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A reactive monocyte subset characterized by low expression of CD91 is expanded during sterile and septic inflammation

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Abstract

Objectives: This study was undertaken to assess CD91 expression on monocytes and changes in monocyte subset distribution during acute tissue damage and bloodstream infection (BSI).
Methods: We investigated blood specimens from healthy individuals, trauma and cardiac surgery patients as a model of tissue damage, and patients with BSI, by flow cytometry using a panel of antibodies comprising CD45, HLA-DR, CD14, CD16 and CD91 for the identification of monocyte subsets.
Results: While infrequent in healthy subjects, CD91low/neg monocyte levels were markedly high in BSI, trauma and after cardiac surgery. This monocyte subset expanded up to 15-fold in both patient cohorts, whereas CD14+CD16+ inflammatory monocytes were multiplied by a factor of 5 only. CD14+CD91low monocytes displayed a significantly lower density of HLA-DR and markedly reduced expression of CD300e, compared to the other subsets. They also expressed high levels of myeloperoxidase and showed robust phagocytic and oxidative burst activity.
Conclusions: Expansion of CD91low monocytes is a sensitive marker of acute inflammatory states of infectious and non-infectious etiology.

Keywords: monocytes; CD91; bloodstream infection (BSI); inflammation; sepsis; flow cytometry

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Introduction

It is well known that monocytes migrate to sites of infection or cell injury and have critical roles in pathogen defense, homeostasis and tissue repair. Both sterile and septic inflammation will induce the expansion of monocytes and their subsequent release from the bone marrow into the peripheral blood. Monocytes are categorized by the expression of the central lipopolysaccharide receptor CD14 and the low affinity type III Fcγ receptor CD16 (FcγRIII), into classical CD14+CD16−, intermediate or inflammatory CD14+CD16+, and non-classical CD14lowCD16+ monocytes. This subset classification was endorsed by the Nomenclature Committee of the International Union of Immunological Societies [1]. Studies on specialized function assignment and subset relationships are usually based on this nomenclature. Of special interest, CD14+CD16+ monocytes have been shown to expand in a variety of inflammatory disorders and have proven to be of prognostic relevance in cardiovascular disease [2, 3] and chronic kidney disease [4], among others. Classical monocytes display the most significant phagocytic activity, whereas non-classical monocytes have been reported to patrol the lining of the endothelium for immune nucleic acid complexes and release pro-inflammatory cytokines (tumor necrosis factor (TNF-α), interleukin-1 beta (IL-1β) and CC motif chemokine ligand 3 (CCL-3)) in response to viral infection [5, 6].

CD91 is a member of the low-density lipoprotein receptor (LRP) family binding a wide range of distinct ligands among them α2-macroglobulin, complement component 1q (C1q) and defensins [7, 8]. Although CD91 is known to be expressed on monocytes and dendritic cells [9, 10], it has broad tissue distribution and takes part in lipoprotein metabolism, lysosomal degradation and cellular migration [7, 8, 11, 12]. CD91 has also been shown to be an active receptor for antigenic peptides chaperoned by heat shock proteins released in response to cellular stress induced by oxidative damage [13].

CD91 is expressed by all three monocyte subpopulations, although with lower intensity in non-classical monocytes. As a pan-monocytic marker, CD91 has been shown to be better for the identification of all blood monocytes, compared to CD33 which may vary considerably between

individuals, and to CD14 which is low to negative in some CD16+ monocytes [14, 15]. While this is the case for monocytes in steady-state, it has not been addressed so far whether CD91 is also expressed by all monocytes in inflammatory states. In studying the dynamics of monocyte subset redistribution in pathological conditions, we recently uncovered a significant fraction of monocytes expressing low to negligible amounts of CD91 [16].

In this paper, the morphology, phenotype, phagocytic and oxydative burst activity of the CD91low/– subset of blood monocytes were assessed in sterile and septic inflammation. These cells are characterized by low levels of HLA-DR and CD300, are rare in healthy subjects but expand markedly in both sterile and septic inflammation.

Materials and methods

Patients

The study population involved 60 patients with a bloodstream infection (BSI), 10 trauma patients and 145 patients undergoing cardiac surgery. Forty-six healthy volunteers were recruited among the laboratory staff. Diagnosis of BSI was based on a positive microbiological blood culture as the reference procedure. Cardiac surgery patients were drawn at 2-, 24- and 48-h post-surgery. After 48 h, only those with C-reactive protein (CRP) levels above 180 mg/L were included in the study in order to ensure that phenotypic changes during acute nonseptic inflammation were being maximized. Cardiac surgery patients received cefuroxime prophylactic antibiotic therapy for the first 24 h.

Electronic medical record (EMR) data of BSI patients, i.e., Sequential Organ Failure Assessment Score (SOFA) and Simplified Acute Physiology Score (SAPS) II, lactate levels and temperature were collected from patients in intensive care unit.

