Flow cytometric analysis of monocytes polarization and reprogramming from inflammatory to immunosuppressive phase during sepsis

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ABSTRACT

Sepsis outcome is determined by a balance between inflammation and immune suppression. We aimed to evaluate monocytes polarization and reprogramming during these processes.

We analyzed 93 patients with procalcitonin level >0.5 ng/mL (hPCT) and suspected/confirmed sepsis, and 84 controls by analysis of CD14, CD16 and HLA-DR expression on blood monocytes using fluorescent labeled monoclonal antibodies and BD FACS CANTO II. Complete blood cell count, procalcitonin and other biochemical markers were evaluated.

Intermediate monocytes $CD14^{++}CD16^{+}$ increased in hPCT patients (including both positive and negative culture) compared to controls (13.6% ± 0.8 vs 6.2% ± 0.3, p<0.001), while classical monocytes $CD14^{++}CD16^{-}$ were significantly reduced (72.5% ± 1.6 vs 82.6% ± 0.7, p<0.001). Among hPCT patients having positive microbial culture, the percentage of intermediate

monocytes was significantly higher in septic compared with non-septic/localized-infection patients (17.4% vs 11.5%; p<0.05) whilst the percentage of classical monocytes was lower (68.0% vs 74.5%). Three-four days following the diagnosis of sepsis, HLA-DR expression on monocyte (mHLA-DR) was lower (94.3%) compared to controls (99.4%) (p<0.05). Septic patients with the worst clinical conditions showed higher incidence of secondary infections, long-time hospitalization and lower HLA-DR$^+$ monocytes compared to septic patients with better clinical outcome (88.4% vs 98.6%, p=0.05).

The dynamic nature of sepsis correlates with monocytes functional polarization and reprogramming from a pro-inflammatory CD14^{++}CD16$^+$ phenotype in non-septic hPCT patients to a decrease of HLA-DR surface expression in hPCT patients with confirmed sepsis, making HLA-DR reduction a marker of immune-paralysis and sepsis outcome.

Analysis of monocytes plasticity opens to new mechanisms responsible for pro/anti-inflammatory responses during sepsis, and new immunotherapies.

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INTRODUCTION

A major defense mechanism against infection and tissue injury is provided by the innate immune system through inflammation (1). Circulating monocytes are critical effectors in the immune response, cross-link innate and adaptive immunity, and are involved in pathogenesis of several inflammatory diseases. Based on the differential expression of LPS receptor, CD14, and FcγIIIR, CD16, three subpopulations are defined: classical monocytes as CD14^{++}CD16$^-$, intermediate monocytes as CD14^{++}CD16$^+$ and non-classical monocytes as CD14$^+$CD16^{++} (2). These subsets are associated with specific functions.

Classical monocytes show antimicrobial potential, while CD16 positive monocytes are involved in antigen processing and presentation as well as trans-endothelial migration (3). The expansion of the CD16 positive monocytes has been observed in many different types of disorders, especially infection or inflammatory conditions (4).

The host immune response during sepsis is complex and variable over time. The first phase is characterized by an overwhelming inflammation also known as the "cytokine storm", during which blood monocytes release high levels of pro-inflammatory cytokines (e.g. TNF-α and IL-1) (5). This phase is followed by a stage of immune-suppression in which patients are characterized by persistent inflammation, neutrophilia, lymphopenia and they are more susceptible to secondary infections due to a dysregulation of innate and adaptive immunity (6). In this stage of immune-paralysis, monocytes are functionally deactivated and show decreased release of pro-inflammatory cytokines and decreased antigen presenting capacity because of low HLA-DR surface expression (7).

Human leukocyte antigen DR (HLA-DR) is a cell surface receptor involved in antigen presentation to the adaptive immune system for the purpose of stimulating T cell responses. Many clinical studies have associated the low levels of HLA-DR expression on circulating monocytes with the increased risk of adverse outcome or death of septic patients and increased susceptibility to contracting secondary/nosocomial infections (8-10). For this reason, among all biological parameters, the evaluation of HLA-DR levels on monocytes surface represents the most studied marker to establish their anergy and the general immuno-paralysis state of patients (11-12).

Changes in circulatory cytokines and surface markers can help understanding the mechanisms of response to infection and finding new diagnostic tools. Characterization of cellular component

of the immune system by flow cytometry is a useful tool to evaluate and phenotype immune response during sepsis (10, 11).

In the present study, we investigated the phenotypic and functional changes in different monocyte subsets from inflammatory state to septic state by flow cytometry analysis in order to evaluate monocytes polarization and reprogramming during these processes.

MATERIALS AND METHODS

Patients

The present study has been conducted on 93 patients hospitalized in different medical and surgical wards of the Vito Fazzi Hospital of Lecce (Italy) with high procalcitonin plasmatic levels (PCT > 0.5 ng/mL) and suspected bacterial infection, based on several clinical suspicions. 84 not hospitalized healthy individuals were used as controls.

For each patient, clinical and biological variables were collected. These included demographic characteristics (age and gender), microbiological findings (infection source and the identified microorganisms) and routine markers of inflammation as PCT, C-reactive protein (CRP), complete blood cell count and biochemical markers.

