A high frequency of MDSCs in sepsis patients, with the granulocytic subtype dominating in gram-positive cases

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ABSTRACT
The causative microorganisms dictate the type of MDSC generated in sepsis patients, and a large proportion of PMN-MDSCs in gram-positive sepsis includes immunosuppressive myeloid blasts. MDSCs constitute a heterogeneous population of immature myeloid cells that potently suppress immune responses. They were identified originally in cancer patients and have since been reported to occur also in chronic inflammation, autoimmunity, and even bacterial infections. Human MDSCs are commonly divided into Mo-MDSCs and granulocytic (PMN-MDSCs) subtypes. To what extent the bona fide cancer MDSCs are representative of the proposed MDSCs found in other diseases is not well known. PMN-MDSCs have been found previously to be enriched among LDGs in density gradient-centrifuged blood. In this study, we analyzed potential MDSCs in sepsis patients with different causative microorganisms, using total peripheral blood compared with density gradient-centrifuged blood. We found a high frequency of typical CD14−HLA-DRlow Mo-MDSCs in all sepsis patients, whereas the typical PMN-MDSCs, as well as a prominent CD14low PMN-MDSC-like population, appeared preferentially in gram-positive cases. The CD14low PMN-MDSC variant was demonstrated to suppress T cell proliferation in vitro via a ROS-dependent mechanism, to display an increased IL-10:TNF-α ratio, and to present with signs of immaturity: blast morphology and low cytokine levels. We conclude that a spectrum of cells with MDSC features is enriched in sepsis and that the microbial origin of sepsis contributes to the substantial interindividual patient variation in the MDSC pattern. J. Leukoc. Biol. 96: 685–693; 2014.

Introduction
MDSCs are a heterogeneous population of immature myeloid cells with a potent immune-suppressive capacity. Their phenotype differs vastly between mice and humans, but in common, they are cells containing precursors of granulocytes, macrophages, and dendritic cells. The two main subpopulations of human MDSCs are the Mo-MDSCs (CD14+HLA-DR−/low) and the granulocytic or PMN-MDSCs (Lin−CD15+CD11b+CD33+) [1–4]. MDSCs are believed to originate from precursors in the bone marrow [2, 4], although in vitro reprogramming of splenocytes [5] or monocytes [6] into MDSCs has been observed. MDSCs were described originally in cancer patients and have since been investigated in detail as a result of their potent immune-suppressive capacity. Indeed, MDSCs not only suppress the immune response in cancer patients but also promote angiogenesis, invasion, and metastasis of tumors to distant sites [7]. Also systemic increases of circulating MDSCs in cancer patients have been found, indicating a broader function [4, 8, 9]. Lately, the presence of MDSCs has also been investigated in other conditions, such as inflammation, autoimmunity, trauma, and infections. It is not obvious that MDSCs would have the same function in all of these conditions, and the question of whether MDSCs really play a physiological role during acute bacterial conditions, such as sepsis, has still not been answered [4, 10].

Abbreviations: 7-AAD=7-amino-actinomycin D, APC=allophycocyanin, ARG=arginase, CARIS=compensatory anti-inflammatory response syndrome, CBA=cytometric bead array, CRP=C-reactive protein, ECD=energy-coupled dye, FSC=forward-scatter, HC=healthy control(s), HSV-2=herpes simplex virus type 2, L-NNA=N-nitro-L-arginine, LDG=low-density granulocyte, MDSC=myeloid-derived suppressor cell, MFI=mean fluorescence intensity, Mo=monocytic, n=not, NOHA=nor-nitro-L-arginine, PE-Cy7=polymorphonuclear, QC=quality control, ROS=reactive oxygen species, SIRS=systemic inflammatory response syndrome, SSC=side-scatter, WBC=white blood cell

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In sepsis, an early-appearing SIRS is accompanied by a state of CARS. Although a major function of MDSCs in sepsis and septic shock presumably would be a regulatory immunosuppression (thus, forming a part of CARS), it has also been suggested that MDSCs may exert antimicrobial effects during infections [1, 4]. A dysfunctional immune response contributes significantly to sepsis mortality, and a better understanding of MDSC generation and activation during acute bacterial infections may lead to improved treatment of these patients.

