower panels) are shown. (E) Caspase-3 and caspase-9 activities following CD24 ligation were measured by monitoring the arbitrary units of fluorescence released by cleavage of the fluorogenic peptide substrates DEVD-AMC and LEHD-AMC, respectively. (F) Preincubation with NADPH oxidase inhibitor DPI (10 μM) or apocynin (10 μM) efficiently blocked neutrophil death following CD24 ligation after 16 h of incubation. (G) Loss of mitochondrial membrane potential: fresh neutrophils or neutrophils either IC treated or cross-linked with anti-CD24 were stained with JC-1 1% and analyzed by flow cytometry. Results are representative of at least three separate experiments. Mean ± SEM is presented. *p < 0.05, (Mann–Whitney U test).
(Fig. 1F). Cellular death via the apoptotic pathway results from a number of key steps, including dissipation of the mitochondrial membrane potential (ΔΨm) (22). To investigate whether CD24-mediated cell death occurs through depolarization of the mitochondrial inner membrane, we used the mitochondrial potential-sensitive probe JC-1. The uptake of JC-1 into mitochondria is driven by the ΔΨm, and at low membrane potentials, JC-1 exists in the form of a "J-monomer" that produces a green fluorescence; at high membrane potentials, it forms "J-aggregates" with red fluorescence. Fresh neutrophils appeared red as J-aggregates (Fig. 1G, left panel), indicating that mitochondrial membranes retained high voltage. In an isotype control–treated neutrophils, some of the mitochondria had lost their ΔΨm, showing a mixture of red and green mitochondria (Fig. 1G, middle panel). Anti-CD24–treated neutrophils showed more green mitochondria (J-monomers), as seen in Fig. 1G, right panel, indicating that CD24–induced apoptosis is associated with the collapse of mitochondrial membrane potential. Taken together, these findings suggest that the oxygen radical stress pathway contributes to the cell death triggered by CD24 in human neutrophils.

Proinflammatory cytokines and immunosuppressive mediators modulate CD24 expression on neutrophils

CD24 is highly expressed on neutrophils, but little is known about its modulation during infection and inflammation. Therefore, we evaluated the ability of LPS and a large number of cytokines to modulate the expression of this molecule.

First, we examined the effect of LPS on CD24 expression by flow cytometry, both on purified neutrophils and on neutrophils cultured in whole blood from healthy controls. A challenge with 500 ng/ml LPS led to a strong and significant upregulation of CD24 expression at 4 h (Fig. 2A, left panel) and 16 h (Fig. 2A, right panel) for neutrophils in whole-blood assay. In contrast, no effect was observed when purified neutrophils were challenged with LPS. Similar experiments were performed with heat-killed Gram-positive (S. aureus) or Gram-negative (E. coli) bacteria. As observed with LPS, both types of bacteria induced a significant increase in CD24 expression on neutrophils from healthy controls in whole blood, but not on purified neutrophils (Fig. 2A). LPS is known to delay apoptosis in neutrophils, whereas heat-killed bacteria are known to accelerate it (26). Therefore, to avoid bias introduced by the different responsiveness in terms of cellular death, the analysis for this group of experiments was performed on viable cells (27). The early upregulation of CD24 following bacterial exposure in whole blood, but not with isolated neutrophils, suggests that this phenomenon may be dependent on accessory cell–derived cytokines induced by pathogen-associated molecular patterns or whole bacteria. Consequently, we performed monocyte–neutrophil coculture experiments. As shown in Fig. 2A (right panel), the addition of monocytes partially mimics the observation in whole blood, indicating that other accessory cells or plasma factors are involved. Therefore, we also tested the effect of several cytokines produced by accessory cells in response to LPS or heat-killed bacteria. Indeed, TNF-α, IFN-γ, or GM-CSF exposure caused a significant upregulation in CD24 expression (Fig. 2B). Some cytokines such as IL-6, IL-8, and IL-1β did not exert any effect on the expression of CD24. Of interest, although incubation with IL-1β alone did not increase the expression of CD24, coinoculation with lower concentrations of TNF-α or with IFN-γ increased the expression of CD24 to a level similar to that induced by the single cytokine at the highest concentration, showing a significant synergistic effect (Fig. 2B). Moreover, by double labeling the neutrophils, we identified two different populations, CD16high/CD24low and CD16low/CD24high, after 16 h of culture for untreated cells. Interestingly, TNF-α, IFN-γ, or GM-CSF alone or in combination induced the appearance of a third population, CD16high/CD24high, characterized by higher granularity, as detected by side scatter. The appearance of that subset (Fig. 2C, 2D) produced, as a general effect, a significant increase in CD24 expression.