Flow cytometric cell staining and reagents

Leukocyte enumeration was obtained on a Sysmex XS-800 hematology analyzer (Kobe, Japan). For monocyte immunophenotyping, a core combination consisted of anti-CD91 PerCP eFluor710 (Thermo-Fisher, MA, USA, clone A2MR-α2), HLA-DR FITC (Sony Biotechnology, CA, USA, clone L243), CD14 APC-H7 (BD Biosciences, CA, USA, clone MρP9), CD16 PE (BD Biosciences, clone 3G8) and CD45 V500 (BD Biosciences, clone HI30). In addition, to extend the phenotypic characterization of the different monocyte subsets, CD11b APC (Beckman-Coulter, CA, USA, clone Bear1), CD11c PerCP-Cy5.5 (BD Biosciences, clone B-ly6), CD13 PE (BD Biosciences, clone L138), CD14 APC-Cy7 (Sony Biotechnology, clone: HCD14), CD16 Pacific Blue (Sony Biotechnology, clone 3G8), CD16 V450 (BD Biosciences, clone 3G8), CD33 PE-Cy7 (BD Biosciences, clone P67.6), CD36 V450 (BD Biosciences, clone CB38), CD62L V450 (BD Biosciences, clone DREG-56), CD64 PE-Cy7 (BD Biosciences, clone 10.1), CD66b PerCP-Cy5.5 (Sony Biotechnology, clone G10F5), CD86 PE (BD Biosciences, clone 2331), CD91 BV421 (BD Biosciences, clone A2MR-α2), CD91 eFluor 660 (ThermoFisher, clone A2MR-α2), CD121b PE-Vio770 (Miltenyi Biotec, Bergisch Gladbach, Germany, clone REA744), CD300e APC (BD Biosciences, clone UP-H2), HLA-DR FITC (Beckman Coulter, clone B8.12.2), anti-human Lysozyme FITC (Agilent, polyclonal) and anti-human Myeloperoxidase PE (Beckman Coulter, clone CLB-MPO-1) were used for complementary analyses. Median fluorescence intensities of cell surface markers of different monocyte subpopulations and quantitative cell counts (number of cells/μL) were measured. The composition of antibody panels is detailed in Supplemental Material, Appendix 1.

EDTA peripheral blood was stored at room temperature prior to staining and immunostaining was performed within 12 h of blood collection. A volume of 100 μL was incubated with pre-mixed antibodies for 20 min in the dark at room temperature, after which red blood cells were lysed by the addition of BD FACS Lysing Solution (BD Biosciences). Cells were centrifuged and resuspended in phosphate-buffered saline with 1% paraformaldehyde and kept at 4 °C. Fix&Perm® cell permeabilization kit (An Der Grub Bio Research GmbH, Austria) was used for intracellular detection of lysozyme and myeloperoxydase.

Flow cytometry acquisition was performed within 1 h of staining on an FACS Canto II (BD Biosciences). A minimum of 6000 CD14 positive cells were acquired per sample. Cyto-Cal™ calibration beads were used daily to adjust the sensitivity of the flow cytometer. BD cytometer setup and tracking (CS&T) beads were used to ensure day-to-day stability of fluorescence detection overtime. Automatic compensation matrices were generated with BD FACS DIVA software v8.0.1. Flow cytometric data were analyzed with Kaluza software (v2.1, Beckman Coulter).

In sorting experiments, stained cells were run on a BD FACSAria II cell sorter, using appropriate color compensation. Sorted cells were transferred in cytofunnels (Thermo, A78710020) and cytospin slides were prepared with the Thermo Shandon Cytospin-4 centrifuge. Slides were stained with May-Grünwald Giemsa.

Measurement of phagocytic activity and oxidative burst

The percentages of monocytes and granulocytes achieving phagocytosis were evaluated using the Phagotest kit (CELONIC, Heidelberg, Germany) using FITC-stained *E. coli*. Phagocytic activity was quantified by the Median Fluorescence Intensity (MFI) of the FITC channel, which correlates with the number of bacteria ingested per cell. The oxidative capacity of monocyte subsets was measured by the Phagoburst test (CELONIC). This assay measures the percentage of cells that produce reactive oxidants by assessing the conversion of dihydrorhodamine 123 to rhodamine 123 in presence of phorbol 12-myristate 13-acetate.

Statistics

Graphs and statistical tests were carried out with Graphpad Prism 8 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± standard error of the mean (SEM). The significance between two variables was established using the Wilcoxon signed-rank test. Normalized monocyte count/μL was defined as the ratio of the mean of the different subject groups to the mean of healthy controls. Normalized counts were used to assess the extent of monocyte subset redistribution in trauma, BSI and cardiac surgery patients. Monte Carlo simulations were performed to derive SEMs of average fold changes in normalized monocyte cell counts. A p-value <0.05 was considered statistically significant.