In this study, we show that sepsis patients have increased levels of circulating Mo-MDSCs, whereas PMN-MDSC were found predominantly in the gram-positive sepsis patient group. More importantly, we also observed a population with a surface phenotype similar to PMN-MDSCs but with the addition of a CD14low expression among the granulocytes contaminating the Ficoll-enriched mononuclear fraction of blood from sepsis patients. Already in 2001, Gabrilovich and coworkers [10] found granulocytes in this fraction of blood from cancer patients and defined them as immature myeloid cells with a potent immune-suppressive capacity. These cells have also been termed LDGs [11–13]. The CD14low variant form of PMN-MDSCs, observed by us, was appearing preferentially in gram-positive sepsis patients and could be demonstrated to suppress T cell proliferation via a ROS-dependent mechanism and to display low cytokine levels and a blast morphology. We propose that a spectrum of MDSCs is generated in patients with sepsis and that the causative microorganisms influence the types of MDSCs generated.

MATERIALS AND METHODS

Subjects

Patients over the age of 18 years admitted to the Skane University Hospital (Malmo, Sweden), were included in the study after informed, written consent. Patients with gram-negative, gram-positive sepsis and septic shock and patients with different viral infections were included. A control group consisting of healthy individuals was also recruited as part of the study. The samples from the admitted patients were obtained within 4 days of admission to the hospital; the majority within 2 days. Their medical history was then <1 week. The final diagnosis was based on a combination of clinical symptoms and conventional testing using Swedish national QC-approved culture, PCR, and serology.

Sepsis was defined using a consensus panel definition: presence of a suspected or microbiologically proven infection together with SIRS. The diagnosis of SIRS required at least two of the following: hypothermia (≤36°C) or hyperthermia (≥38°C), tachycardia (≥90/min), tachypnea (≥20 breaths/min) and/or arterial PCO2 (32 mmHg or lower) and/or mechanical ventilation, and leukocytosis (≥12 000/μL) or leukopenia (≤4000/μL) and/or a left-shifted WBC differential count of 10% or higher. Sepsis shock was defined as sepsis-induced hypotension persisting, despite adequate fluid replacement [14].

The following patients, with their indicated microbial agents obtained by culture or by PCR (using Swedish national QC-approved methods), were included: septic shock (n=12; four women and eight men), with blood culture isolate of Escherichia coli (five patients), Klebsiella oxytoca (one patient), Staphylococcus aureus (three patients), and Streptococcus pyogenes (one patient), and in two patients, no microbiological agent was isolated from blood but with suspected gram-negative (one patient) and gram-positive (one patient) etiology (these two patients had clear symptoms from the lungs or the urinary tract, and they responded quickly to gram-negative or -positive antibiotic treatment); gram-positive sepsis (n=19; six women and 13 men), with blood culture isolate of S. aureus (four patients), Streptococcus pneumoniae (nine patients), anaerobic cocci (one patient), Enterococcus faecalis (one patient), and Group B Streptococcus in a wound infection (one patient) and three patients with a probable pneumococcal etiology; gram-negative sepsis (n=25; 15 women and 10 men), with blood culture isolate of E. coli (seven patients), Klebsiella pneumoniae (one patient), Citrobacter koseri (one patient), Pseudomonas aeruginosa (one patient), Serratia marcescens (one patient), and Proteus mirabilis (one patient) and another 11 patients with a significant quantity of E. coli in urine. In two patients, no microbiological agent was isolated, because the patient was already on antibiotics when the cultures were taken, but the patients had clear symptoms from the urinary tract and also responded quickly to gram-negative antibiotic treatment. The virois cases (n=11; eight women and three men) were one influenza A, four influenza B, two influenza H1N1 (one coinfected with influenza A), one hepatitis A, one acute hepatitis B, and two HSV-2 patients. HC were also included (n=18; 15 women and five men).

Ethical approval

An ethical permit was obtained from the local Ethical Committee at Lund University (Dnr 2015/358, Dnr 288/2007, Dnr 2010/477, and Dnr 2010/135), and a written, informed consent was given from the participating patients.