Sepsis diagnosis has been established according to Society of Critical Care Medicine and the European Society of Intensive Care Medicine (13). Written informed consent was obtained from the patients or, if not possible, from their relative as designed by ethic committee.

Hematochemical investigation

Complete blood count was performed on a Sysmex XE-2100 Automatic Hematology Analyzer (Sysmex, Kobe, Japan). Plasmatic levels of PCT were determined by Enzyme-Linked Fluorescence Assay (ELFA) with the VIDAS® B.R.A.H.M.S. PCT™ system (Biomerieux, Marcy-l'Etoile, France), ac-

cording to manufacturer recommendations and expressed in ng/mL.

Serum levels of CRP were measured immune-turbidometrically on the analyzer Roche/Hitachi MODULAR P, according to manufacturer recommendations (Roche Diagnostic GmbH) and expressed in mg/L.

All others biochemicals parameters analyzed were performed on Modular Cobas® 8000 according to manufacturer directions (Roche Diagnostic GmbH).

Microbiological cultural assay

Microbial cultures were used to determine the type of organism present in different biological specimens derived from different site (respiratory tract, urinary tract, abdomen, and others) according to clinical suspicion and test requested to the laboratory by standardized microbiological cultural assays procedures.

Blood cultures were performed in patients with clinical symptoms of bloodstream infections prior the administration of antimicrobial therapy. For each patient, two bottles sets were used for each septic episode; approximately 10 mL of blood was inoculated in the aerobic and anaerobic bottle (BACT/ALERT Culture Media, Biomerieux, Marcy-l'Etoile,France); the bottles were entered in the BACT/ALERT 3D System for the incubation and measure of the color change in response to shift in pH as a result of rising of CO_2 levels produced by microorganisms. In positive samples, bacteria and yeasts were identified on the Vitek 2 system (Biomerieux, Marcy-l'Etoile, France), according to manufacturer directions.

Flow cytometrical analysis

For monocytes phenotypic analysis, 100µL of EDTA anticoagulated whole blood from patients and controls were incubated with the following combination of monoclonal antibodies: 20 µL of

anti CD45-PerCP (BD, clone 2D1), 20 µL of anti CD14-PE (BD, clone MφP9), 20 µL of anti CD16-PE-Cy7 (BD, clone B73.1) and 20 µL of anti HLA-DR-APC-Cy7 (BD, clone L243) for 30 minutes in the dark. All samples were stained within 1.5 hours after blood collection.

After staining, red blood cells were lysed for 15-20 minutes with 1:10 dilution of BD FACS™ lysing solution (containing 30.0% diethylene glycol, 9.99% formaldehyde and 3.51% methanol), and centrifugated for 5 minutes at 300xg. Cell pellet was resuspended in 450 µL of BD FACSFlow™ Sheath Fluid solution and acquired on a BD FACS CANTO II flow cytometer.

A minimum of 3500 monocyte events were recorded for each sample based on a gate created on scatter plot of CD45-PerCP vs side scattered light signals. The three monocytes subsets, $CD14^{++}CD16^{-}$, $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$, were identified by different surface expression of LPS receptor CD14 and the FcγIIIR CD16, among $CD45^{+}$ gated monocytes.

Moreover, in setting conditions, we used also markers for other leucocytes populations (as CD3 for T lymphocytes, CD19 for B lymphocytes or other specific markers of granulocytes lineage in combination with physical and dimensional parameters) and we confirmed the accurate gating of monocytes even in absence of additional markers (data not shown).

For mHLA-DR quantitation, total monocyte events were recorded for each sample on a gate created on scatter plot of CD14-PE vs side scattered light signals. mHLA-DR expression was reported as a percentage of HLA-DR positive monocytes out of the total $CD14^{+}$ monocyte population and as the Mode of Florescence Intensities (MFI) of the analyzed monocyte's population. Samples were collected after 3-4 days from sepsis diagnosis. Data were analyzed by BD Facs DIVA 8.0.1 software (Becton Dickinson).

Statistical analysis

Statistical analysis was performed by MedCalc v19.9.1 statistical software. Data were presented as the mean ± SEM (Standard Error of the Mean). The Student's *t*-test was used for comparison between patients and controls; statistically significant differences were established by *p* value (< 0.05).

RESULTS

The population of 93 hospitalized patients analyzed in the present study was enrolled according to high plasmatic level of procalcitonin (PCT > 0.5 ng/ml, hPCT) while 84 not hospitalized healthy individuals were used as controls. Demographic and clinical characteristics of all individuals are shown in Table 1.