Results

Patient characteristics

After cardiac surgery, CRP levels at 2, 24 and 48 h were 3.4 ± 0.9, 69.3 ± 3.8, 242 ± 6.5 respectively. In the positive blood culture patients, CRP was 218 ± 15 mg/L. Among them, 17 patients had clinical sepsis and eight were in septic shock with mortality of 17 and 50 % respectively. Cardiac surgery patients collected at 2, 24, and 48 h postoperatively were treated by prophylactic cefuroxime antibiotherapy for the first 24 h. For each subject, the clinical profile, CRP, and blood cell count were recorded (Table 1).

Identification of the CD91low monocyte subset

We set up a monocyte core panel with CD14, CD16, CD45 and CD91. The time gate was used to control the stable flow of cells (Figure 1A, a). The forward scatter area (FSC-A) and forward scatter height (FSC-H) were used for exclusion of cell doublets (Figure 1A, b). Next, an SSC/CD45 dot plot was used to select mononuclear cells and exclude the bulk of neutrophils and debris (Figure 1A, c). Then, by combining all CD14+ cells and all CD91+ cells, the whole blood monocyte population was selected (Figure 1A, e–n), while excluding remaining CD91–/CD14– lymphocytes and neutrophils. This allowed us to identify CD14+CD91low monocytes as a distinct subset (gate 1). Although there were very few CD91low monocytes in healthy subjects as previously reported [14], a significant increase of these cells was observed after trauma, heart surgery and in systemic infection. CD91+ cells (gate 2) were further visualized on a CD14/CD16 plot, wherein a rectangular gating strategy was used to delineate the three monocyte subsets (Figure 1A, f–o): classical CD14+CD16– (gate 3), intermediate CD14+CD16+ (gate 4) and non-classical CD14lowCD16+ monocytes (gate 5). Gating of the intermediate population was fixed relative to the upper limit of CD16 expression and the lower limit of CD14 expression in the classical subset. This sequential gating was carried out in healthy patients (Figure 1A, e–g), trauma patients (Figure 1A, h–j), heart surgery patients (Figure 1A, k–m) and BSI patients (Figure 1A, n–p). Backgating of the CD91low subset on the CD14/CD16 dot plot was used to illustrate their relationship to the common monocyte populations: CD91low monocytes co-localize mostly with classical CD14+/CD16– monocytes, with respect to the CD14 and CD16 expression.

Monocytes belonging to the four monocyte subsets were sorted separately and stained on cytospin slides (Figure 1B). Compared to classical, intermediate and non-classical monocytes, nuclei of CD91low monocytes display a more uniform and regular shape.

CD91low monocytes are increased in systemic inflammation and tissue injury

We explored the dynamic redistribution of the four monocyte subsets in patients with BSI, assessed by a positive blood culture, in trauma subjects and in cardiac surgery patients after 2, 24 and 48 h, as a model of sterile inflammation (Figure 2). In absolute counts, classical CD14+CD16– monocytes predominate in all conditions (Figure 2A). The reference range for CD14+CD91low cell counts in 46 healthy subjects is narrow, not exceeding 24 cells/µL with an average of 9 cells/µL. In relative count, the average is 1.7 % with a maximum of 4.8 % of total monocytes.

In cardiac surgery patients, CD14+CD91low monocytes increased ($p<0.05$) as early as 2 h after the operation, before any significant change in the other blood cell subsets, and prior to CRP elevation. In absolute counts, the CD91low monocytes peaked at 24 h and then declined afterwards while the CD14+CD16+ intermediate subset was still expanding at 48 h (Figure 2A). As percent of total monocytes, CD14+CD91low monocytes rose to 15.4 % 2 h after operation and to 14.1 % at 24 h, and then declined to 2.9 % at 48 h (Figure 2B). In trauma patients, a similar increase in CD91low monocytes was found, reaching 19.6 % of total monocytes. When looking at fold-change compared to baseline (Figure 2C), variations of CD91low monocytes were more pronounced by far among the different monocyte subsets, and thus should be considered as the earliest monocyte change in acute inflammation.

In subjects with BSI, we noticed a major increase of CD91low ($p<0.05$) and, to a lesser extent, of intermediate monocytes, while classical and non-classical subsets showed less variation compared to healthy controls. BSI patients had 10.3 % of CD14+CD91low monocytes, indicating a significant increase compared to healthy individuals (Figure 2B). Intermediate monocytes were found to be particularly high compared to healthy subjects with a proportion of monocytes exceeding 50 % of the total number in five of 60 infected patients. Again, in fold-change analysis, CD14+CD91low cell counts rose drastically among BSI patients ($p<0.05$) while the elevation of the other subsets was of lesser magnitude (Figure 2C).

