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RESEARCH

Prospective flow cytometry analysis of leucocyte subsets in critically ill patients who develop sepsis: a pilot study

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Abstract

Purpose Sepsis in critically ill patients with injury bears a high morbidity and mortality. Extensive phenotypic monitoring of leucocyte subsets in critically ill patients at ICU admission and during sepsis development is still scarce. The main objective of this study was to identify early changes in leukocyte phenotype which would correlate with later development of sepsis.

Methods Patients who were admitted in a tertiary ICU for organ support after severe injury (elective cardiac surgery, trauma, necessity of prolonged ventilation or stroke) were sampled on admission (T1) and 48–72 h later (T2) for phenotyping of leukocyte subsets by flow cytometry and cytokines measurements. Those who developed secondary sepsis or septic shock were sampled again on the day of sepsis diagnosis (Tx).

Results Ninety-nine patients were included in the final analysis. Nineteen (19.2%) patients developed secondary sepsis or septic shock. They presented significantly higher absolute monocyte counts and CRP at T1 compared to non-septic patients (1030/µl versus 550/µl, $p = 0.013$ and 5.1 mg/ml versus 2.5 mg/ml, $p = 0.046$, respectively). They also presented elevated levels of monocytes with low expression of L-selectin ($CD62L_{neg}$ monocytes) (OR[95%CI] 4.5 (1.4–14.5), $p = 0.01$) and higher SOFA score ($p < 0.0001$) at T1 and low mHLA-DR at T2 (OR[95%CI] 0.003 (0.00–0.17), $p = 0.049$). Stepwise logistic regression analysis showed that both monocyte markers and high SOFA score (> 8) were independently associated with nosocomial sepsis occurrence. No other leucocyte count or surface marker nor any cytokine measurement correlated with sepsis occurrence.

Conclusion Monocyte counts and change of phenotype are associated with secondary sepsis occurrence in critically ill patients with injury.

Keywords Injury · Sepsis · Flow cytometry · Monocytes · HLA-DR · L-selectin

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Abbreviations

AUC	Area under the curve
BSI	Blood stream infection
CI	Confidence interval
CLABSI	Central line associated blood stream infection
CRP	C-reactive protein
HAP	Hospital-acquired pneumonia
HLA-DR	Human leucocyte antigen
ICU	Intensive care unit
IQR	Interquartile range
LOS	Length of stay
MFI	Median fluorescence index or median of fluorescence intensity
OR	Odds ratio
PE	Phycoerythrin-linked
PerCP	Perinidin-chlorophyll protein-linked
ROC	Receiver operating characteristic

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SOFA	Sequential Organ Failure Assessment
SSTI	Surgical site and soft tissue infection
TNF-α	Tumor necrosis factor α
VAP	Ventilator-associated pneumonia

Background

It is estimated that 25–35% of critically ill patients develop sepsis which is associated with increased length-of-stay (LOS), morbidity and mortality [1–4]. As in sepsis-induced immunosuppression, immune alterations affecting patients with critical injuries such as trauma, major surgery or burns, have been associated with increased susceptibility to secondary infections and mortality [5–8]. The first reports of monocyte anergy and endotoxin tolerance date back to the 70's in major surgical and burn patients [9, 10]. Since then, most studies relying on flow cytometric analysis of peripheral blood cells have focused on single and restricted types of immune cells defects such as T-lymphocytes, monocytes and neutrophils [11–14]. The most commonly studied parameter of immune dysfunction associated with injury is the low HLA-DR expression on monocytes (mHLA-DR), which induces an impaired functional state of these cells. The latter feature has been associated with secondary sepsis and sometimes outcome in severe trauma, burn and postoperative patients [15–21]. Targeted treatment has been tempted in that context. Older studies have shown contrasted clinical outcomes after immunotherapy, based on GM-CSF or IFNγ administration, despite efficacious restoration of mHLA-DR and/or IFNγ endogenous secretion [22–24]. In a hypothesis-driven approach, other markers such as elevated levels of regulatory T-helper cells (T_{regs}) were recently shown to be predictive of nosocomial sepsis in combination with low levels of mHLA-DR and neutrophil CD88 in an ICU patient population comprising but not restricted to trauma and postoperative patients [25]. So far, only three studies relying on wide flow cytometry panels to predict secondary sepsis in critically ill patients have been conducted and the first two included only septic patients [26–28]. These authors showed that clinical deterioration at 48 h could be predicted in septic patients with circulating immature granulocytes which induced T-cell lymphopenia after enrichment. A very recent study focused on the overtime changes of the injury-induced immune profile in a large cohort of septic, trauma and surgical patients during the first week of ICU admission [28]. The authors used a restricted number of immune markers determined by flow cytometry, combined with transcriptomic and functional tests to show that the initial adaptive immune response to injury, whatever the etiology, was not associated with a risk of secondary infections. Moreover, only a subset of patients exhibiting late combined immune alterations (such as low CD3D, CD74 messenger RNA and mHLA-DR and high S100A9 messenger RNA at days 5–7) developed secondary infections. Our study aimed at describing the temporal changes of various leucocyte surface markers, via flow cytometric analysis, in non-septic patients, after critical injury, in association with nosocomial sepsis occurrence. The studied panel included subsets of B and T lymphocytes, as well as monocyte and neutrophil characterization.

Materials and methods

Study patients

This single-center, prospective, observational study was conducted in 3 tertiary ICUs over a 7-month period at CHU de Liège. The institutional ethics committee approved the study (Belgian number: B707201111981) and written informed consent was obtained from the patient or his/her legal representative. Inclusion criteria included: age over 18 years, elective cardiac surgery (CABG or valve replacement), trauma, acute ischemic or hemorrhagic stroke and invasive ventilation (> 48 h) for reasons other than infection. Exclusion criteria were: life expectancy of less than 48 h, systemic or oral antibiotic therapy for active infection, active hematological or solid organ proliferative disease, HIV (+) status, chronic viral hepatitis B and C and use of any immunosuppressive therapy. Upon admission to ICU, the following demographic characteristics were recorded: gender, age, type of admission (surgical or medical) and treatment with vasopressors. The sequential organ failure assessment score (SOFA) score was calculated [29]. For each patient, the following data were also collected: length of ICU and hospital stay (days), duration of ventilation (days), administration of vasopressors prior to and during ICU stay, antibiotic treatment, site of infection and microbiological documentation, necessity of hemofiltration or intermittent hemodialysis during and/or after ICU stay. All patients included were followed up until 1 year after inclusion in the study or death. In case of death, time was recorded.