We also were interested to know whether anti-inflammatory cytokines or immunosuppressive mediators such as corticosteroids or PGs were able to affect CD24 expression. We did not observe any effect in terms of alteration of cell surface CD24 on neutrophils following incubation with IL-10, TGF-β, or PGE2 (data not shown). In contrast, a modest downmodulation of CD24 expression was observed on neutrophils exposed to low concentrations of hydrocortisone, which reached statistical significance at highest concentration (Fig. 3A). In addition, the coinoculation with 1 μM RU486, a glucocorticoid receptor antagonist, fully abolished the inhibitory effect of hydrocortisone (Fig. 3A, 3B). By double labeling, we determined that the downregulation of CD24 expression by hydrocortisone was due to the shift of the CD16low/CD24high cells to a CD16high/CD24low population (Fig. 3B). In another set of experiments, we explored whether hydrocortisone might counterregulate the CD24 upregulation induced by proinflammatory cytokines. For that purpose, purified neutrophils were cultured in the presence of GM-CSF (the most potent proinflammatory cytokine for CD24 upregulation) in combination with hydrocortisone or 1 h pretreated or 1 h posttreated with hydrocortisone. Of note, hydrocortisone was able to completely inhibit CD24 upregulation in all the examined experimental conditions and abolished the appearance of the CD16high/CD24high population induced by GM-CSF (Fig. 3A). A representative experiment is shown in Fig. 3B. Moreover, in agreement with the results obtained by flow cytometry, hydrocortisone induces decreased CD24 mRNA expression in neutrophils, as assessed by quantitative RT-PCR (Fig. 3C).

Early and persistent downregulation of CD24 expression on neutrophils from sepsis patients