Considering the whole monocyte population, CD91 was significantly downregulated in trauma patients, in heart-operated patients at H2, H24, and during BSI ($p<0.05$). At 48 h

Table 1: Laboratory and demographic characteristics of patients.

Characteristics	Healthy (n=46)	Trauma (n=10)	Cardiac surgery (n=145)			BSI (n=60)
			H_2 (n=22)	H_{24} (n=80)	H_{48} (n=43)	
C-reactive, protein mg/L (mean ± SEM)	<5	67.4 ± 19.2	3.4 ± 0.9	69 ± 3.8	242 ± 6.5	218 ± 15
Neutrophil, count/µL (mean ± SEM)	3,368 ± 113	7,947 ± 1,433.8	8,687 ± 633	9,944 ± 334	8,706 ± 387	13,543 ± 891
Lymphocyte, count/µL (mean ± SEM)	2016 ± 72	1,062 ± 223.5	1792 ± 155	922 ± 48	1,296 ± 69	999 ± 84
Monocyte, count/µL (mean ± SEM)	561 ± 23	759 ± 131.6	682 ± 67	1,071 ± 46	1,173 ± 57	1,040 ± 88
(Mean ± SEM)						
Age, median years	43	58	69			66
Gender, M/F, %	58/42	70/30	72/28			62/38
Hospitalization length, median days	–	–	12			31
Decease after admission, median days	–	–	–			61
Hospitalized mortality	–	–	5.3 %			30.5 %
Trauma: Cause of hospital admission						
Highway accident						60 %
Domestic incident						30 %
Firearm						10 %
Cardiac surgery: Cause of hospital admission						
Coronary artery bypass graft						57.5 %
Valve replacement						33.5 %
Other						9 %
BSI: Cause of hospital admission						
Localized infection – sepsis – septic shock						33 %
Postsurgery monitoring						10 %
Renal insufficiency						8 %
Heart failure						8 %
Hematological malignancy						7 %
Respiratory failure						7 %
Trauma						7 %
Digestive tract hemorrhage						7 %
Cerebrovascular accident						7 %
General deterioration						3 %
Carcinoma						3 %
BSI: Site of infection						
Urinary tract						25 %
Other						22 %
Lung						18 %
Abdominal						15 %
Endocarditis						10 %
Surgical site						7 %
Catheter						3 %

Data of healthy, cardiac surgery and BSI patients were previously reported in [16].

post-surgery, CD91 expression recovered to levels similar to healthy individuals (Figure 2D).

A decline of HLA-DR expression by monocytes during infection has been reported by many groups and suggested to be associated with immune dysregulation and poor outcome [17–19]. We assessed HLA-DR expression in the four monocyte subsets for all patients. Regardless of the increase in CD14+CD91low, all patients experienced a decline in HLA-DR expression at all time points. In healthy subjects, HLA-DR MFI was highest in intermediate monocytes, followed by non-classical, classical and CD91low in that order. In BSI patients, HLA-DR decline was at least of 50 % in all monocyte subsets, except for non-classical monocytes where a 40 % decrease was observed. In heart surgery patients, HLA-DR downregulation progressed at each time point to reach a nadir at 48 h, of more than 50 % reduction in