Blood samples

Blood from sepsis patients, breast cancer patients, and healthy blood donors were drawn in EDTA tubes and analyzed immediately. A lysis-wash protocol or the automated Beckman Coulter TQ-Prep machine was used for non-Ficoll-enriched blood samples. In-house solutions for lysis and fixation were used. For Ficoll-enriched blood samples, blood was diluted immediately 1:2 in PBS (EDTA/sucrose) and overlaid on Ficoll-Paque PLUS. Leukocyte concentration was determined using an LH750 machine (Beckman Coulter, Hialeah, FL, USA) and plasma levels of CRP with an automated immune turbidimetric assay system (IMMAGE immunochemistry system, Beckman Coulter, Bromma, Sweden) with a minimum detectable concentration of 0.2 mg/L, both using Swedish national QC-approved clinical diagnostic methodology.

Flow cytometry

Acquisition and analysis of non-Ficoll-enriched cells were made using CXP software (Beckman Coulter, Hialeah, FL, USA) as part of another study. The stainings were performed in a routine flow cytometry laboratory at Skane University Hospital, with internal controls for isotype or staining variations. Whole blood (50 μL) in each of the four tubes was incubated at room temperature with antibodies conjugated with the fluorochromes FITC, PE, PE-Texas Red, APC, or Alexa-Fluor 647 and PE-Cy7 to permit up to five-color analysis. All of the following antibodies used in flow cytometry, i.e., negative control IgG1-PE or -FITC (clone 679.1Mc7), CD45-ECD (clone J33), CD8-ECD (clone SFC127Thy2D3), CD14-PE-Cy7 (clone RM052), and CD11c-PE (clone B1U5), were obtained from Beckman Coulter (Beckman Coulter, Hialeah, FL, USA), except CD3-APC, PE, and -ECD or PE-Cy5 (clone UCHT1) from DakoCytomation (Carpinteria, CA, USA); HLA-DR-FITC (clone L243) from Becton Dickinson (San Diego, CA, USA); and CD40-PE (clone MAB89) and CD64-FITC (clone 22) from Immunotech Beckman Coulter (Cedex, France). The combination of antibodies used was as follows for each tube: negative control-FITC/negative control-PE/CD45-PE/Texas Red/CD3-APC; HLA-DR-FITC/CD3-PE/CD8-PE-Texas Red/cholera toxin B-Alexa Fluor 647/CD14-PE-Cy7; HLA-DR-FITC/CD40-PE/CD3-PE-Texas Red/cholera toxin B-Alexa Fluor 647/CD14-PE-Cy7; and CD64-FITC/CD11c-PE/CD45-PE-Texas Red/cholera toxin B-Alexa Fluor 647/CD14-PE-Cy7. The results for cholera toxin B and CD40 were scored as part of a separate study. A lysis-wash protocol or the automated Beckman Coulter TQ-Prep machine was used. In-house solutions for lysis and fixation were used. At least 1000 events were analyzed in an FC500 Beckman Coulter flow cytometer using both lasers. Acquisition and analysis were made using CXP software (Beckman Coulter, Hialeah, FL, USA).
The Ficoll-enriched LDGs were analyzed using a FACScalibur (BD Biosciences, San Jose, CA, USA). PBMCs were collected, washed once in PBS and once in FACS buffer, and then stained with the indicated antibodies and analyzed by flow cytometry, as described below. All of the antibodies used in flow cytometry, i.e., CD14 PerCP (clone 61D3), CD33-FITC (clone HIM3-4), CD33-PE and FITC (clone M5E2), CD64-PE (clone 10.1), HLA-DR-PE and APC (clone WM53), CD15-PE (clone HI98), CD11b-APC (clone ICRF44), CD11c (HLA-DR) or PMN-MDSCs (CD14low/H11022), were obtained from Becton Dickinson. Analyses were performed using 7-AAD (eBioscience, San Diego, CA, USA) as a dead cell marker. CD14low PMN-MDSCs [CD33+/H11002] were characterized by CD14low/H11002 PMN-MDSCs (CD14low PMN-MDSCs [CD33+/H11002]) 

**RESULTS**

A total of 56 sepsis patients were included: gram-negative sepsis (n=25), gram-positive sepsis (n=19), and septic shock (n=12). Patients with different viral infections (n=11) and a control group consisting of healthy individuals (n=18) were also included (Table 1).