Blood samples were collected within 24 h (T1) of admission, 48 h (T2) after admission and on the day of diagnosis of sepsis and/or septic shock (Tx). The Sepsis-3 definition [30] was used for this study. Definitions of infection were based on Center for Disease Control (CDC) criteria [31–33]. Our institution does not recommend routine use of selective digestive tract decontamination. Patients were compared to an age-matched (> 50 years) cohort of healthy controls ($n = 18$).

Immunophenotyping

Automated blood counts were obtained using the Sysmex XS-800 hematology analyzer (Kobe, Japan) for

quantification of the absolute cell counts. Immunophenotyping was performed by adding combinations of monoclonal antibodies to 100 µl of whole blood, incubated for 20 min at 4 °C in the dark, after which red cell lysis was achieved by adding BD FACS Lysing Solution. Cells were centrifuged and resuspended in HBSS 1% formaldehyde. Flow cytometric data were acquired on a FACS Verse flow cytometer (BD Biosciences). The daily setup procedure involved a one-step performance check, using BD FACSuite™ CS&T Research Beads to adjust photomultiplier tube voltages. This ensured that the target MFI (Mean Fluorescence Intensity) values were held constant from day to day.

The following combinations of monoclonal antibodies (BD Biosciences, San Jose, CA, USA) were used. For NK cells and T lymphocytes: anti-CD3-FITC (clone SK7, 5 µl), CD4-PerCP (clone SK3, 5 µl), CD8-APC-H7 (clone SK1, 5 µl), CD14-V450 (clone MφP9, 5 µl), CD45-V500 (clone HI30, 5 µl), CD56-PE-Cy7 (clone B159, 5 µl), CD69-APC (clone L78, 5 µl) and CD279-PE (clone MIH4, 20 µl). For B and regulatory T lymphocytes: anti-CD3-FITC (clone SK7, 5 µl), CD4-PerCP (clone SK3, 5 µl), CD19-PE-Cy7 (clone SJ25C1, 5 µl), CD25-PE (clone 2A3, 20 µl), CD45-V500 (clone HI30, 5 µl) and CD127-AlexaFluor 647 (clone HIL-7RM21, 20 µl). For monocytes: anti-CD14-V450 (clone MφP9, 5 µl), CD16-AlexaFluor647 (clone 3G8, 5 µl), CD45-V500 (clone HI30, 5 µl), CD64-PE-Cy7 (clone 10.1, 5 µl), CD279-PE (clone MIH4, 20 µl), and HLA-DR-PerCP (clone G46-6, 10 µl). Monocytes were categorized into classical (CD14 + +/CD16-), non-classical (CD14 + /CD16 + +) and intermediate (CD14 + +/CD16 +) according to the level of expression of CD14/CD16, respectively [34]. For neutrophils: anti-CD11b-PE (clone D12, 20 µl), CD11c-PE (clone B-ly6, 5 µl), CD16-PE (clone 3G8, 20 µl), CD45-V500 (clone HI30, 5 µl), CD62L-APC (clone DREG-56, 20 µl) and CD64-PE-Cy7 (clone 10.1, 5 µl).

Doublet exclusion with the FSC-A/FSC-H (Fig. S1A) was followed by selection of granulocytes, monocytes and lymphocytes with the CD45/SSC-A dot plot (Fig. S1B, lymphocytes: 1, orange; monocytes: 2, green; granulocytes: 3, blue). Total lymphocytes were first characterized on a CD3/CD56 biplot for the NK (Fig. S1C1) and NKT (Fig. S1C2) subsets. CD3 positive lymphocytes (Fig. S1D2) were subsequently classified into CD4 + (Fig. S1E1) or CD8 + (Fig. S1E2) population. CD69 and CD279 expression from these subsets was assessed (Fig S1F1 and F2, respectively). B cells were separated according to the expression of CD19 (Fig. S1D1) as well as CD25 (Fig. S1G). Regulatory lymphocytes (Tregs) were isolated from CD4 + T cells (Fig. S1E1). Tregs were then stained as CD4 + CD25highCD127- cells (Fig. S1H). Monocytes identification involves a first step of detection of HLA-DR + cells (Fig. S1I). In Fig. S1I, monocytes and some lymphocytes can be seen where monocytes are strongly positive for CD14 with some HLA-DR expression together with cells weakly positive for CD14 with a high HLA-DR signal. Figure S1J shows a dot plot for CD14 and CD16 that contains all gate I events. Classical (CD14 + CD16-), intermediate (CD14 + CD16 +), and nonclassical (CD14lowCD16 +) monocytes are defined by gates J1, J2, and J3, respectively. All J1-3 gate events are next displayed in the CD62L/CD64 dot plot (Fig. S1K). In this figure, CD62L negative monocytes can be identified. The CD16 marker is also expressed by neutrophil granulocytes located in gate L (Fig. S1L). Cells known not to express any of the antigens against which antibodies are present in the panels were used as unstained controls.

Cytokine measurements

Plasma was prepared from citrated whole blood samples to quantify plasma levels of TNFα, IL-10, IL-17A, IL6, IL-7 and IFNγ. Cytokine levels were measured using multiplex Cytometric Bead Arrays (BD Biosciences) on the FACSVerse System. Analysis was performed with the FCAP Array™ software (BD Biosciences).

Statistical analysis

Results were expressed as mean and standard deviation (SD) for quantitative data and as median and interquartile range (IQR) for durations. For categorical findings, frequency tables were used. Comparisons between septic and non-septic patients' characteristics were done by the ANOVA or Kruskal–Wallis test for continuous variables and Chi-square or Fisher exact test for categorical variables. The predictive value of sepsis was assessed for each baseline variable by logistic regression analysis on log-transformed biological variables. The variables significant at $p < 0.10$ were combined in a stepwise multivariate logistic regression analysis to identify independent baseline predictors of sepsis. The odds ratio (OR) with 95% confidence interval [95%CI] and ROC (receiving operating curve) curve analysis with area under the curve (AUC) were used to quantify the ability of the selected predictors to discern between septic and non-septic patients. The Youden method was applied to define an optimal cut-off point for those predictors. Data recorded on the same patients but at different time points were compared by the Wilcoxon signed rank test. Results were considered significant at the 5% critical level ($p < 0.05$). All statistical calculations were performed with SAS (version 9.4) and R (version 3.0.3).