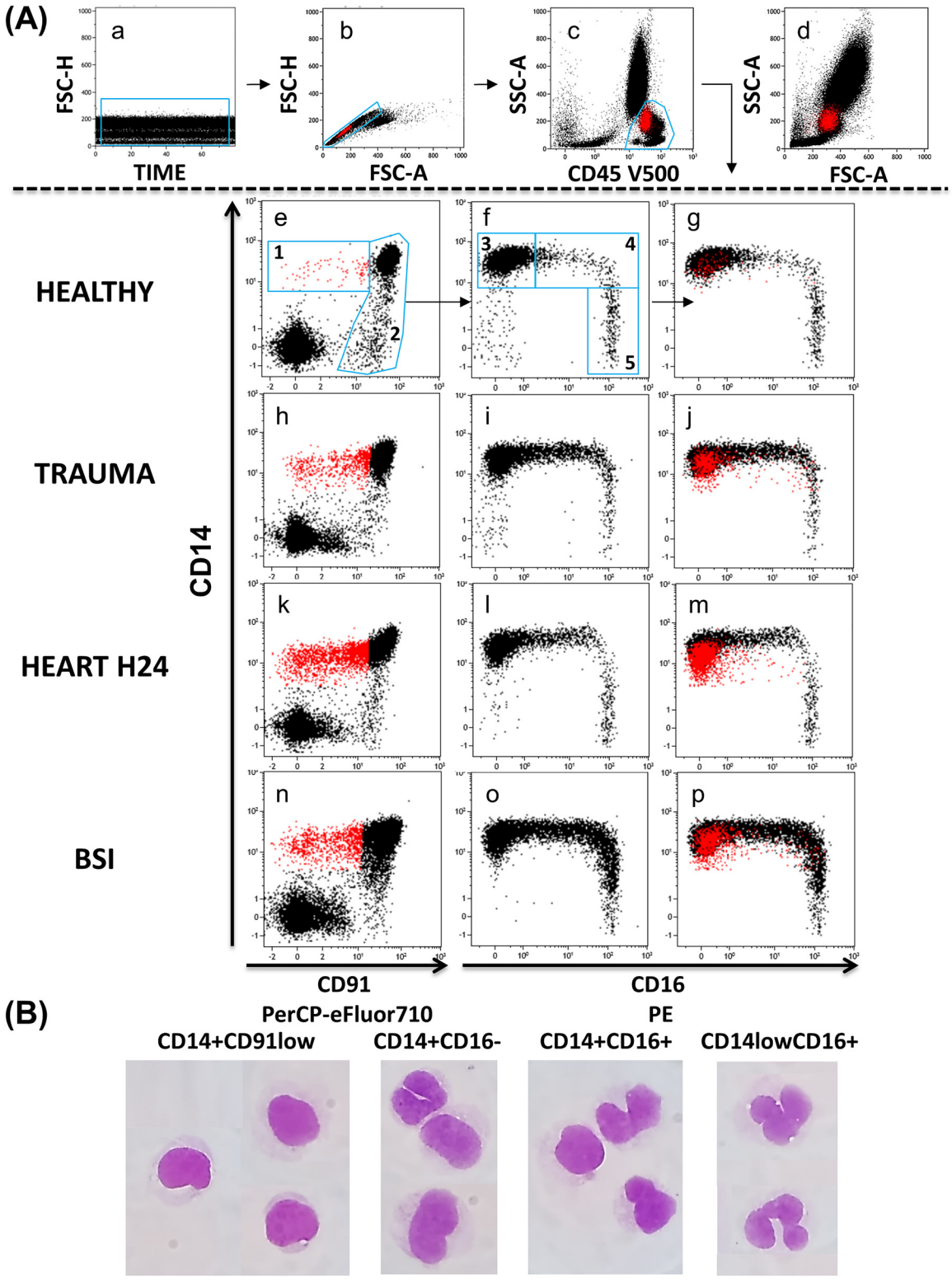

Figure 1: Sequential gating for classical, intermediate and non-classical monocytes. (A) Gating strategy for the identification of CD14+CD91low monocytes. (a) Time gate; (b) doublet exclusion; (c) mononuclear cell gating; (d) representative FSC-A/SSC-A plot; (e–g) sequential gating allowing delineation of (1) CD91low monocytes, (2) CD91+ monocytes, (3) classical, (4) intermediate; (5) non-classical in a healthy control; (h–j) monocyte subsets in trauma; (k-m) monocyte subset distribution 24 h after heart surgery; (n–p) monocyte subsets in bloodstream infection. The CD91low monocyte fraction is backgated in red on all plots. (B) May-Grunwald-Giemsa morphology of FACS-sorted monocytes.