Because we showed that CD24 expression can be affected by proinflammatory cytokines or immunosuppressive mediators and because delayed neutrophil apoptosis is regularly detected in inflammatory conditions such as sepsis (8–10, 28), we decided to investigate whether CD24 was modulated in neutrophils from sepsis patients. Patients with a confirmed diagnosis of sepsis were enrolled in the study. The demographic and clinical characteristics of the cohort are presented in Table I. We assessed CD24 expression on circulating neutrophils from 27 consecutive sepsis patients, 19 SIRS patients, and 21 healthy controls by flow cytometry. The patients were analyzed upon suspicion of sepsis (day 0) and again after 24 h (day 1) and 48 h (day 2). Neutrophils were gated using side scatter dot plots versus CD66 expression. All neutrophils express CD24. The expression level was quantified as mean fluorescence intensity (MFI). Sepsis patients displayed a decrease in CD24 expression, in comparison with healthy controls (Fig. 4A). This phenomenon appeared to be persistent, because 48 h after the onset of the disease, neutrophils from sepsis patients still showed a significant decreased CD24 expression compared with those from healthy controls. Of interest, CD24 downregulation is a unique feature of sepsis because neutrophils from SIRS patients, compared with those from healthy controls, display no major changes in terms of CD24 expression. In fresh neutrophils from healthy controls, we were able to define two populations by double labeling: CD16high/CD24low (region A1) and CD16low/CD24high (region A2). As shown in Fig. 4B, in sepsis patients, the CD16high/CD24high population was undetectable, leading to a general decreased expression of CD24 and CD16.
FIGURE 2. Proinflammatory cytokines induce CD24 upregulation. (A) Purified blood neutrophils from healthy controls, neutrophils cultured in whole blood (100 μl), or cocultures of neutrophils and monocytes (20:1) were challenged with LPS (500 ng/ml) or heat-killed bacteria (HKEc, heat-killed E. coli; HKSa: heat-killed S. aureus; 10⁸ CFU/ml) for 4 h or 16 h, and CD24 expression was assessed by flow cytometry, gating on CD66-positive cells and excluding apoptotic cells. (B) Purified blood neutrophils from healthy controls were challenged with proinflammatory cytokines, 10 or 100 ng/ml of TNF-α, IL-1β, IL-6, IL-8, GM-CSF, IFN-γ, and a combination of these, and quantification (MFI) of CD24 expression is reported. (C) A representative experiment shows the subpopulations of in vitro cultured neutrophils defined by CD16 and CD24. (D) Percentages of CD16<sup>high</sup>/CD24<sup>high</sup> and CD16<sup>high</sup>/CD24<sup>low</sup> after cytokine stimulation. Results are presented as mean ± SEM and are representative of n = 3–5 separate experiments. *p < 0.05, **p < 0.01 (one-way ANOVAs with a Tukey posttest).
CD24 is known to support a P-selectin–mediated interaction with platelets (29). In sepsis, an increased percentage of circulating neutrophil–platelet aggregates has been reported (30). Therefore, to exclude the possibility that the CD24 decreased expression observed in sepsis patients was a result of Ag masking by platelets, we measured the expression of CD24 both on neutrophil–platelet complexes and on platelet-free neutrophils by flow cytometry. Circulating neutrophil–platelet complexes were detected as neutrophils positive for the platelet marker CD42 (Fig. 4C), which is present on the surface of resting and activated platelets. Even though we did observe an increase in the percentage of the aggregates in sepsis patients (data not shown), in agreement with previous reports (30), no difference was found for the CD24 expression level in the two populations (Fig. 4D). Taken together, these results indicate that if the aggregation of platelets and neutrophils occurs through CD24, binding does not affect the recognition of the binding site by the staining Ab. To determine whether the alteration in CD24 protein expression was due to its transcriptional inhibition, we used quantitative real-time PCR. In agreement with the results obtained by flow cytometry, the patients, in comparison with healthy controls, showed a decrease in CD24 mRNA expression (Fig. 4E).

FIGURE 3. Hydrocortisone counterbalances the proinflammatory cytokine–mediated effects. (A) Purified blood neutrophils from healthy controls were challenged with hydrocortisone (HC) at 20 or 200 μM, ± 1 μM RU486 or ± GM-CSF for 16 h, and CD24 expression was analyzed by flow cytometry. (B) A representative experiment shows that the appearance of the CD16high/CD24high subpopulation by GM-CSF is completely inhibited in the presence of hydrocortisone in all the examined conditions. (C) CD24 mRNA expression was measured by quantitative real-time PCR and normalized against that of actin in purified, not treated, neutrophils. Results are expressed as the n-fold difference of the expression in HC-treated neutrophils compared with that in neutrophils not treated. Relative CD24 expression of those not treated was set as 1. Results are presented as the mean ± SEM and are representative of n = 3–7 separate experiments. *p < 0.05, **p < 0.01 (one-way ANOVAs with a Tukey posttest).
**Table I. Demographic characteristics of patients included in the study**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SIRS, n = 19</th>
<th>Sepsis, n = 27</th>
</tr>
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<tbody>
<tr>
<td>Age (y), mean ± SD</td>
<td>65.7 ± 19.5</td>
<td>63.9 ± 24.8</td>
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<tr>
<td>Male, n (%)</td>
<td>10 (52.6%)</td>
<td>15 (55.6%)</td>
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<tr>
<td>Female, n (%)</td>
<td>9 (47.3%)</td>
<td>12 (44.4%)</td>
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<tr>
<td>BMI, mean ± SD</td>
<td>28.4 ± 5.2</td>
<td>24.8 ± 8.6</td>
</tr>
<tr>
<td>Comorbidity, n (%)</td>
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<td></td>
</tr>
<tr>
<td>Hypertension, isolated</td>
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<td>0 (0.0%)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>0 (0.0%)</td>
<td>3 (11.1%)</td>
</tr>
<tr>
<td>Ischemic cardiomyopathy</td>
<td>3 (15.8%)</td>
<td>5 (18.5%)</td>
</tr>
<tr>
<td>COPD</td>
<td>5 (26.3%)</td>
<td>3 (11.1%)</td>
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<tr>
<td>Chronic renal failure</td>
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<td>1 (3.7%)</td>
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<td>Liver failure</td>
<td>1 (5.3%)</td>
<td>0 (0.0%)</td>
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<tr>
<td>Diabetes</td>
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<td>7 (25.9%)</td>
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<tr>
<td>Ongoing cancer</td>
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<td>6 (22.2%)</td>
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<td>Infection site, n (%)</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Positive blood culture</td>
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<td>1 (3.7%)</td>
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<tr>
<td>Unknown</td>
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<td>0 (0.0%)</td>
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<tr>
<td>Temperature (˚C), mean ± SD</td>
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<tr>
<td>Heart rate (bpm), mean ± SD</td>
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<tr>
<td>Blood pressure (mm Hg), mean ± SD</td>
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<tr>
<td>Respiratory rate (c/min), mean ± SD</td>
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<tr>
<td>Leukocytes (10^3/mm^3), mean ± SD</td>
<td>14 ± 8.1</td>
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<td>SAPS II at ICU admission, mean ± SD</td>
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<td>51.1 ± 16.1</td>
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<td>McCabe 0, n (%)</td>
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<tr>
<td>Length of stay in hospital (d), median ± SD</td>
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<td>46.8 ± 43.5</td>
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<tr>
<td>Length of stay in ICU, median ± SD</td>
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<td>18.5 ± 20.3</td>
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<td>Hospital survival, n (%)</td>
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<td>Microorganism responsible for infection</td>
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<tr>
<td>Gram-positive bacteria</td>
<td>–</td>
<td>10 (37.0%)</td>
</tr>
<tr>
<td>Gram-negative bacteria</td>
<td>–</td>
<td>10 (37.0%)</td>
</tr>
<tr>
<td>Polymicrobial, Gram-positive and Gram-negative bacteria</td>
<td>–</td>
<td>4 (14.8%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>–</td>
<td>3 (11.1%)</td>
</tr>
</tbody>
</table>