Total white blood cells, monocytes and neutrophils counts were significantly higher in patients compared to controls (Table 1), while lymphocytes count was significantly reduced (Table 1). 24,7% of hPCT patients with negative cultural assay (Cult-NEG, n=23, Table 2) showed a condition defined "strong inflammatory state" characterized by alteration of white blood cells count ($13.0 ± 1.6 \times 10^{9}$/L, reference range 4.0-10.0 x 10^{9}/L) and hematological parameters, increased C-reactive protein plasmatic levels (115.0 ± 16.5 mg/L, reference range 0.0-10.0 mg/L), as well as some liver markers (i.e. AST 54.4 ± 18.8 U/L, reference range <45 U/L; GGT 85.9 ± 26.1 U/L, reference range <45 U/L) and total bilirubin (3.0 ± 1.9 mg/ dL, reference range <1.25 mg/dL).

The remaining 75,3% of hPCT patients showed positive cultural assay demonstrating the presence of "infection" in different site as blood, respiratory tract, urinary tract, abdomen, and others (Cult-POS, n=70, Table 2). Among infection-positive patients, 68.6% were infected by Gram negative bacteria, 21,4% by Gram positive bacteria and 10% by Candida.

Table 1	Demographic and clinical characteristics of hPCT patients (procalcitonin > 0.5 ng/mL) and controls		
	hPCT patients (n=93)	**Controls (n=84)**	**T-test**
Gender	Male n= 53 Female n= 40	Male n= 41 Female n= 43	-
Age (years)	65.9±1.8 62.5 to 69.5 34.2 to 89.0	50.4±1.5 47.3 to 53.4 26.5 to 69.5	-
WBC (10^9 cell/L)	12.9±0.8 11.4 to 14.5 3.0 to 27.0	6.5±0.2 6.2 to 6.8 4.2 to 9.1	p<0.001
Platelets (10^9 cell/L)	220.5±19.7 181.4 to 259.7 28.0 to 520.3	224.5±7.3 210.0 to 239.0 133.2 to 337.0	p=0.85
Monocytes (10^9 cell/L)	0.9±0.07 0.8 to 1.1 0.2 to 2.4	0.5±0.02 0.5 to 0.6 0.3 to 0.8	p<0.001
Neutrophils (10^9 cell/L)	10.6±0.7 9.1 to 12.0 2.0 to 24.0	3.7±0.1 3.4 to 3.9 2.0 to 5.8	p<0.001
Lymphocytes (10^9 cell/L)	1.3±0.08 1.1 to 1.5 0.3 to 2.8	2.1±0.06 2.0 to 2.2 1.2 to 3.0	p<0.001
PCT (ng/mL)	13.6±3.3 7.0 to 20.2 0.5 to 80.0	<0.05	-
CRP (mg/L)	141.9±12.5 116.9 to 166.9 12.9 to 346.0	<10	-

Data are presented as mean ± SEM, 95% CI (mean) and 5-95 percentiles.

Table 2	Grouping of analyzed patients

Analyzed patients			
hPCT patients n=93			Controls n=84
INFLAMMATION (Cult-NEG) n=23	INFECTION (Cult-POS) n=70		
	BC+	BC-	
	n=44	n=26	

hPCT, patients with procalcitonin >0.5ng/mL; Cult-NEG, hPCT patients with negative cultural assay; Cult-POS, hPCT patients with positive cultural assay in different site; BC+, hPCT patients with positive blood culture; BC-, hPCT patients with positive cultural assay in different site.

Bloodstream infection was demonstrated by positive blood culture (BC+, n=44, Table 2) in CultPOS patients with clinical evaluation correlated with sepsis (13).

In a small number of septic patients (n=23), biochemical and hematological parameters were monitored at admission (time 1), at blood culture request (time 2), after 3-4 days from diagnosis (time 3), as well as after 6-7 days from diagnosis (time 4).

The samples were retrospectively categorized into two groups characterized by better clinical outcome (group BO) or worst clinical outcome (group WO) based on time of hospitalization, recovery time from primary infection, appearance of secondary/nosocomial infections and death. Clinical characteristics of subgroups with better and worst clinical outcome are shown in Table 3.

Blood monocyte subsets

Monocytes subsets were differentiated by flow cytometrical analysis on the basis of CD14 and CD16 surface expression.

Intermediate monocytes $CD14^{++}CD16^{+}$ were significantly increased in hPCT patients compared to controls (13.6% ± 0.8 and 6.2% ± 0.3 respectively with p<0.001), while classical monocytes $CD14^{++}CD16^{-}$ were significantly reduced (72.5% ± 1.6 vs 82.6% ± 0.7, p<0.001). Non-classical monocytes didn't show significant differences between patients and controls (5.5% ± 0.9 and 5.1% ± 0.3 respectively with p=0.6).

The differences between subsets of monocytes from hPCT patients with infection (Cult-POS) and with inflammatory state (Cult-NEG) were not significant: in both patient groups, intermediate monocytes remained significantly increased compared to healthy controls (Figure 1) highlighting a pro-inflammatory phenotype for all hPCT patients.

Cult-POS infected patients included both septic patients (BC+) and non-septic patients (BC-)presenting infection in other body sites, excluding bloodstream infection).