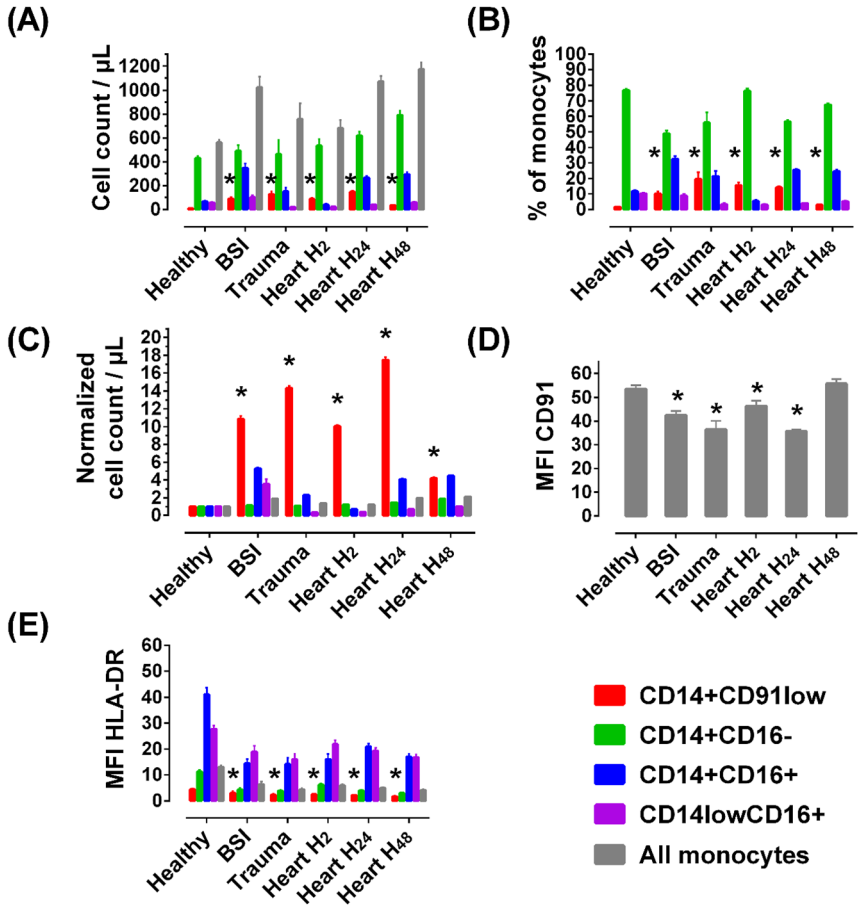

Figure 2: Comparison of monocyte subsets, HLA-DR and CD91 expression level in healthy, trauma, 2-, 24- and 48-h post-surgery and BSI patients. (A) Monocyte cell count/µL. *: p<0.05, CD91low count compared to healthy subjects. (B) Percentage of monocyte subsets relative to total monocytes. *: p<0.05 compared to healthy subjects. (C) Normalized cell count/µL expressed as fold-change compared to healthy subjects. *: p<0.05, CD91low count compared to healthy subjects. (D) MFI of CD91. Data are represented as mean ± SEM. *: p<0.05 compared to normal subjects. MFI: Mean fluorescence intensity. (E) MFI of HLA-DR. Data are represented as mean ± SEM. *: p<0.05, CD91low vs. all other monocyte subsets.

CD91low, classical and intermediate subpopulations. HLA-DR downregulation was somewhat less pronounced in non-classical monocytes (Figure 2E). Thus, CD91low monocytes was the subset with the lowest HLA-DR density in all subject groups (p<0.05).

The correlation of CD14+CD91low monocyte count with CRP was evaluated in all study groups (Table 2). Interestingly, while intermediate and non-classical monocytes were positively correlated with CRP as expected (p<0.05), an inverse correlation was found between CRP and the CD14+CD91low subset (correlation –0.43, p<0.05).

Temporal variation of monocyte subsets with respect to CRP was evaluated in patients undergoing cardiac surgery (Figure 3). While classical and intermediate monocyte peak levels were not reached at 48 h, CD14+CD91low monocytes increase was steeper and maximal around 24 h, making it the most precocious change in monocyte subset redistribution during acute inflammation, detected as early as 2 h and before the onset of CRP elevation. The decline of the CD14+CD91low monocytes beyond 24 h, while CRP is still increasing, is reflected by the negative correlation of –0.37. Thus, the kinetics of the CD14+CD91low monocytes are clearly distinct from those of the traditional monocyte subsets.

Table 2: Spearman correlation between CRP and monocyte subsets (all patient groups).

Monocyte subset	Corr coefficient
CD91low, µL	–0.43[a]
CD14+CD16–, µL	0.11
CD14+CD16+, µL	0.44[a]
CD14lowCD16+, µL	0.41[a]

[a]p<0.05.

In addition, SOFA score, SAPS II score, lactate levels, temperature, and mortality were evaluated in the BSI

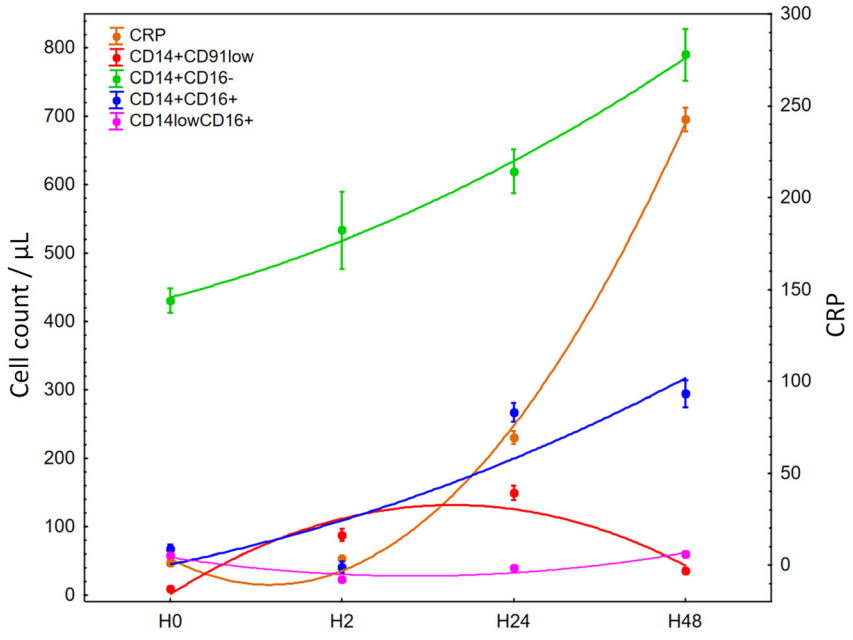

Figure 3: Time course of CRP and monocyte subset changes during acute inflammation in cardiac surgery patients. Monocyte cell counts are expressed as # cells/µL (left Y-axis). CRP levels are indicated in mg/L (right Y-axis). Data are represented as mean ± SEM, polynomial regressions with time are indicated in solid lines.