**Sepsis patients have increased proportions of Mo-MDSCs**

Human MDSCs can generally be divided into Mo-MDSCs (CD14+HLA-DR−/low) or PMN-MDSCs (Lin−CD15+/lowCD11b+CD33+) [1-4]. We first set out to analyze the proportions of typical Mo-MDSCs (CD14+HLA-DR−/low) in Ficoll-enriched blood from sepsis patients with different causative origin. HLA-DR is often used as a marker of immunosuppression, and MDSCs should express absent/low levels of this activation marker. The HLA-DR expression levels (MF1) on CD14+CD64ılan Mo cells (Fig. 1A) showed a significant decrease in patients with gram-negative sepsis particularly. Also, patients with gram-positive sepsis had decreased levels of monocyte HLA-DR expression (Fig. 1A). In line with this, we could also see that in patients with gram-negative sepsis, a significantly increased proportion of typical Mo-MDSCs (CD14+HLA-DR−/low; for gating, see Fig. 1B) in relation to mononuclear cells (percent of total; Fig. 1C,

**Statistical analysis**

Mean and median were calculated. Kruskal-Wallis multiple comparison test was used for the nonparametric data unless stated otherwise, and P<0.05 was considered significant. GraphPad Prism 6 and SPSS were used as software. The HC data were considered to show normal distribution.

**Cytokine analysis**

Sorted LDG CD3+CD14+/CD64+ or CD3+CD14+CD64+ was cultured ex vivo in Opti-MEM for 24 h, after which, the supernatants were harvested. For Becton Dickinson CBA, the Human Inflammatory Cytokines Kit was used. TGF-β ELISA was from R&D Systems (Minneapolis, MN, USA).
Mo-MDSCs particularly increase in gram-negative sepsis patients. 

(A) MFI of HLA-DR expression levels on the CD14^low^CD64^low^ population in Ficoll-enriched blood samples from sepsis patients with gram-positive or -negative sepsis compared with HC indicates that cells from gram-positive and -negative patients have decreased HLA-DR levels. All cells were defined as viable by the 7-AAD dead cell discriminator: gram-positive sepsis (n=9), gram-negative sepsis (n=13), HC (n=5); *P < 0.05; **P < 0.01, Kruskal-Wallis multiple comparison test. Error bars represent SEM. (B) Mo-MDSCs were analyzed in Ficoll-enriched blood samples from patients with gram-positive or -negative sepsis compared with HC, as indicated by Gate A and the dashed line in the right dot plots (CD14^+^CD33^+^HLA-DR^+^). The dot plot represents a gram-positive case. (C) The Mo-MDSC population was increased significantly in the gram-negative sepsis patients sample (percent of total; left), but when compared with gated monocytes (percent of CD14^+^ gated cells), gram-positive and -negative sepsis patients showed increased levels of Mo-MDSCs (right): gram-positive sepsis (n=9), gram-negative sepsis (n=13), HC (n=5); *P < 0.05; **P < 0.01; ***P < 0.001, Kruskal-Wallis multiple comparison test. Error bars represent SEM.

A fraction of LDGs increases in patients with sepsis. 

In cancer patients, PMN-MDSCs have been recovered from the mononuclear and the granulocyte fraction of density gradients [3]. During the work of two ongoing studies, where we analyzed immune cell populations in sepsis and breast cancer patients (refs. [17, 18] and unpublished work), we observed a prominent granulocytic cell population in our Ficoll-enriched mononuclear fractions compared with HC, where this population was absent (Fig. 2A). Initially, we therefore analyzed the presence of typical PMN-MDSCs (CD14^+^HLA-DR^-^CD15^+^/CD64^-^-CD11b^-^-CD33^-^-) in the same samples of Ficoll-enriched blood from sepsis patients with different causative origin as described above. Based on a high FSC/SSC profile (Fig. 2A, circle) and a typical PMN-MDSC (CD14^+^HLA-DR^-^CD15^+^/CD11b^-^-CD33^-^-) phenotype (gates presented in Supplemental Fig. 1B), we found that PMN-MDSCs were increased in gram-positive and -negative sepsis patients but only significantly increased in gram-positive sepsis compared with HC (Fig. 2B). The increases of the respective cell populations were not a result of relative increases of monocytes (for Mo-MDSCs) or granulocytes (for PMN-MDSCs) in the sample preparations (Supplemental Fig. 1A).