Intermediate monocytes were significantly increased in BC+ compared to BC- (17.4% ± 2.1

Table 3	Clinical characteristics of BC+ patients according to clinical outcome		
	Better Outcome, BO (n=13)	**Worst Otcome, WO (n=10)**	**T-test**
WBC (10^9 cell/L)			
Time 1	10.2±2.2	11.3±1.5	p=0.72
Time 2	11.5±2.7	14.4±2.8	p=0.47
Time 3	12.1±2.3	16.4±2.9	p=0.25
Time 4	8.4±0.9	14.0±3.0	p=0.99
Platelets (10^9 cell/L)			
Time 1	198.5±30.3	237.1±43.0	p=0.46
Time 2	209.9±32.7	105.9±26.9	***p=0.02***
Time 3	216.3±33.5	113.8±34.1	***p=0.04***
Time 4	291.6±42.6	175.4±46.3	p=0.08
Monocytes (10^9 cell/L)			
Time 1	0.6±0.09	0.5±0.09	p=0.20
Time 2	0.7±0.09	0.7±0.1	p=0.99
Time 3	0.8±0.09	1.1±0.3	p=0.28
Time 4	0.7±0.07	0.9±0.2	p=0.29
Neutrophils (10^9 cell/L)			
Time 1	8.3±2.3	9.4±1.5	p=0.70
Time 2	9.1±2.5	12.7±2.7	p=0.33
Time 3	9.7±2.2	14.2±2.7	p=0.21
Time 4	5.7±0.6	11.6±2.7	p=0.06
Lymphocytes (10^9 cell/L)			
Time 1	1.2±0.2	1.3±0.2	p=0.58
Time 2	1.5±0.4	0.9±0.1	p=0.18
Time 3	1.5±0.2	1.1±0.2	p=0.16
Time 4	1.7±0.2	1.4±0.3	p=0.38
Secondary/nosocomial infections	n=2	n=4	
Death	/	1	

Data are presented as mean ± SEM. Time 1, admission; time 2, at blood culture request; time 3, after 3-4 days from diagnosis; time 4, after 6-7 days from diagnosis.

Figure 1   Percentage of monocytes subsets in Cult-POS, Cult-NEG and Ctrl patients
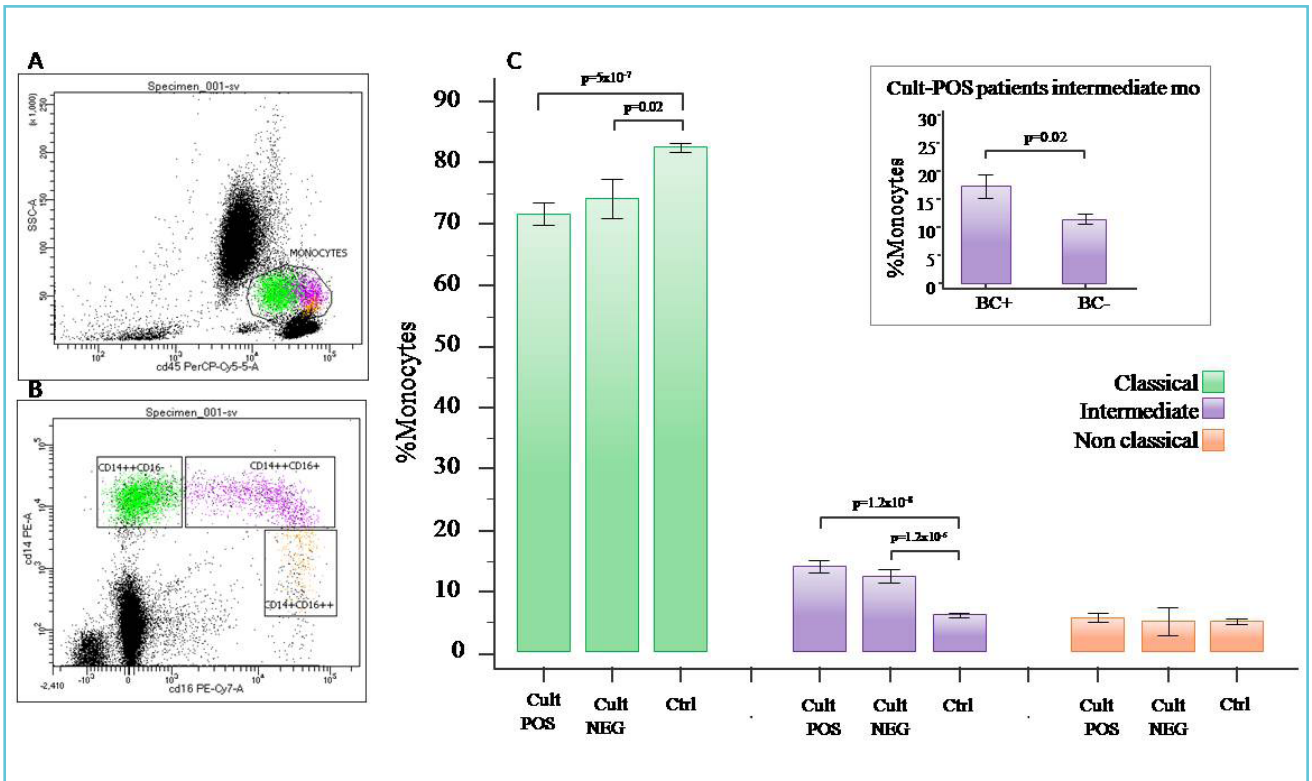
A: *Flow cytometric analysis of monocytes on a gate created on scatter plot of CD45-PerCP vs side scattered light signals.*