BMI, body mass index; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; SAPS, simplified acute physiology score; –, not found.

**CD24 ligation fails to induce death in neutrophils from sepsis patients: ex vivo study suggests a potential role in sepsis**

To assess whether the decreased CD24 expression observed in sepsis patients might have an impact on neutrophil functionality, we investigated the response of sepsis neutrophils to anti-CD24–mediated cell death ex vivo. Neutrophils of patients at the onset of sepsis (day 0) were cross-linked with anti-CD24, and the percentage of apoptotic cells was evaluated. No statistically significant change was noted between the CD24 mAb and the isotype control treatment (Fig. 6A), indicating a complete unresponsiveness after CD24 engagement, which may represent a further mechanism contributing to the elevated accumulation and delayed neutrophil apoptosis recorded in sepsis. The dot plot of a representative patient is shown in Fig. 6B.

Nevertheless, in inflammatory disorders such as sepsis, granulocytes from the marginal and bone marrow pool are supplied to the site of infection, with a significant increase in circulating immature neutrophils (31). To estimate the potential impact of immature granulocytes on the unresponsiveness of the sepsis neutrophils to CD24 ligation, we double labeled the neutrophils with anti-CD16 and anti-CD11b Ab to classify neutrophils as promyelocytes and myelocytes (CD11b^+/CD16^−), metamyelocytes (CD11b^+/CD16^−), or mature neutrophils (CD11b^+CD16^+), and we measured the CD24 and CD66 expression on each population (32). CD24 expression was exclusively observed on CD11b^+ neutrophils, indicating that immature neutrophils gain CD24 expression not before the myelocyte stage but before CD16 expression. More importantly, no difference in terms of CD24 expression was found between metamyelocytes and mature granulocytes (data not shown).