Results

Patients baseline characteristics

A total of 99 adult patients with complete data were included in the final analysis. The demographic and clinical

characteristics at admission are presented in Table 1. There were predominantly male patients (60.6%) with a mean age of 64 ± 15 years. The type of admission was mainly surgical (86.9%) and cardiac surgery accounted for most patients (68.7%). Ten (10.1%) patients received vasopressors before admission, 67 (67.7%) received prophylactic antibiotics during surgery. The median admission SOFA score was 5 [IQR 4–8].

Sepsis occurrence

Nineteen (19.2%) patients developed sepsis or septic shock during follow-up, after a median time of 5 [IQR 3–7] days and 80 did not. As shown in Table 1, age, gender, category of admission, history of diabetes and use of vasopressor prior to ICU admission were not associated with sepsis occurrence. By contrast, higher SOFA score, admission for brain injury and lack of prophylactic antibiotics were predominant in patients who developed sepsis. Moreover, septic patients displayed higher hospital and ICU length-of-stay compared to non-septic patients (26 days [16–71] versus 11 days [9–16], $p < 0.0001$ and 15 days [10–22] versus 3 days [2–4], $p < 0.0001$, respectively). Septic patients also displayed a higher 28-day and 90-day mortality compared to non-septic patients (36.8% versus 7.5%, $p = 0.0028$ and 38.9% versus 8.9%, $p = 0.0038$, respectively). Infections sites and microbiological documentation are shown in Table S1.

Standard laboratory tests and cytokines

Comparison of standard laboratory tests and cytokine levels obtained within 24 h after admission to the ICU is shown in Table 2. Absolute monocyte counts and CRP were significantly higher in patients who developed sepsis compared to non-septic patients (1030/µl versus 550/µl, $p = 0.013$ and 5.1 mg/ml versus 2.5 mg/ml, $p = 0.046$, respectively). Monocyte counts did not add to the performance of SOFA score alone (AUC 0.84 with a cut-off level > 8) for prediction of secondary sepsis as shown in Fig S1. Cytokine measurements were not different between patients who would later develop sepsis versus those who would not.

Leucocytes cell surface markers

When considering leucocytes subsets at T1 against healthy controls, elevated absolute counts of classical, intermediate and total monocytes, increased levels of monocytes exhibiting a low expression of L-selectin (identified here as $CD62L_{neg}$ monocytes) and low expression of HLA-DR in total and intermediate monocytes were shown to be associated with further sepsis development in univariate analysis (Table 3). When all potential predictors of sepsis ($p < 0.10$) recorded at ICU admission (T1) were combined into a stepwise logistic regression, only the absolute count of $CD62L_{neg}$ monocytes was independently associated with sepsis occurrence (OR[95%CI]

Table 1 Demographic and clinical characteristics of the patients at ICU admission ($N = 99$)

	Total $N = 99$	Nonseptic $N = 80$	Septic $N = 19$	p value
Age (years)	64 ± 15	65 ± 15	62 ± 15	0.46
Gender: male	60 (60.6)	48 (60.0)	12 (63.2)	0.80
Surgical admission	86 (86.9)	70 (87.5)	16 (84.2)	0.70
Reason for admission				0.0022
Cardiac surgery	68 (68.7)	61 (76.2)	7 (36.8)	
Acute brain injury	12 (12.1)	6 (7.5)	6 (31.6)	
Trauma	13 (13.1)	10 (12.5)	3 (15.8)	
Ventilation > 48 h	6 (6.1)	3 (3.8)	3 (15.8)	
SOFA at ICU admission	5 (4–8)	4 (3–7)	10 (8–12)	< 0.0001
Diabetes	17 (17.2)	13 (16.2)	4 (21.0)	0.74
Cardiovascular disease	79 (79.8)	68 (85.0)	11 (57.9)	0.021
Vasopressor before admission	10 (10.1)	6 (7.5)	4 (21.0)	0.096
Prophylactic antibiotics	67 (67.7)	61 (76.2)	6 (31.6)	0.0002
Total hospital LOS (days)	11 (9–19)	11 (9–16)	26 (16–71)	< 0.0001
ICU LOS ((days)	3 (2–7)	3 (2–4)	15 (10–22)	< 0.0001
28-days mortality	13 (13.1)	6 (7.5)	7 (36.8)	0.0028
90-days mortality ($N = 97$)	14 (14.4)	7 (8.9)	7 (38.9)	0.0038

Results are expressed has mean ± SD, median (IQR), or n(%) as appropriate and p-values from ANOVA, Kruskal–Wallis, Chi-square or Fischer exact tests, respectively

ICU intensive care unit, *SOFA* sequential organ failure assessment, *LOS* length of stay

Table 2 Comparison of biological parameter levels recorded upon admission to ICU according to later occurrence of sepsis ($n=99$ patients)

	Non-septic $n=80$	Septic $n=19$	p value
CRP (mg/ml)	2.5 (1.1–9.1)	5.1 (2.5–17.4)	0.046
Fibrinogen (g/l)	2.4 (2.0–3.0)	3.0 (2.0–3.7)	0.13
Platelet count (k/µl)	134 (105–166)	169 (117–213)	0.12
White blood cells count (k/µl)	9.0 (7.0–12.2)	9.8 (6.8–16.3)	0.47
TNFα (pg/ml)	< 3.8 (< 3.8–< 3.8)	< 3.8 (< 3.8–< 3.8)	–
IL10 (pg/ml)	4.2 (0.0–11.8)	3.8 (0.0–10.1)	0.95
IL17A (pg/ml)	4.9 (0.76–11.6)	2.0 (0.0–7.8)	0.17
IL6 (pg/ml)	97.0 (34.8–189.2)	105.7 (39.3–240.3)	0.75
IL7 (pg/ml)	1.4 (0.17–4.3)	1.2 (0.21–1.5)	0.28
IFNγ	< 3.8 (< 3.8–< 3.8)	< 3.8 (< 3.8–< 3.8)	–
Neutrophils (counts/µl)	7045 (5704–9344)	6405 (5919–7298)	0.62
Monocytes (counts/µl)	550 (320–873)	1030 (430–1600)	0.013
Lymphocytes (counts/µl)	1200 (810–1715)	1180 (990–1470)	0.97

Results are expressed as median and interquartile range (IQR). p value of Kruskal–Wallis test; < 3.8 values for TNFα and IFNγ correspond to values under the level of detection (3.8 pg/ml)