B: *Monocytes subsets, CD14++CD16-, CD14++CD16+ and CD14+CD16++, are identified by different surface expression of CD14 and CD16, among CD45+ gated monocytes.*

C: *Bar graph of percentage of three monocytes subsets in Cult-POS, Cult-NEG and healthy control (Ctrl) patients.*

Cult-POS patients showed significantly higher percentage of intermediate monocytes compared to Cult-NEG patients and Ctrl, and lower percentage of classical monocytes.

The difference between blood culture positive and negative Cult-POS patients are showed in the box. (P values are showed in figure; where not indicated, difference was not statistically significant).

vs 11.5% ± 0.9, respectively with p<0.05, as in the Figure 1 box) and compared to the controls (17.4% ± 2.1 vs 6.2% ± 0.3, respectively with p<0.001).

According to the role of CD16 positive monocytes in modulating inflammatory response, a positive correlation trend between procalcitonin level and intermediate $CD14^{++}CD16^{+}$ monocytes percentages was observed; on the other hand, classical monocytes showed an opposite trend of correlation with the increase of procalcitonin (Figure 2).

HLA-DR monocytes surface expression in septic patients

HLA-DR monocytes surface (mHLA-DR) expression was analyzed by flow cytometrical analysis (Figure 3 A, B, C). Percentage of mHLA-DR and MFI in non-septic patients (BC-) showed no significant difference compared to controls (99.1% ± 0.3 vs 99.4% ± 0.2 respectively with p=0.3). In septic patients, HLA-DR expression on all monocytes subsets was significantly lower (BC+, 94.3% ± 2.2) compared to healthy controls (99.4% ± 0.2, with p<0.05) (Figure 3D). As

Figure 2 — Serum levels of PCT and percentage of monocytes subsets
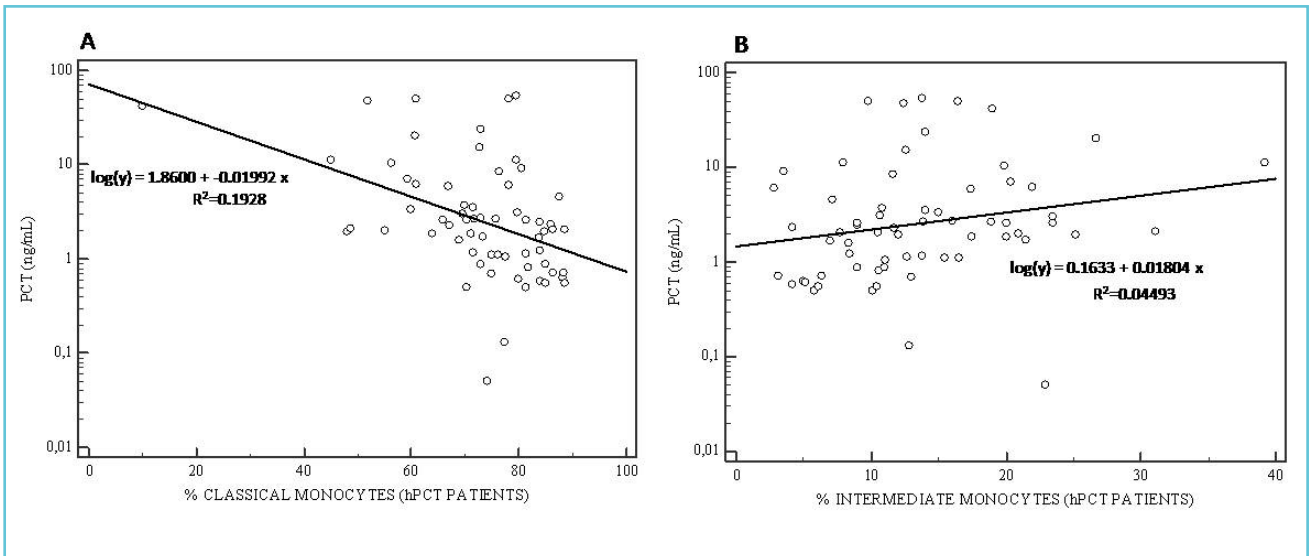
A: Classical monocytes.
B: Intermediate monocytes. Intermediate monocytes increased as levels of PCT increased. Classical monocyte show an opposite trend. PCT values are showed in semilogarithmic scale.

previously reported (11), in septic patients mH-LA-DR was estimated 3-4 days after diagnosis of sepsis. mHLA-DR expression in BC+ patients was significantly lower than BC- patients (94.3% ± 2.2 vs 99.1% ± 0.3 with p<0.05) (Figure 3D). No statistical significance was observed for mHLA-DR MFI between BC+, BC- and control patients.