When we analyzed the LDG population, also formerly called LDGs [11–13] in more detail, however, we could show that a large proportion of the LDGs (Fig. 2A, circle) had a CD33^-^-CD14^low^-^-CD64^low^-^- phenotype (Fig. 2C, Gate B) and were not CD14^-^- as the previously proposed PMN-MDSCs. It has been suggested previously that CD64 can be used as a marker for activated granulocytes in neonatal sepsis [19], and therefore, we suspected the population to be granulocytes. The CD14^low^- LDG population increased significantly in sepsis patients of gram-positive and -negative origin compared with HC (Fig. 2D). We decided to investigate the CD14^low^- LDG surface phenotypes in more detail and found them to be CD16^-^- and CD11c^-^-expressing cells (Supplemental Fig. 1C). By first gating on CD33^-^-CD15^-^- cells in the total CD14^-^-/Ficoll-enriched fraction (Supplemental Fig. 1D, Gate D), we could verify that these cells expressed CD11b and also were found in the LDG FSC/SSC area (Supplemental Fig. 1D, right).

To evaluate if the CD14^low^- LDGs could be CD14^low^- PMN-MDSCs recovered from the mononuclear layer with a slightly elevated CD14 expression (CD14^low^-CD15^-^-CD11b^-^-CD33^-^- in the LDG region) or simply immature promonocytes, we next investigated their presence and function in blood from sepsis patients. With the use of the same antibodies as in the PMN-MDSCs (CD14^-^-CD11b^-^-CD33^-^-), we could verify that these cells expressed CD11b and also were found in the LDG FSC/SSC area (Supplemental Fig. 1D, right).

Increased proportion of Mo-MDSCs in gram-negative sepsis patients could not only be caused by these relative increases in the monocyte population in the prepared samples (Fig. 1C, right, and Supplemental Fig. 1A).
PMN-MDSCs out of gated CD11b+ cells are shown in Supplemental Fig. 2A and B.

**Functional characteristics of CD14<sub>low</sub> LDGs**

To evaluate whether the CD14<sub>low</sub> PMN-MDSCs in sepsis patients were similar to PMN-MDSCs functionally, we next aimed at investigating a potential immune-suppressive function of the CD14<sub>low</sub> PMN-MDSCs. We therefore sorted out the CD14<sub>low</sub>CD64<sub>low</sub>CD33<sup>+</sup> [confirmed to be LDGs only (Supplemental Fig. 2C and D, purple cells)] and CD15<sup>+</sup> (Supplemental Fig. 1C) LDGs from patients with gram-positive or -negative sepsis using a FACSAria cell sorter and performed in vitro assays measuring the capacity to suppress T cell activation (Fig. 3A) and measuring the cytokines produced of the isolated CD14<sub>low</sub> PMN-MDSCs in vitro (Fig. 3B and C). The isolated CD14<sub>low</sub> PMN-MDSCs from gram-positive (red) and -negative (light blue) sepsis patients suppressed T cell activation, as measured by 3H-thymidine incorporation of cocultures of anti-CD3/CD28-stimulated T cells (Fig. 3A). However, the CD14<sub>low</sub> PMN-MDSCs from gram-positive sepsis patients (red) were significantly more potent suppressor cells compared with CD14<sub>low</sub> PMN-MDSCs from gram-negative (light blue) sepsis patients (Fig. 3A). Also, the heterogeneous, Mo CD14<sup>+</sup>CD64<sup>+</sup>CD33<sup>+</sup> population (consisting of HLA-DR<sup>+</sup> and HLA-DR<sup>+</sup>/low monocytes), isolated from gram-positive (orange) and -negative (dark blue) sepsis patients, suppressed T cell proliferation slightly (Fig. 3A). The MDSC-induced suppression of T cell proliferation can be caused by the release of suppressive cytokines or by other factors (e.g., ARG1, iNOS, and ROS) [2]. Mo-MDSCs typically suppress T cell proliferation via inhibitory cytokines (IL-10 and TGF-β) or by production of iNOS and ARG1, whereas PMN-MDSCs preferentially use ROS as suppressor mechanisms [2]. To investigate the functional mechanism behind the CD14<sub>low</sub> PMN-MDSCs found in sepsis patients, we next performed MLRs with different chemical inhibitors added (Fig. 3B). Neither ARG1 nor iNOS inhibitors could relieve the T cell suppression induced in the MLRs from gram-positive sepsis patients; however, catalase, a ROS inhibitor, did (Fig. 3B, red bars). Interestingly, the ROS inhibitor did not affect the MLRs with sorted CD14<sub>low</sub> PMN-MDSCs.
from gram-negative sepsis patients (Fig. 3B, blue bars). We then analyzed the cytokine profiles of the CD14low PMN-MDSCs. The sorted CD14low PMN-MDSCs produced low amounts of cytokines, including the immunosuppressive IL-10 and TGF-β (Fig. 3C-E and Supplemental Fig. 3A-C). This could be a result of exhaustion mechanisms [20] or more likely, reflecting the PMN-like cell character. This is supported by the fact that the most abundant cytokine produced was IL-8 (Fig. 3C). IL-6 was slightly more produced by CD14low PMN-MDSCs from gram-positive patients, although not significantly (Fig. 3C).