**Discussion**

CD24 is a small mucin-like protein localized at the outer cell surface and expressed on a broad range of hematopoietic cells (12). Data from the current study define the function of CD24 on human neutrophils in both normal and inflammatory or sepsis conditions. In this article, we demonstrate that cross-linking of CD24 with specific mAb induces the apoptosis of human neutrophils. Indeed, under those conditions, neutrophils showed several hallmarks of apoptosis, such as exposure of phosphatidylserine on the cell surface and CD16 shedding. However, following CD24 ligation, phosphatidylserine transition to the cell surface was completely abolished when the cells were pretreated with the flavoprotein inhibitor DPI, indicating that the apoptotic pathway triggered by CD24 was induced by NADPH oxidase–derived ROS. During apoptosis, a cross-talk between ROS and the maintenance of normal mitochondrial membrane potential has been reported (33). Elevated intracellular levels of ROS affect the mitochondrial membrane potential by increasing mitochondrial membrane permeability (33). Indeed, our data showed that mitochondrial membrane integrity is impaired following CD24 ligation. Moreover, following cross-
FIGURE 4. Decreased CD24 expression on neutrophils from sepsis patients. (A) CD24 expression on neutrophils was measured by flow cytometry. Patients were analyzed during the first 48 h following the onset of sepsis (day 0). Quantification of CD24 expression (MFI) from healthy donors \( (n = 21) \), sepsis patients \( (n = 27) \), and SIRS patients \( (n = 19) \). Data are shown as median and interquartile range. *** \( p < 0.001 \) (one-way ANOVAs with a Tukey posttest). (B) Representative dot plot of CD16 and CD24 expression on neutrophils of a healthy individual (left panel) and for a sepsis patient on day 0 (right panel). Gates are determined by isotype-stained controls. (C) Peripheral blood cells were stained with anti-CD66 and anti-CD42. Platelet-free neutrophils were identified as CD66\(^+\)/CD42\(^-\) events indicated as (B1), whereas platelet–neutrophil aggregates were identified as CD66\(^+\)/CD42\(^+\) events indicated as (B2). A representative dot plot from a sepsis patient is shown. (D) Quantification of CD24 expression (MFI) on CD66\(^+\)/CD42\(^-\) and CD66\(^+\)/CD42\(^+\) from 10 sepsis patients. (E) CD24 mRNA expression was measured by quantitative real-time PCR and normalized against that of actin in purified neutrophils from 10 sepsis patients and 5 healthy controls. Results are expressed as the \( n \)-fold difference of the expression in sepsis patients compared with healthy individuals. Relative CD24 expression of healthy donors was set as 1. Mean ± SEM is presented. ** \( p < 0.01 \) (one-way ANOVAs with a Tukey posttest).
ligation, caspase-3–like and caspase-9–like activities were detected, indicating that CD24 triggers cell death via depolarization of the mitochondrial membrane, ROS generation, and activation of pro-caspase-9 in human neutrophils.

Neutrophils are constitutively apoptotic cells. Indeed, within the circulation, the half-life of human neutrophils is 7–10 h (3). In contrast, neutrophils from critically ill patients show profoundly delayed rates of apoptosis (8–10). Mediators such as GM-CSF or pre-B cell enhancing factor, which can be detected in plasma from sepsis patients, have been shown to delay neutrophil apoptosis (34, 35). In such a context, we wanted to address the impact of CD24-mediated killing in a pathologic condition associated with deregulated apoptosis as sepsis. Sepsis is defined as a systemic reaction to an overwhelming infection (36). During sepsis, the immune status of circulating leukocytes undergoes a profound transcriptional reprogramming, an effect that extends to phagocytic cells including neutrophils (37, 38). In this article, we report for the first time, to our knowledge, that in the early stage of the disease, neutrophils from sepsis patients display a persistent decrease of CD24 expression, both at the mRNA and at the protein level.

It is unclear which factors play a role in the regulation of CD24 on neutrophils during inflammatory processes. Increased glucocorticoid levels are regularly detected during sepsis (39, 40). Consistent with our in vivo observation, we showed that hydrocortisone decreases the expression of CD24 on neutrophils of healthy controls. (A) A representative experiment is shown. (B) After incubation with sepsis plasma from five different patients (SP1, SP2, SP3, SP4, SP5) or plasma collected from four healthy controls (HCP), CD24 expression (MFI) on different neutrophil subsets was assessed by flow cytometry. (C) CD24 mRNA expression was measured by quantitative real-time PCR after overnight sepsis plasma treatment, and levels were normalized to that of the housekeeping gene actin. Results are presented as mean ± SEM of three separate experiments. **p < 0.01 (Mann–Whitney U test).