Among septic patients, the subgroup WO showed worst conditions, characterized by higher incidence of secondary/nosocomial infections, often polymicrobial, as well as long time hospitalization (>30 days).

We separately analyzed subgroup WO and, in agreement with the known sepsis-induced immune system alterations, we found that these patients had lower percentage of HLA-DR positive monocytes compared with better outcome patients (BO subgroup) (88.4% ± 4.5 and 98.6% ± 0.3, respectively, with p=0.05, 10% reduction, Figure 3E) associated with lower mHLA-DR MFI (Mode 1193.6 ± 219.6 and 2819.4 ± 591.1, respectively, with p=0.022, 58% reduction) (Figure 3F), and also lower Median of Florescence Intensities

(1623.7 ± 416.3 and 2730.8 ± 332.5, respectively, with p=0.05) or Mean of Florescence Intensities (2344.0 ± 590.2 and 3837.4 ± 398.1, respectively, with p=0.05). Similarly, significant 11% reduction of mHLA-DR percentage and 46% reduction of mHLA-DR MFI was obtained by comparing worst condition and control patients (Figure 3E and F).

Interestingly, the worst condition patients didn't show a tendency towards restoration of normal values of white blood cells, platelets, monocytes, neutrophils and lymphocytes counts like better outcome patients (Figure 4, Table 3) as well as BC- patients, which however showed more stable hematological parameters than BC+ (data not shown).

DISCUSSION

Impaired immune response following sepsis originates from delayed restoration of immunologic homeostasis between pro- and anti- inflammatory responses and determines higher risk for adverse outcome including secondary

Figure 3 HLA-DR monocytes surface expression in BC+, BC- and Ctrl patients
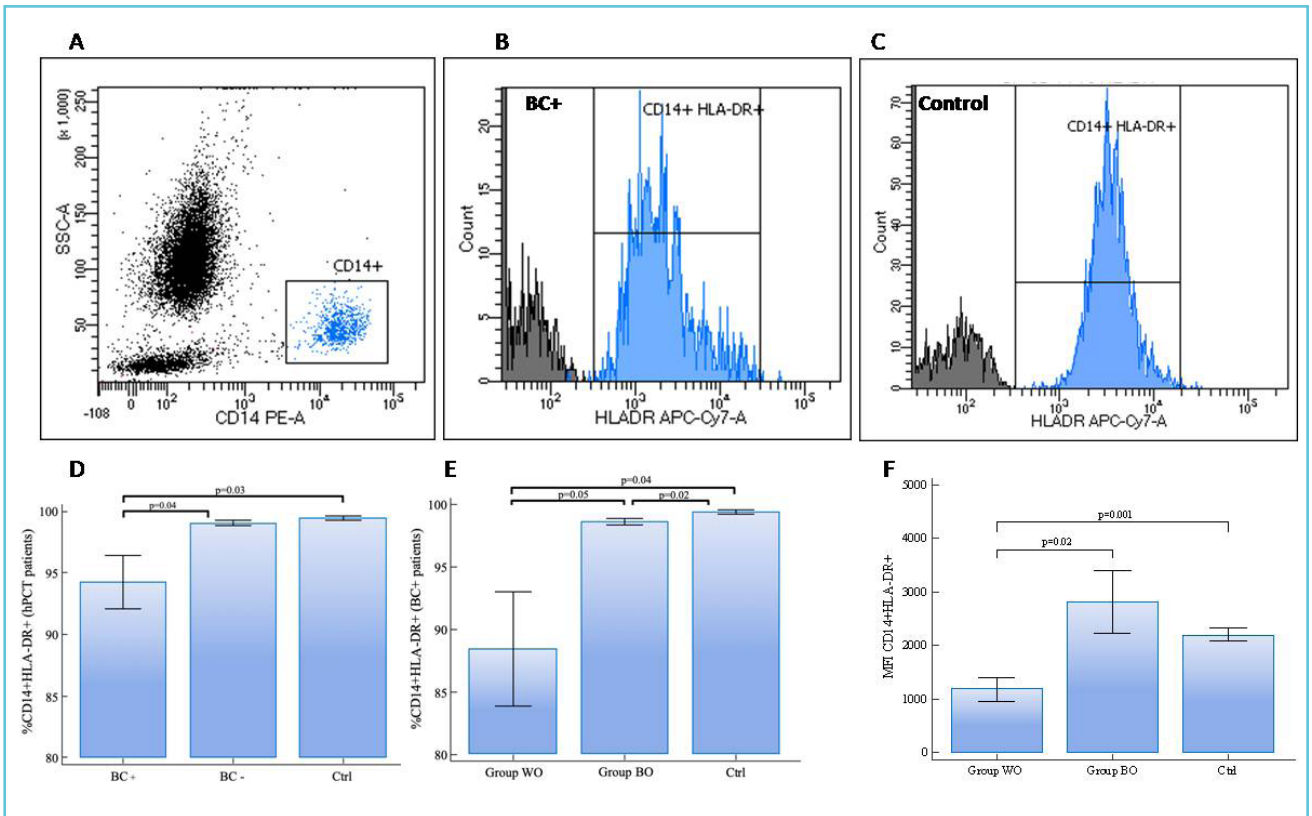
A: *Flow cytometric analysis of monocytes on a gate created on scatter plot of CD14-PE vs side scattered light signals.*