Interestingly, the IL-10:TNF-α ratio was increased in the sepsis CD14low PMN-MDSCs prepared from gram-positive (red) and -negative (light blue) sepsis patients, compared with monocytes from the same patients (Fig. 3D). However, the CD14low PMN-MDSCs produced less of the immunosuppressive cytokine TGF-β (Fig. 3E). Importantly, the IL-10:TNF-α ratio and the TGF-β levels were similar to sorted PMN-MDSCs from gram-positive sepsis patients (Fig. 3D and E, black bars). In summary,
The isolated CD14low LDGs in gram-positive sepsis patients are a heterogeneous population

In cancer patients, the typical morphology of PMN-MDSCs is believed to be the classic ring-shaped nuclei MDSCs also seen in mice. The more immature PMN-MDSCs are more blast-like and tend to increase over time in inflammatory disorders [1]. To analyze the morphology of CD14low PMN-MDSCs from sepsis patients with different causative microorganisms and severity, we prepared cytopsins of the isolated population from patients with gram-negative or -positive sepsis, breast cancer (Fig. 4, left), and septic shock (Supplemental Fig. 3D, upper). The corresponding CD33+CD14CD64monocyte population from each patient is shown as control (Fig. 4, right). The blood was drawn at <2 days from onset of sepsis and ≤1 day at onset of septic shock. It was clear that in patients with gram-positive sepsis, the CD14low PMN-MDSCs were very heterogeneous with few ring-shaped MDSCs but with large numbers of blasts (monoblasts, promonocytes, or immature neutrophils; Table 2). Also, CD14low PMN-MDSCs from gram-positive shock patients had immature blasts (Supplemental Fig. 3D, upper). In contrast, the majority of cells in the CD14low PMN-MDSC population from the gram-negative sepsis patients was morphologically similar to mature PMN cells with some typical ring-shaped CD14low monocytes present (Fig. 4 and Table 2). As a control, PMN-MDSCs were sorted from sepsis patients (Supplemental Fig. 3D, lower). Also, this population presented with a heterogeneous morphology of PMN cells and blasts (Supplemental Fig. 3D, lower). As a typical MDSC control, the same cells were sorted from a patient with newly diagnosed, recurrent breast cancer (Fig. 4 and Table 2). It is difficult to discriminate among monoblasts, promonocytes, or immature neutrophils based on surface phenotype or morphology; hence, “immature myeloid cell” is probably still the best terminology. In the clinic, the term “left shift” of the bone marrow is commonly used for the presence of blasts in the blood. This phenotype has recently been suggested to reflect an emergency myelopoiesis that is induced upon an acute systemic infection [1].

Granulocyte CD11c bright expression in gram-negative sepsis patients

We finally investigated whether the CD14low PMN-MDSCs could be visualized in non-Ficoll-enriched peripheral blood that commonly is used for biomarkers in the clinics. To this end, we analyzed the presence of CD14lowCD64low cells in the total granulocyte gate (CD45/FSC) of non-Ficoll-enriched peripheral blood cells in a larger cohort of patients with septic shock, gram-negative sepsis, gram-positive sepsis, and severe