MFI median fluorescence intensity

4.5[1.4–14.5], $p=0.011$) (Fig. 1A). By ROC curve analysis (Fig. 1B), a cut-off value of 180/µl (AUC 0.69) was derived for $CD62L_{neg}$ monocytes at T1 to discriminate septic from non-septic patients. The $CD62L_{neg}$ monocytes count did not add to the performance of SOFA score alone for secondary sepsis prediction, as seen in Fig. 1B. In the 12 patients available for complete data at T1, T2 and Tx, there was no temporal change in the numbers of $CD62L_{neg}$ monocytes (Fig. S2). When considering leucocyte subsets at T2, low expression of mHLA-DR by classical and intermediate monocytes and low levels of CD4 + CD279 + lymphocytes were associated with sepsis development in univariate analysis (Table 4). When all potential predictors of sepsis ($p<0.10$) recorded at T2 were combined into a stepwise logistic regression, only low expression of HLA-DR by intermediate monocytes was independently associated with sepsis development (Fig. 2A) (OR[95%CI] 0.003[0–0.17], $p=0.049$). By ROC curve analysis (Fig. 2B), a cut-off level of 1090 MFI (AUC 0.74) was derived for mHLA-DR to discriminate septic from non-septic patients. The level of mHLA-DR did not add to the performance of SOFA score alone for secondary sepsis prediction, as seen in Fig. 2B. In the 7 septic patients available for complete data at T1, T2 and Tx, there was no temporal change in the levels of the marker (Fig. S3).

The temporal change (delta T2-T1) of the two monocyte markers, i.e. $CD62L_{neg}$ monocytes absolute count and HLA-DR expression by intermediate monocytes, was not predictive of sepsis occurrence (data not shown).

Discussion

In this single-center study, we showed that, in critically ill injured adults, increased levels of absolute monocyte counts and of $CD62L_{neg}$ monocytes at ICU admission and reduced mHLA-DR in intermediate monocytes 48–72 h later, were independently associated with later sepsis occurrence. To the best of our knowledge, such a wide leucocyte panel including 63 flow cytometry markers exploring innate and adaptive immunity has not been reported in critical injury [35]. Despite the pivotal role of monocytes as sentinel cells in sepsis, the prognostic and diagnostic value of their absolute counts is conflicting in the literature [36]. Small observational trials including mainly trauma and sepsis patients have shown elevated or low monocyte counts to be associated with sepsis occurrence or outcome [37–40]. A very recently published observational study including more than 300 severely injured patients (out of which a third were already septic patients) looked into 30 immune markers, among which 12 were determined by flow cytometry [28]. The authors showed that monocyte count was not associated with secondary infection acquisition.

Considering the downregulation of L-selectin, identified here as increased numbers of $CD62L_{neg}$ monocytes, little is known in terms of sepsis prediction apart from conflicting data in neonates [41–43]. In newborn infants with suspected bacterial infection, L-selectin expression was significantly reduced in both granulocytes and

Table 3 Impact of parameters at ICU admission (T1) on the risk of sepsis

	Nonseptic ($N=80$)			Septic ($N=19$)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1; Q3)	N	Mean ± SD	Median (Q1; Q3)	OR (95%CI)	p value
HLA-DR MFI—total monocytes	80	1293 ± 632	1145 (805; 1682)	19	909 ± 477	776 (469; 1382)	0.030 (0.003–0.35)	0.0052
CD14 MFI—total monocytes	69	15,709 ± 6886	13,787 (11,585; 19,230)	12	15,838 ± 7310	14,613 (8432; 20,030)	082 (0.022–31)	0.92
CD16 MFI—total monocytes	69	149 ± 167	111 (78; 171)	12	147 ± 70	150 (84; 190)	2.1 (0.28–15.2)	0.48
CD64 MFI—total monocytes	80	25,273 ± 7449	23,702 (19,603; 29,378)	19	25,813 ± 5149	24,679 (23,119; 27,938)	4.0 (0.052–301)	0.53
CD279 MFI—total monocytes	80	18 ± 100	− 8.7 (− 39; 43)	19	55 ± 127	33 (− 24; 112)	2.9 (0.73–12)	0.13
Classical monocytes/µl	69	472 ± 324	419 (256; 598)	12	746 ± 433	742 (343; 1077)	11 (1.01–122)	0.049
Intermediate monocytes/µl	69	151 ± 171	82 (35; 221)	12	326 ± 221	392 (79; 502)	4.7 (1.2–19)	0.029
Non-classical monocytes/µl	57	22 ± 32	7.8 (3.3; 25)	10	36 ± 34	28 (5.7; 55)	2.7 (0.81–9.2)	0.11
CD279 MFI—classical monocytes	69	− 16 ± 76	− 23 (− 58; 6.1)	12	3.8 ± 118	− 20 (− 58; 20)	1.1 (0.17–7.5)	0.90
HLA-DR MFI—classical monocytes	69	1126 ± 595	1030 (690; 1539)	12	756 ± 467	481 (373; 1155)	0.025 (0.001–0.47)	0.014
CD64 MFI—classical monocytes	69	25,751 ± 7066	24,756 (20,707; 29,028)	12	25,712 ± 6122	24,923 (22,944; 27,241)	1.3 (0.005–321)	0.93
CD279 MFI—intermediate monocytes	69	45 ± 109	11 (− 7.4; 74)	12	103 ± 207	26 (− 37; 164)	3.2 (0.60–17)	0.18
HLA-DR MFI—intermediate monocytes	69	1643 ± 791	1380 (1180; 2022)	12	1382 ± 756	1196 (647; 2053)	0.08 (0.003–2.1)	0.13
CD64 MFI—intermediate monocytes	69	25,335 ± 7530	23,912 (19,508; 29,242)	12	26,032 ± 5305	25,458 (22,585; 29,091)	4.4 (0.025–777)	0.58
CD279 MFI—non-classical monocytes	69	166 ± 133	142 (103; 211)	12	172 ± 85	192.3 (108; 221)	1.4 (0.21–8.9)	0.73
HLA-DR MFI—non-classical monocytes	69	6615 ± 4883	6328 (1962; 10,108)	12	7973 ± 4160	6745 (4431; 11,246)	4.0 (0.64–24.8)	0.14
CD64 MFI—non-classical monocytes	69	12,141 ± 8841	8343 (5272; 16,776)	12	12,653 ± 7537	10,944 (6659; 16,946)	1.9 (0.25–14)	0.55
CD62Lneg monocytes/µl	80	91 ± 94	48 (24; 131)	19	185 ± 196	179 (44; 247)	4.5 (1.4–14.5)	0.011
Total neutrophils/µl	80	7838 ± 3815	7045 (5365; 10,160)	19	8601 ± 4456	7310 (4720; 12,670)	2.1 (0.19–23)	0.55
CD62L MFI—neutrophils	80	7571 ± 2585	7677 (5704; 9344)	19	6658 ± 1751	6405 (5919; 7298)	0.21 (0.010–4.4)	0.32
CD16 MFI—neutrophils	69	1773 ± 654	1720 (1466; 2176)	12	1623 ± 395	1661 (1384; 1820)	0.49 (0.013–19)	0.70
CD64 MFI—neutrophils	80	1517 ± 1040	1293.5 (890; 1801)	19	1490 ± 876.1	1285 (699; 1849)	1.1 (0.20–6.4)	0.89
CD11b MFI—neutrophils	80	11,569 ± 6583	9645 (7279–14,752)	19	11,000 ± 5935	9057 (6674–15,051.)	0.59 (0.061–5.7)	0.65
CD11c MFI—neutrophils	80	723 ± 350	622.8 (522; 788)	19	853 ± 423	813 (476; − 982)	7.9 (0.47–131)	0.15
CD62Lneg neutrophils/µl	80	1067 ± 925	819.6 (245; 1613)	19	758 ± 698	487 (204; 1197)	0.54 (0.20–1.5)	0.24
Total lymphocytes/µl	80	1303 ± 688	1200 (810; 1715)	19	1261 ± 481	1180 (990; 1470)	1.2 (0.12–12)	0.87
CD4 + lymphocytes/µl	80	620 ± 342	610 (346.0; 829.1)	19	605 ± 232	612 (432; 779)	1.5 (0.21–11)	0.69
CD8 + lymphocytes/µl	80	281 ± 271	204 (143.7; 362.3)	19	261 ± 179	208 (150; 323)	0.88 (0.15–5.0)	0.88