**B** and **C:** *Histogram plot of HLA-DR-APC-Cy7 among CD14+ gated monocytes.*

D: *mHLA-DR expression in hPCT-patients; in septic patients (BC+), HLA-DR expression is significantly lower compared to non-septic patients (BC-) and healthy controls (Ctrl).*

E and **F:** *subgroup of septic patients presenting worst outcome (BC+, group WO) shows a significant reduction of mHLA-DR percentage and relative MFI compared to patients with better clinical outcome (group BO) and healthy controls (Ctrl). (P values indicated in figure; where not indicated, difference was not statistically significant).*

infection and death (7). The innate immune cells (including monocytes) are involved in the initial immunologic response to critical illness with the adaptative response being more prevalent in the subacute phase of illness. In the present study, we analyzed monocytes polarization and reprogramming from inflammatory to immuno-suppressive phase in critical ill patients with high procalcitonin serum levels (>0.5 ng/mL). These patients showed a significant shift to a proin-flammatory phenotype of monocytes with the expansion of the CD14++CD16+ subpopulation (intermediate monocytes) and the decrease of

classical CD14++CD16- monocytes subpopulation (Figure 1C). Intermediate monocytes mainly ex-ert a pro-inflammatory role. CD16+ monocytes are recognized to be involved in antigen pro-cessing and presentation as well as trans-endo-thelial migration (3) and to be increased during inflammation (e.g., cancer, sepsis and stroke), infections such as HIV (14-17), tuberculosis (18) and other pathogens (19, 20). Accordingly, in our study, intermediate monocytes were signifi-cantly increased during both "strong inflamma-tory state" (Cult-Neg) and "infection state", iden-tified by a positive microbiological cultural assay

| Figure 4 | Evaluation of hemato-chemical markers during hospitalization time |
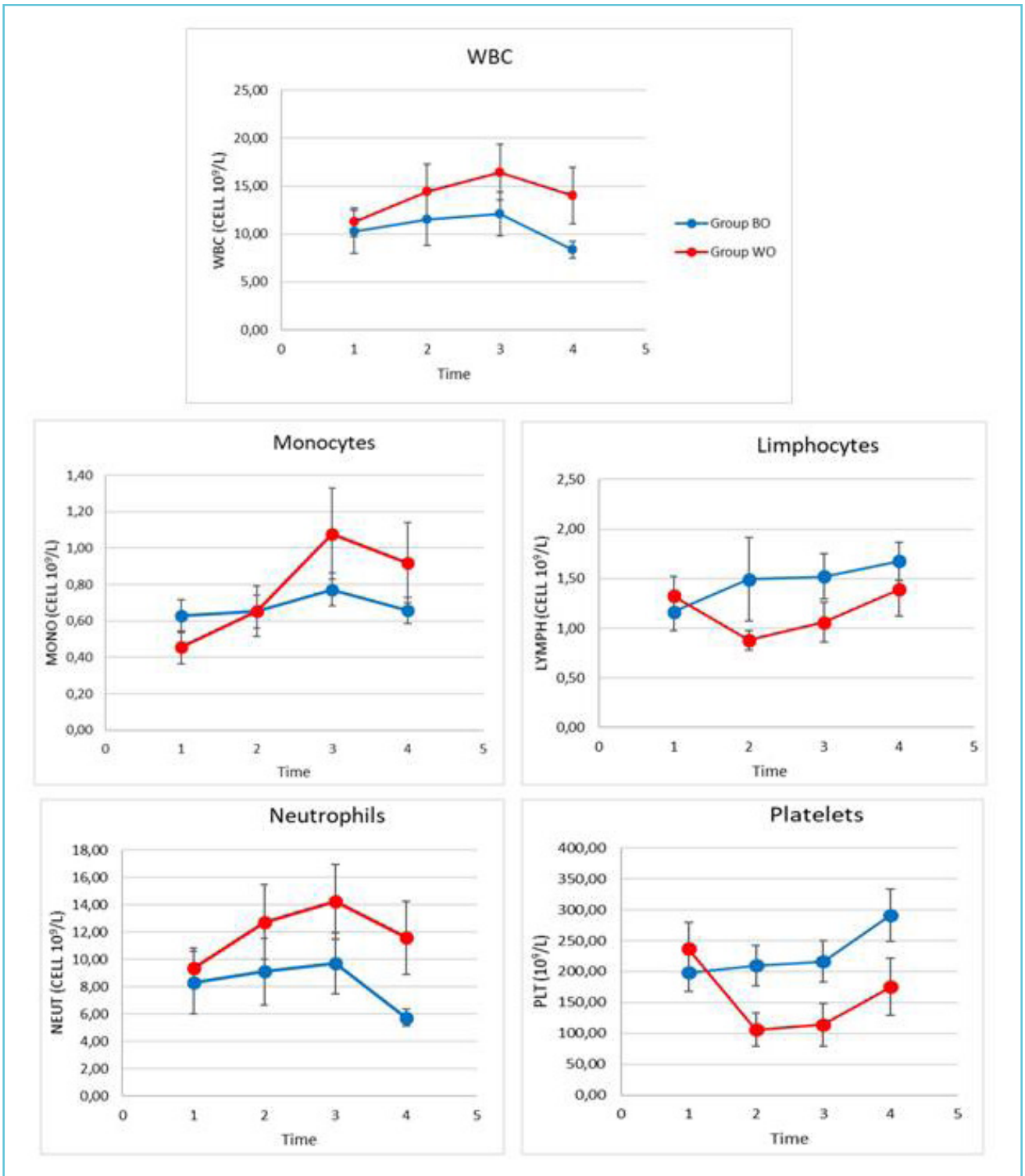
Trends values of white blood cells, monocytes, lymphocytes, neutrophils and platelets count in better (BO) and worst (WO) clinical outcome patients. Parameters were monitored at admission (time 1), at blood culture request (time 2), after 3-4 days from diagnosis (time 3) and after 6-7 days from diagnosis (time 4).