patients. A positive correlation was observed between CD14+CD91low monocytes and lactate levels. No correlation was found for CD14+CD91low monocytes with prognostic markers, SAPS II and SOFA scores, or between temperature and mortality (Table 3).

Phenotypic characterization of CD91low monocytes

To further characterize CD91low monocytes, we tested the expression of cellular receptors/proteins in cells obtained from healthy individuals, cardiac surgery patients at H24 and BSI patients on all monocyte fractions, along with neutrophils and lymphocytes as controls.

Expression profiles of monocyte-associated markers CD11b, CD13, CD33, CD36, CD45, CD64, CD86 and CD300e were established in each monocyte population. We observed that CD14+CD91low monocytes strongly express CD33, a recognized monocyte lineage marker. As for CD300e expression, it is variable among monocytes, and CD14+CD91low monocytes express lower levels of CD300e than the other fractions. The CD14+CD91low cells do not express the granulocyte markers CD15 and CD66b, or the lymphocyte markers CD3 and CD19 (data not shown), confirming their monocytic lineage.

Lysozyme expression in CD91low monocytes was equivalent to that of the classical fraction, while their myeloperoxidase content was higher. CD86 and CD121b, the interleukin-1 receptor type 2, were lower on CD91low monocytes compared to other fractions. CD62L, involved in leucocyte-endothelial interactions during monocyte trafficking, was higher in CD91low and classical subpopulations compared to other monocyte subsets. The expression profile of monocytes was very similar between healthy subjects, heart surgery and BSI patients, as far as this marker selection was concerned (Figure 4). Overall, the expression levels of all markers tested in CD91low monocytes was different

Table 3: Spearman correlation between CD14+CD91low cell count, CRP, SAPS II, SOFA, lactate, temperature and mortality (BSI patients).

	CD14+CD91low cell count, µL	CRP	SAPS II score	SOFA score	Lactate, mg/L	Temperature, °C	Mortality
CD14+CD91low, cell count/µL	1						
CRP	−0.37[a]	1					
SAPS II score	0.10	0.22	1				
SOFA score	0.03	0.20	0.73[a]	1			
Lactate, mg/L	0.32[a]	0.07	0.36[a]	0.35[a]	1		
Temperature, °C	−0.18	0.27[a]	0.03	0.02	−0.03	1	
Mortality	0.04	0.01	0.38[a]	0.41[a]	0.13	−0.35[a]	1

[a]$p<0.05$.