Table 3 (continued)

	Nonseptic ($N=80$)			Septic ($N=19$)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1; Q3)	N	Mean ± SD	Median (Q1; Q3)	OR (95%CI)	p value
CD4 + CD69 + lymphocytes/µl	80	61 ± 62	45 (30.0; 67.0)	19	71 ± 46	54 (43; 97)	2.5 (0.52–12)	0.25
CD4 + CD279 + lymphocytes/µl	80	168 ± 89	156 (101.8; 215.1)	19	189 ± 149	167 (113; 199)	1.5 (0.19–11)	0.71
CD8 + CD69 + lymphocytes/µl	80	61 ± 102	33 (17.4; 53.7)	19	96 ± 125	51 (28; 137)	2.8 (0.93–8.2)	0.069
CD8 + CD279 + lymphocytes/µl	80	89 ± 65	76 (46.8; 100.9)	19	103 ± 100	77 (47; 111)	1.5 (0.28–8.6)	0.62
CD69 MFI—CD4 + CD69 + lymphocytes	80	369 ± 95	359 (320; 414)	19	343 ± 76	338 (280; 388)	0.042 (0.001–9.2)	0.25
CD69 MFI—CD8 + CD69 + lymphocytes	80	683 ± 867	483 (397; 688)	19	1030 ± 1601	624 (504; 822)	4.4 (0.72–27)	0.11
CD279 MFI—CD4 + CD279 + lymphocytes	80	232 ± 54	218 (193; 255)	19	236 ± 37	232 (206; 251)	4.3 (0.016–999)	0.61
CD279 MFI—CD8 + CD279 + lymphocytes	80	269 ± 95	236 (201; 293)	19	310 ± 109	277 (247; 343)	26 (0.74–901)	0.073
B lymphocytes/µl	80	203 ± 242	149 (89; 233)	19	187 ± 166	147 (67; 225)	1.1 (0.28–4.0)	0.93
CD25 + B lymphocytes/µl	80	59 ± 218	16 (7.4; 37)	19	49 ± 91	19 (9.4; 40)	1.3 (0.51–3.0)	0.63
CD25 MFI—Tregs	80	3473 ± 691	3434 (2939; 3917)	19	3744 ± 980	3704 (291; 4682)	25 (0.096–999)	0.26
CD127 MFI—CD4 + lymphocytes	80	1378 ± 372	1313 (1111; 1660)	19	1420 ± 330	1496 (1095; 1671)	3.9 (0.05–304)	0.54
CD127 MFI—Tregs	80	209 ± 65	197 (162; 246)	19	211 ± 78	198 (161; 237)	0.89 (0.022–37)	0.95
Tregs/µl	80	59 ± 34	55 (32; 75)	19	56 ± 223	60 (39; 71)	1.0 (0.13–7.9)	0.99

monocytes of infected newborns compared with controls [42]. L-selectin is a leucocyte surface glycoprotein which mediates extravasation and recruitment of white blood cells to sites of inflammation. Its downregulation in vitro had been shown in murine and human neutrophils and this was the first report of in vivo downregulation of L-selectin on granulocytes and monocytes [44–47]. Authors postulated that bacterial stimuli such as FMLP (*N*-formyl-methionyl-leucyl-phenylalanine)-related peptides or lipopolysaccharides or host-derived soluble mediators such as those released during acute systemic inflammatory response syndrome (cytokines, C5a, leukotriene B4) may have triggered L-selectin downregulation. Furthermore, a more recent study focusing on regional and systemic immune responses before, during and after major splanchnic surgery showed that intraoperative splanchnic hypoperfusion and mucosal acidosis led to monocyte deactivation [48]. In that study, 20 patients who underwent resection for cancer of the esophagus had no difference in monocyte marker expression in the preoperative period. They were categorized into 3 groups according to the nadir perioperative intestinal pH. Those who developed postoperative sepsis (5/20) had the lowest intestinal pH, a persistently lower postoperative expression of L-selectin and m-HLA-DR and a more acute phase response (higher CRP) compared to non-sepsis patients, similar to our findings. The authors concluded that severe mucosal acidosis, secondary to splanchnic hypoperfusion and increased intestinal permeability during major surgery, was associated with regional and systemic immune suppression predisposing to sepsis.