(Cult-Pos), compared to controls (Figure 1). In Cult-Pos patients, a positive trend of correlation has been observed for procalcitonin values and percentage of intermediate monocytes, while a negative trend was found for classical monocytes (Figure 2). PCT has already been demonstrated to have diagnostic and prognostic use in shock patients; peak levels have been shown to closely follow those of TNF-α and IL-6 (21). Moreover, we found that the increase of intermediate monocytes was significantly associated with the presence of bloodstream infection (BC+) in Cult-Pos patients (Figure 1, rectangle box) presenting also other clinical signs of sepsis as defined by the third international consensus definition of sepsis (13) on the basis of sequential organ failure assessment (SOFA) score. In hPCT patients analyzed in this study, especially those with infection, monocytes showed to be polarized toward a specific increase of intermediate subpopulations that is indicative of a more pronounced inflammatory environment. Other studies demonstrated that infection triggers expression of CCR2 by intermediate monocytes, which promote their migration into the lesions where increased levels of its ligand CCL2 protein are present; moreover, intermediate monocytes produce TNF-α, thus enhancing the inflammatory response (20). Leukocytes trafficking occurs in presence of increased vascular permeability and endothelial activation during sepsis; these processes are controlled by molecular mediators in the context of the regulation of the pro- anti- inflammatory response (22).

When severe and persistent compensatory anti-inflammatory response follows critical illness, patient is exposed to high risk for adverse outcome. As known, monocytes normally recognize and process the pathogen and present the antigens on their cell surface via human leukocytes antigen HLA-DR molecules; when activated, monocytes secrete proinflammatory cytokines, such as TNF-α, which amplify the immune response. As critical condition progresses, lower mHLA-DR expression determines a reduced monocytes antigen presenting capacity and releasing of pro-inflammatory cytokines in response to bacterial compounds and immune-suppressed state (9, 23, 24). In this setting, we examined mHLA-DR expression in hPCT-patients population of the present study, and we found a significant percentage reduction in septic patients compared to controls and to BC- patients (Figure 3D). Notably, subgroup of septic patients presenting worst outcome (BC+, group WO) showed a significant reduction of mHLA-DR percentage (Figure 3E) and of mHLA-DR quantification as MFI (mode of florescence intensity) which is an indicator of a reduced number of HLA-DR molecule per monocytes (Figure 3F). Time course evaluation of hemato-chemical markers showed worsening of the clinical conditions of WO-patients compared to restoration of same parameters in BO-patients (Figure 4, Table3). Recently, four phenotypes of sepsis have been derived from clustering analysis of multiple data set (25, 26), and currently not included in consensus definition of sepsis (13), which are potentially related to biomarker variation and clinical outcome of patients. Difference between the 4 phenotypes derives from different pattern of organ dysfunction, demographic data and laboratory values: broad differences were observed in the distribution of the host immune response biomarkers across phenotypes; in general there was an increase in the markers of inflammation and of endothelial dysfunction in γ and/or δ high-mortality phenotypes compared with the α or β phenotypes with better clinical outcome (25, 26). From our results, HLA-DR appears to be a good marker for classification of adverse clinical outcome in septic patients. The clinical outcome of the patients includes the successive involvement of adaptive immune response. First available marker of it is the absolute lymphocytes count; in fact, lymphocyte apoptosis

causes lymphopenia during sepsis and has been associated with mortality and secondary infection risk (27-29). We found that lymphocytes absolute count showed a substantial drop in WO-patients (Figure 4), while both BO-patients (BC+) and BC- patients showed a more stable trend.

Further studies are in progress to evaluate degree of T cell dysfunction (i.e. activation in presence of bacterial lysate) and regulatory T cell (i.e. immune-suppressive subset of T cells) in the perspective of better understanding of mechanisms responsible for pro/anti-inflammatory responses during sepsis, and develop new approaches of immuno-monitoring strategies and immunotherapies.

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