The ex vivo death response after CD24 ligation in neutrophils from sepsis patients. (A) Neutrophils from sepsis patients or healthy controls were cultured in the presence of anti-human CD24 (500 ng/ml) and secondary Ab (500 ng/ml) or a matched isotype control for 16 h and then were stained with Annexin V and propidium iodide. Results are presented as mean ± SEM and are representative of five patients. (B) A representative experiment shows that the cells fail to undergo death after CD24 cross-linking.

FIGURE 5. Plasma from sepsis patients decreases the expression of CD24 on neutrophils of healthy controls. (A) A representative experiment is shown. (B) After incubation with sepsis plasma from five different patients (SP1, SP2, SP3, SP4, SP5) or plasma collected from four healthy controls (HCP), CD24 expression (MFI) on different neutrophil subsets was assessed by flow cytometry. (C) CD24 mRNA expression was measured by quantitative real-time PCR after overnight sepsis plasma treatment, and levels were normalized to that of the housekeeping gene actin. Results are presented as mean ± SEM of three separate experiments. **p < 0.01 (Mann–Whitney U test).

FIGURE 6. The ex vivo death response after CD24 ligation in neutrophils from sepsis patients. (A) Neutrophils from sepsis patients or healthy controls were cultured in the presence of anti-human CD24 (500 ng/ml) and secondary Ab (500 ng/ml) or a matched isotype control for 16 h and then were stained with Annexin V and propidium iodide. Results are presented as mean ± SEM and are representative of five patients. (B) A representative experiment shows that the cells fail to undergo death after CD24 cross-linking.
was able to downregulate CD24 expression at both mRNA and protein levels on neutrophils isolated from healthy controls. An inhibitory effect similar to that of hydrocortisone was also observed with the addition of plasma from sepsis patients. Sepsis plasma contains a plethora of molecules, including cytokines and translocated pathogen-associated molecular patterns, mediators of inflammation with anti- and proinflammatory properties. We showed that LPS or heat-killed bacteria did not exert any effect on the expression of CD24 on purified neutrophils, whereas proinflammatory cytokines such as TNF-α, GM-CSF, and IFN-γ alone or in combination were able to induce CD24 upregulation. Accordingly, Kobayashi et al. (35) also reported GM-CSF as a potent inducer of CD24 upregulation. Because plasma from patients with sepsis contains increased circulating levels of proinflammatory cytokines, such as TNF-α and IL-1β, one could argue that the presence of those mediators should induce upregulation of CD24 instead of causing decreased expression. However, it is accepted that those circulating cytokines are largely inactive owing to an excess of natural inhibitors (sTNFRI and II, IL-1Ra) (41). In fact, the plasma collected from sepsis patients is actually recognized as an immunosuppressive milieu also containing many anti-inflammatory molecules (42). Finally, we showed that hydrocortisone by itself was able to completely dampen the proinflammatory cytokine-mediated effects, indicating that corticosteroids could contribute to sustaining the CD24 downregulation observed in sepsis patients. However, it is unlikely that in vivo, corticosteroids are the only mediator playing a role in the downregulation of CD24 observed in sepsis patients. Additional studies are required to further define the cross-talk between CD24 and immunosuppressive molecules that occurs in sepsis.

We finally investigated CD24-induced mediated death response in neutrophils from sepsis patients ex vivo to verify whether the decreased CD24 expression corresponded to a loss of functional response. Indeed, we report a complete unresponsiveness toward CD24-mediated neutrophil death in sepsis. Neutrophils might downregulate CD24 expression to bypass the signals delivered through it, contributing to the delayed rate of apoptosis observed in these patients.

The refractory response to CD24-induced cell death and the CD24 level of expression of neutrophils represent new features of sepsis-induced reprogramming. Furthermore, because both phenomena are occurring at the early stage of the disease and can be detected by flow cytometry–based methods, they may represent a new way to monitor the immune status of patients during sepsis and to distinguish them from SIRS patients. Our results might contribute to an early and accurate diagnosis of sepsis, improving the management and outcome of the patients.

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Disclosures

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