Our results are not in accordance with an observational study including 41 severely traumatized patients who underwent sampling and staining of 3 leucocyte subsets for CD62L, 1 h and 20 h after trauma [49]. The authors found that monocytes, lymphocytes and neutrophils showed an early increase in CD62L cell surface expression and that this persisted in the later samples up to 20 h. However, association with subsequent sepsis occurrence was not an endpoint in the latter study. In a more recent study aiming at guiding the optimal timing of non-lifesaving orthopedic surgery for trauma, authors hypothesized that neutrophils

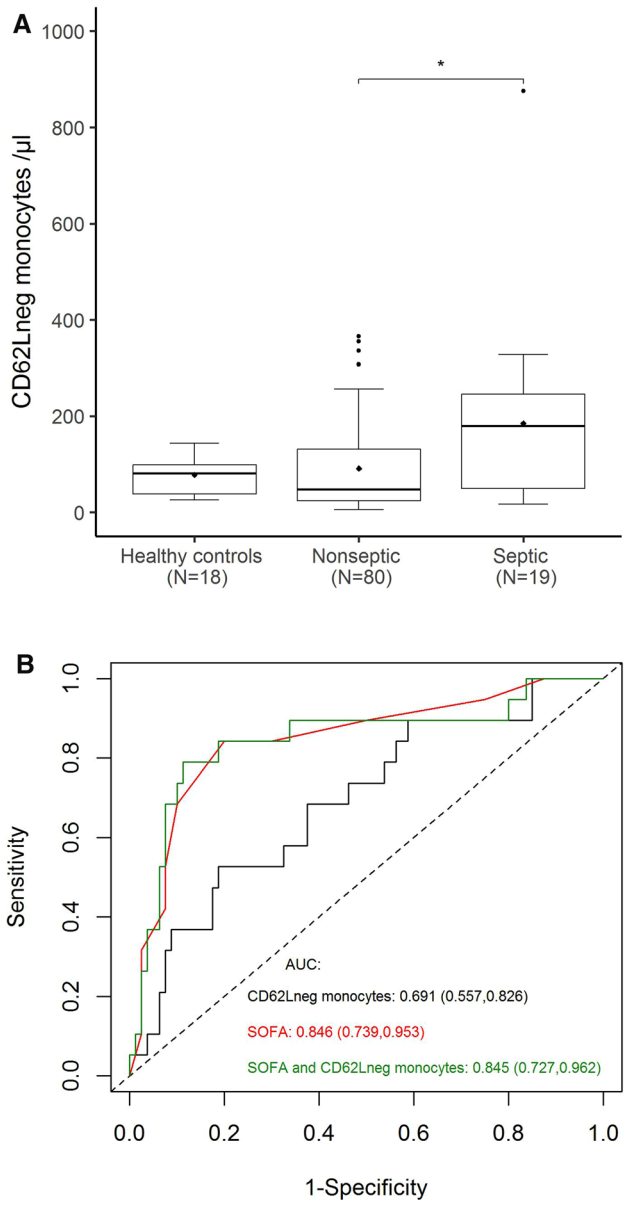

Fig. 1 **A** Measurements at ICU admission in nonseptic and septic patients and in healthy controls (> 50 years). (*$p < 0.05$). **B** Predictive value of $CD62L_{neg}$ monocytes absolute count (/µl) obtained at T1. ROC curve analysis of sepsis occurrence based on levels of $CD62L_{neg}$ monocytes and SOFA is shown

and monocytes express activation markers prior to sepsis development [50]. They found that in the perioperative period, elevated monocyte L-selectin (AUC 0.76 [95%CI 0.63–0.89] was a significant predictor of sepsis, thereby precluding urgent surgery. However, these patients were not critically ill.

Considering expression of mHLA-DR, our results confirm those of older single-center single-biomarker studies [20, 21] and of two more recent multi-center studies [25, 28]. The first multi-center study validated a combined immune dysfunction score associated with sepsis development in a cohort of patients described as requiring organ support for more than 48 h in the ICU [25]. Trauma and surgery were among the inclusion criteria but sepsis patients were also included. The score encompassed low mHLA-DR (Youden index optimal cutoff < 10,000 molecules/cell), elevated T_{regs} and low neutrophil CD88. In our study, T_{regs} were not found to be predictive of sepsis probably because of earlier serial sampling and different case-mix. Indeed, elevation of T_{regs} was only seen 6–10 days after ICU admission in the aforementioned study and sepsis patients were included, contrary to our study. Elevated levels of these suppressor cells have frequently been reported in sepsis patients, reflecting severity of disease and predisposition to secondary infections, but very seldomly in injury, such as in our study, prior to the occurrence of a primary infection [51–53]. The second recent large multicenter study explored mHLA-DR and ex vivo TNF-α release in sepsis, trauma and postoperative patients in association with adverse clinical outcome (death or secondary infection) [19, 28]. It showed persistent decreases of both markers at days 5–7 post ICU admission to be associated with both outcomes, whatever the type of injury.

Finally, our results are partly corroborated by a recent study investigating the potential of HLA-DR expression by monocyte subsets in diagnosing sepsis in cardiac surgery patients [54]. The authors showed that that there was a significant downregulation, in the postoperative period, of mHLA-DR on both intermediate ($p = 0.0477$) and non-classical monocytes ($p = 0.033$). However, in contrast to our findings, it is the combination of the reduced preoperative count and postoperative HLA-DR expression of the non-classical compound that was found to be associated with sepsis occurrence at 48 h post cardiac surgery, with a 100% sensitivity and 69.2% specificity.

Concerning cytokine measurements and despite controversy regarding their contribution to diagnosis and prognostication of sepsis and systemic inflammation, our results are in line with those of Venet et al. [28, 55]. Indeed, these authors report similar median values of IL-6 and IL-10 at day 1 in the cohorts of trauma and surgical patients compared to our patients. Furthermore, they observed no difference between secondarily septic and non-septic patients. Samplings of TNF-α and IFN-γ were done ex vivo, contrary to ours, and were found to be weakly elevated.