![Figure 4. The morphology of sorted CD14low PMN-MDSCs varies with causative microorganisms. Cytopsins of sorted CD14low PMN-MDSCs (left) or CD14CD64 monocytes (right; 99% purity) were analyzed using H&E staining. Gram-positive patients (n=5) had an increased amount of blasts (green arrows), and typical ring-shaped MDSCs were also present (red arrow). Gram-negative sepsis patients (n=3) had the ring-shaped MDSCs (red arrow) but did not have blasts. The same population from a breast cancer patient is shown as a control (Fig. 4, right). The blood was drawn at <2 days from onset of sepsis and ≤1 day at onset of septic shock. It was clear that in patients with gram-positive sepsis, the CD14low PMN-MDSCs were very heterogeneous with few ring-shaped MDSCs but with large numbers of blasts (monoblasts, promonocytes, or immature neutrophils; Table 2). Also, CD14low PMN-MDSCs from gram-positive shock patients had immature blasts (Supplemental Fig. 3D, upper). In contrast, the majority of cells in the CD14low PMN-MDSC population from the gram-negative sepsis patients was morphologically similar to mature PMN cells with some typical ring-shaped CD14low monocytes present (Fig. 4 and Table 2). As a control, PMN-MDSCs were sorted from sepsis patients (Supplemental Fig. 3D, lower). Also, this population presented with a heterogeneous morphology of PMN cells and blasts (Supplemental Fig. 3D, lower). As a typical MDSC control, the same cells were sorted from a patient with newly diagnosed, recurrent breast cancer (Fig. 4 and Table 2). It is difficult to discriminate among monoblasts, promonocytes, or immature neutrophils based on surface phenotype or morphology; hence, “immature myeloid cell” is probably still the best terminology. In the clinic, the term “left shift” of the bone marrow is commonly used for the presence of blasts in the blood. This phenotype has recently been suggested to reflect an emergency myelopoiesis that is induced upon an acute systemic infection [1].

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| TABLE 2. Proportions of Cells Presented in Fig. 4 and Supplemental Fig. 3D with Different Morphological Features (PMN, Blast, Ring-Shaped) in Sorted CD14low PMN-MDSCs (Proportion, %±sd)/Field and Sample |
|-----------------|--------------|--------------|--------------|-----------------|-----------------|
|                 | HC           | Gram+ sepsis | Gram− sepsis | Gram+ shock     | Breast cancer   |
| PMN (%)         | 96 ± 1       | 81 ± 9       | 94 ± 1       | 92 ± 3          | 92 ± 1          |
| Blast (%)       | 3 ± 1        | 16 ± 7       | 2 ± 1        | 10 ± 3          | 5 ± 1           |
| Ring-shaped (%) | 0.7 ± 0.2    | 2.5 ± 1.8    | 4.5 ± 1.3    | 0.4 ± 0.6       | 2.3 ± 0.6       |

*P < 0.001 in PMN and Ring-shaped groups; P < 0.01 in Blast group, using Kruskal-Wallis tests.
virosis. As shown in Fig. 5A, the population of CD14	extsuperscript{low}CD64	extsuperscript{low} cells within the total granulocyte gate of non-Ficoll-enriched peripheral blood cells was increased significantly only in the gram-positive sepsis group.

Recently, a novel subset of CD11c	extsuperscript{bright}-suppressive neutrophils was discovered in human systemic inflammation [21]. They were induced upon endotoxin challenge and also expressed high levels of CD11b and CD16. Interestingly, when we analyzed the level of CD11c expression in our patient cohort, we found that the CD11c expression was only increased significantly in the gram-negative patient group (Fig. 5B, left). This might reflect the data from Pillay et al. [21], where only endotoxin challenge with LPS (gram-negative) was given to the subjects.

The neutrophil/granulocyte expression of CD64 on cells in the granulocyte gate (CD45/FSC) of non-Ficoll-enriched peripheral blood was higher in the three sepsis groups compared with the HC ($P<0.001$; Fig. 5B, middle). The CD64 expression was higher in the patient with septic shock than in gram-positive sepsis patients ($P<0.05$) but lower compared with the gram-negative sepsis patients ($P<0.05$). High levels of CD64 expression were also seen in the DNA and the RNA viral infections compared with HC ($P<0.001$). CD64 was positive in a few viral infections (hepatitis A and B and HSV-2) when they, at the same time, were negative for CRP.

The levels of CD14 in the granulocyte gate (CD45/FSC) of non-Ficoll-enriched peripheral blood did not differ significantly among the patient groups (Fig. 5B, right).