Conclusion

Our study has several limitations among which, a single-center design and a small sample size. Furthermore, due to its exploratory nature, there was no a priori planned hierarchical clustering of surface markers, rendering consistency

Table 4 Impact of parameters 48–72 h after ICU admission (T2) on the risk of sepsis

	Nonseptic ($N=80$)			Septic ($N=19$)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1; Q3)	N	Mean ± SD	Median (Q1; Q3)	OR (95%CI)	p value
Total monocytes/µl	79	992 ± 452	950 (650; 1240)	15	1247 ± 997	980 (810; 1440)	4.5 (0.27–73)	0.30
HLA-DR MFI—total monocytes	79	1146 ± 556	972 (766; 1544)	15	690 ± 303	685 (435; 824)	0.004 (0.000–0.10)	0.0011
CD14 MFI—total monocytes	69	19,581 ± 6805	18,541 (15,644; 23,293)	9	19,714 ± 5996	18,758 (14,917; 25,446)	1.4 (0.011–169)	0.90
CD16 MFI—total monocytes	69	198 ± 127	171 (113; 240)	9	191 ± 88	174 (126; 198)	1.5 (0.077–28)	0.80
CD64 MFI—total monocytes	79	31,472 ± 7345	32,326 (26,274; 36,512)	15	28,749 ± 7858	27,011 (22,435; 36,294)	0.042 (0.000–4.7)	0.19
CD279 MFI—total monocytes	79	51 ± 160	24 (− 21; 74)	15	100 ± 119	91 (20; 152)	2.9 (0.74–11)	0.13
Classical monocytes/µl	69	633 ± 328	589 (393; 828)	9	624 ± 245	562 (419; 747)	1.5 (0.054–43)	0.80
Intermediate monocytes/µl	69	271 ± 164	226 (143; 383)	9	283 ± 97	259 (195; 324)	3.3 (0.17–63)	0.43
Non-classical monocytes/µl	57	61 ± 47	50 (29; 75)	8	66 ± 31	68 (45; 83)	2.5 (0.25–26)	0.43
CD279 MFI—classical monocytes	69	6.6 ± 142	− 19 (− 39; 25)	9	62 ± 137	33 (− 15; 43)	1.4 (0.32–5.8)	0.67
HLA-DR MFI—classical monocytes	69	976 ± 460	874 (642; 1296)	9	579 ± 228	560 (443; 761)	0.006 (0.000–0.26)	0.0081
CD64 MFI—classical monocytes	69	31,749 ± 7633	32,367 (26,010; 36,052)	9	29,332 ± 7667	26,825 (23,234; 36,640)	0.080 (0.000–26)	0.39
CD279 MFI—intermediate monocytes	69	89 ± 205	60 (− 2.0; 115)	9	181 ± 191	154 (39; 178)	1.2 (0.35–4.0)	0.79
HLA-DR MFI—intermediate monocytes	69	1744 ± 769	1678 (1195; 2224)	9	1131 ± 733	941 (591; 1694)	0.003 (0.000–0.17)	0.0049
CD64 MFI—intermediate monocytes	69	33,603 ± 7755	34,545 (27,854; 37,880)	9	32,401 ± 9496	30,484 (24,179; 38,814)	0.20 (0.000–82)	0.60
CD279 MFI—non-classical monocytes	69	184 ± 124	160 (112; 232)	9	221 ± 97	206 (192; 241)	2.9 (0.29–30)	0.37
HLA-DR MFI—non-classical monocytes	69	8213 ± 3628	7898 (5738; 10,756)	9	5929 ± 3234	5663 (3055; 7805)	0.074 (0.004–1.2)	0.070
CD64 MFI—non-classical monocytes	69	18,781 ± 6999	18,782 (14,162; 23,492)	9	17,638 ± 6443	18,175 (12,364; 23,814)	0.57 (0.018–19)	0.76
CD62Lneg monocytes/µl	79	158 ± 100	143 (82; 202)	15	170 ± 133	157 (74; 197)	0.94 (0.13–7.0)	0.95
Total neutrophils/µl	79	9057 ± 3048	8470 (7160; 10,430)	15	8766 ± 3611	8040 (6780; 10,560)	0.31 (0.007–13)	0.54
CD62L MFI—neutrophils	79	6618 ± 1519	6787 (5603; 7695)	15	6548 ± 2394	5984 (5057; 7099)	0.34 (0.002–50)	0.67
CD16 MFI—neutrophils	69	1956 ± 689	1872 (1442; 2318)	9	1863 ± 611	2027 (1233; 2495)	0.52 (0.007–40)	0.77
CD64 MFI—neutrophils	79	1901 ± 926	1619 (1263; 2305)	15	2281 ± 1689	1688 (1018; − 2787)	2.1 (0.16–27)	0.57
CD11b MFI—neutrophils	79	12,027 ± 6866	9577 (7330; 16,463)	15	13,650 ± 7155	13,145 (8932; 19,791)	2.3 (0.23–23)	0.48
CD11c MFI—neutrophils	79	1220 ± 641	1056 (748; 1512)	15	1329 ± 687.0	1250.7 (666; 1926)	1.8 (0.13–26)	0.66
CD62Lneg neutrophils/µl	79	853 ± 849	512 (324; − 974)	15	827 ± 1153	551.3 (271; 893)	0.78 (0.19–3.2)	0.73
Total lymphocytes/µl	79	1220 ± 620	1140 (830; 1540)	15	1011 ± 336	1120 (760; 1210)	0.21 (0.013–3.5)	0.28
CD4 + lymphocytes/µl	79	524 ± 240.8	490 (356; 639)	15	434 ± 175	421 (291; 568)	0.15 (0.009–2.7)	0.20
CD8 + lymphocytes/µl	79	255 ± 172.9	206 (135; 344)	15	213 ± 131	183 (90; 308)	0.49 (0.084–2.9)	0.43

Table 4 (continued)

	Nonseptic ($N=80$)			Septic ($N=19$)			Univariate logistic regression	
	N	Mean ± SD	Median (Q1; Q3)	N	Mean ± SD	Median (Q1; Q3)	OR (95%CI)	p value
CD4 + CD69 + lymphocytes/µl	79	60 ± 34.8	51 (33; − 75)	15	57 ± 30	61 (35; 70)	0.87 (0.10–27.6)	0.90
CD4 + CD279 + lymphocytes/µl	79	179 ± 98	159 (116; 217)	15	121 ± 59	107 (97; 121)	0.044 (0.003–0.69)	0.026
CD8 + CD69 + lymphocytes/µl	79	53 ± 89	27 (19; − 53)	15	71 ± 65	46 (24; − 97)	2.8 (0.75–11)	0.12
CD8 + CD279 + lymphocytes/µl	79	95 ± 70	77 (50; 126)	15	80 ± 62.2	55 (31; 106)	0.56 (0.10–3.1)	0.50
CD69 MFI—CD4 + CD69 + lymphocytes	79	329 ± 56	324 (289; 356)	15	343 ± 81	319 (287; 381)	11 (0.008–999)	0.51
CD69 MFI—CD8 + CD69 + lymphocytes	79	771 ± 1162	527 (410; 731)	15	733 ± 441	605 (490; 725)	1.7 (0.19–16)	0.64
CD279 MFI—CD4 + CD279 + lymphocytes	79	258 ± 58	247 (212; 283)	15	247 ± 24	253 (221; 257)	0.25 (0.001–226)	0.69
CD279 MFI—CD8 + CD279 + lymphocytes	79	300 ± 91	289 (235; 346)	15	319 ± 79	311 (241; 385)	8.2 (0.092–724)	0.36
B lymphocytes/µl	79	216 ± 297	159 (108; 223)	15	191 ± 252	116 (56; 235)	0.50 (0.11–2.3)	0.38
CD25 + B lymphocytes/µl	79	66 ± 285	167 (7.5; 31)	15	55 ± 147	14 (7.4; − 35)	0.85 (0.29–2.5)	0.76
CD25 MFI—Tregs	79	3970 ± 1060	3818 (3319; 4600)	15	4150 ± 942	4135 (3593–4633)	6.2 (0.039–999)	0.48
CD127 MFI—CD4 + lymphocytes	79	1193 ± 373	1181 (878; 1493)	15	1230 ± 337	1195 (997; 1551)	2.9 (0.049–171)	0.61
CD127 MFI—Tregs	79	184 ± 69	176 (145; − 217)	15	197 ± 65	204 (133–237)	4.7 (0.12–180)	0.41
Tregs/µl	79	53 ± 27	46 (34; 72)	15	41 ± 16	37 (30–48)	0.12 (0.008–2.0)	0.14

and fit-of-the model arguable. Validation of the two monocyte markers and of sampling times in a bigger cohort of patients could help to identify an optimal combination for sepsis prediction. Third, sampling times were limited and evolution of the biomarkers cannot be inferred past the third day of ICU admission. Furthermore, in patients who went on to develop sepsis, there are missing data in 7/19 for $CD62L_{neg}$ monocytes and 12/19 for mHLA-DR, respectively, thereby hindering interpretation of the biomarkers' levels time course. Fourth, potential confounders affecting the immune response to injury, such as blood transfusions and general anesthetics, were not taken into account at this stage [56]. Fifth, sepsis occurrence was lower than expected (19% versus 25–35% in other studies) probably owing to the predominance of cardiac surgery patients who received prophylactic antibiotic therapy. Finally, we cannot exclude that some patients might have been in a pre-septic condition although high expression of neutrophil CD64, which is a recognized marker of bacterial infection, was not found at ICU admission [57–60]. Furthermore, CRP and fibrinogen levels were within normal ranges at ICU admission. It must be emphasized that procalcitonin was purposely not included in the design of the study because of known poor specificity as a diagnostic marker of sepsis in injured patients, as shown previously by our group [61].

In conclusion, this preliminary study showed that, in a selected population of critically injured patients, monocytes either in absolute count or via downregulation of specific surface markers, are predictive of subsequent sepsis development upon ICU admission and 48 h later. Further validation in a bigger cohort of patients, perhaps in combination with recently published biomarkers, is warranted before envisaging a preventive immunomodulatory approach of sepsis in injured patients [62]. In clinical practice, the latter approach could be feasible thanks to the readily available complete blood count and to a recent proof-of-concept study showing promising results for mHLA-DR bedside monitoring [63].

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s15010-023-01983-3.

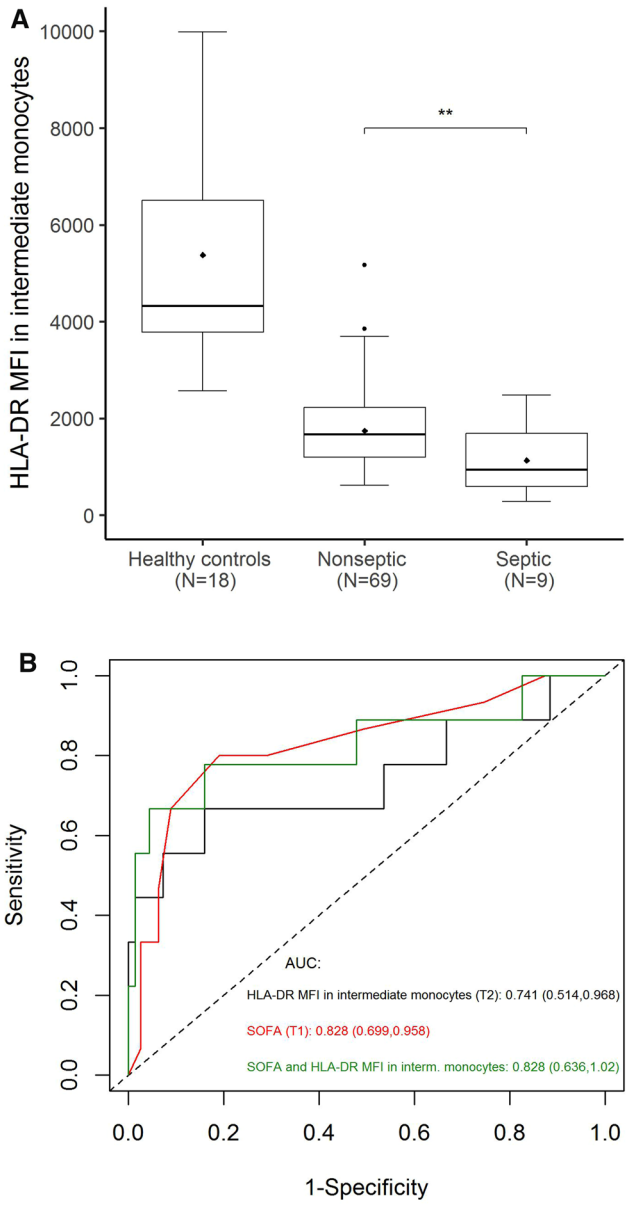

Fig. 2 **A** Measurements at T2 in nonseptic and septic patients and in healthy controls (>50 years). (**$p<0.001$). **B** Predictive value of intermediate (CD14++CD16+) monocyte expression of HLA-DR (MFI) obtained at T2. ROC curve analysis of sepsis occurrence based on HLA-DR MFI levels in intermediate monocytes obtained at T2 and calculation of SOFA score at T1 is shown

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Availability of data and materials The datasets used and/or analysed (beyond those included in the supplementary files) during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no competing interests.

Ethics approval and consent to participate The study was appointed the Belgian number B707201111981 by the local ethics committee of University Hospital of Liège (number 707) and written informed consent was obtained from the patient or his/her legal representative.

Consent for publication Not applicable.

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