**DISCUSSION**

MDSCs were identified originally as a population of immature myeloid cells enriched in cancer patients and have since been investigated in detail as a result of their potent immune-suppressive capacity. Human MDSCs can generally be divided into Mo-MDSCs (CD14	extsuperscript{+}HLA-DR	extsuperscript{+}/low) or PMN-MDSCs (Lin	extsuperscript{−}CD15	extsuperscript{+}/lowCD11b	extsuperscript{+}CD33	extsuperscript{−}) [1–4]. In cancer patients, MDSCs are a problematic part of the pathological response, where they induce immune suppression and promote angiogenesis, invasion, and metastatic spread [7]. Lately, the presence of MDSCs has also been investigated in inflammation, autoimmunity, trauma, and infections. However, the question of whether these cells are similar to cancer MDSCs or really do exist as a physiological response to acute bacterial conditions, such as sepsis, is still not answered [4, 10].

In this study, we show that a CD14	extsuperscript{low} LDG population is increased, primarily in patients with gram-positive sepsis. Except for the low expression of CD14, the population has a surface and functional phenotype that is similar to that of PMN-MDSCs (i.e., CD14	extsuperscript{−} PMN-MDSCs). A similar population of low-density CD14	extsuperscript{low} immature neutrophils has also been described recently by Drifte et al. [22]. It has been shown previously that different antibody clones directed toward CD14 might give rise to different patterns or detection levels or might even cross-react with unknown epitopes [23]. This suggests that exclusion of CD14	extsuperscript{low} cells upon analyses of PMN-MDSCs prompts a risk of underestimating cells with immune-suppressive properties in patients with severe infectious diseases. It is likewise an important finding that certain MDSCs are more easily detected using density gradient centrifugation and not whole blood as the routines are in the clinics. We cannot assert that low-density PMN-MDSCs (including CD14	extsuperscript{low} PMN-MDSCs) are the only functionally relevant PMN-MDSCs, but the presence of a potential high-density PMN-MDSC population is difficult to detect using whole-blood samples from sepsis patients. The CD14	extsuperscript{low} PMN-MDSCs potentially suppress T cell proliferation via a ROS-dependent mechanism and primarily produce IL-8, thus strengthening the PMN-MDSC-like phenotype. Despite the seemingly homogenous surface phenotype of CD14	extsuperscript{low} PMN-MDSCs isolated from gram-negative and -positive sepsis patients, they differ in morphology and function depending on the causative microorganism. One obvious difference between gram-positive and-negative bacteria is their respective TLR ligands (TLR2 and TLR4) that are proposed to
be important for MDSC generation [24]. The notion that different types of bacteria would give rise to or activate distinct MDSC populations is fascinating and would indicate that indeed, MDSCs are part of a functional immune response also in patients with sepsis. In addition, as CD14<sup>+</sup> PMN-MDSCs in gram-positive sepsis patients show an immature blast-like character, while suppressing T cell functions more efficiently than CD14<sup>+</sup> PMN-MDSCs from gram-negative sepsis patients, it is tempting to speculate that the immature cells that are being released from the bone marrow in gram-positive sepsis are not only immature but have a distinct function. This highlights the bone marrow as an effector organ and could be interpreted as gram-positive but not gram-negative sepsis induces the export of immature myeloid cells that are functionally and phenotypically related to PMN-MDSCs. Indeed, in the clinics, it is the gram-positive sepsis group that has the worst prognosis, and more investigation on how to improve the treatment of these patients is needed [25]. We propose that a spectrum of cells with MDSC features is generated during sepsis and that the microbial origin of sepsis contributes to the substantial interindividual patient variation in the MDSC pattern.

AUTHORSHIP

H.J. and M.W. were responsible for the sepsis patient contact and clinical parameters. H.J. performed all experiments with help from C.B. R.A. helped to prepare cells for the MLR and performed some experiments. A.M.L. and L.R. were responsible for the routine flow cytometry and some clinical parameters. K.L. was responsible for the experimental design, writing the manuscript, and interpreting data. H.J., C.B., and A.B. corrected the manuscript and helped interpret data.

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DISCLOSURES

The authors declare no conflict of interest.

REFERENCES


KEY WORDS:

MDSC • cancer • granulocyte • CD14 • CD16c • Ficolll
A high frequency of MDSCs in sepsis patients, with the granulocytic subtype dominating in gram-positive cases

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