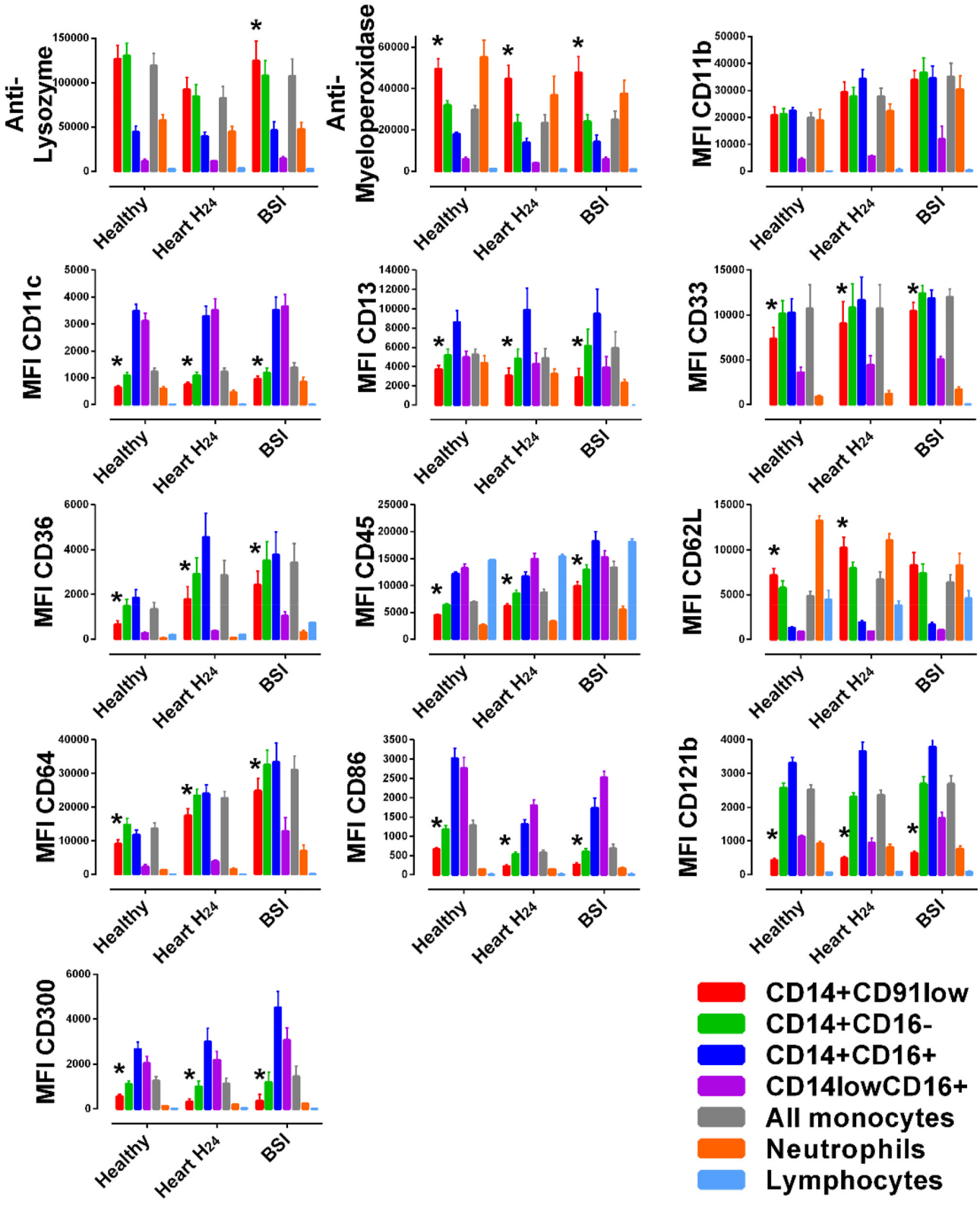

Figure 4: Expression profile (MFI) of cell surface markers on monocyte subsets, neutrophils and lymphocytes. Data are shown as mean ± SEM of MFI (n=6 healthy controls, n=6 patients post-cardiac surgery at H24 and n=9 for BSI patients). *: p<0.05, CD91low vs. classical monocytes.

compared to classical monocytes in heart surgery patients ($p<0.05$), except for CD11b and lysozyme, as well as compared to BSI patients ($p<0.05$), except for CD11b and CD62L, emphasizing that CD91low monocytes represent a distinct cell subset.

Oxidative burst and phagocytosis capacity of CD91low monocytes

Monocyte subsets were analyzed in cardiac surgery patients after 24 h and in BSI patients to assess their phagocytosis and oxidative capacity. Overall, the CD91low fraction showed robust phagocytic activity as well as classical and intermediate monocytes, whereas non-classical monocytes were less potent (Figure 5A). Oxidative burst assays revealed a similar pattern with intermediate and CD91low fractions being the most active (Figure 5B).

Discussion

Passlick et al. [20] initially proposed a distinction of monocyte fractions differing by the expression of the Fcγ receptor CD16. Subsequent work detailed CD16+ monocytes into functionally distinct subpopulations [21]. Later, subsetting of the CD16+ population was proposed by Ancuta et al. [22] into defined phenotypes. Recently, single-cell expression profiling studies confirmed the distinct functions of classical (CD14+CD16−), intermediate (CD14+CD16+) and non-classical (CD14lowCD16+) monocytes [4, 6, 23]. There is increasing evidence that additional levels of heterogeneity are yet to be identified [24].

Since a significant proportion of monocytes express low to negligible amounts of CD14, and given that other cell subsets, namely NK cells and granulocytes, are also CD16 positive, a pan-monocyte marker is needed to define the whole monocyte population, on which to apply the CD14/CD16-based segregation. Many authors have used HLA-DR in that context [5, 25, 26], since it is negative on NK cells and granulocytes. However, in the context of inflammation, HLA-DR cannot be used as a positive selection marker of monocytes since it is significantly decreased. CD64 has been proposed as a pan-monocyte gating marker, for the assessment of paroxysmal nocturnal hemoglobinuria [27]. However, in acute inflammation, both septic and non-septic, CD64 is not an appropriate monocyte selection marker because its density is decreased on non-classical monocytes, and thus overlaps with that of lymphocytes (data not shown). CD91 was proposed by Hudig as a stable pan-monocyte marker [14], which could be used for accurate determination of total blood monocytes. In the present paper, we combined CD14 and CD91 positivity in the inclusion gate for all monocytes.

CD91 is the low-density lipoprotein receptor-related protein 1 (LRP-1) which binds a variety of ligands including proteases, extracellular matrix proteins, viral proteins and

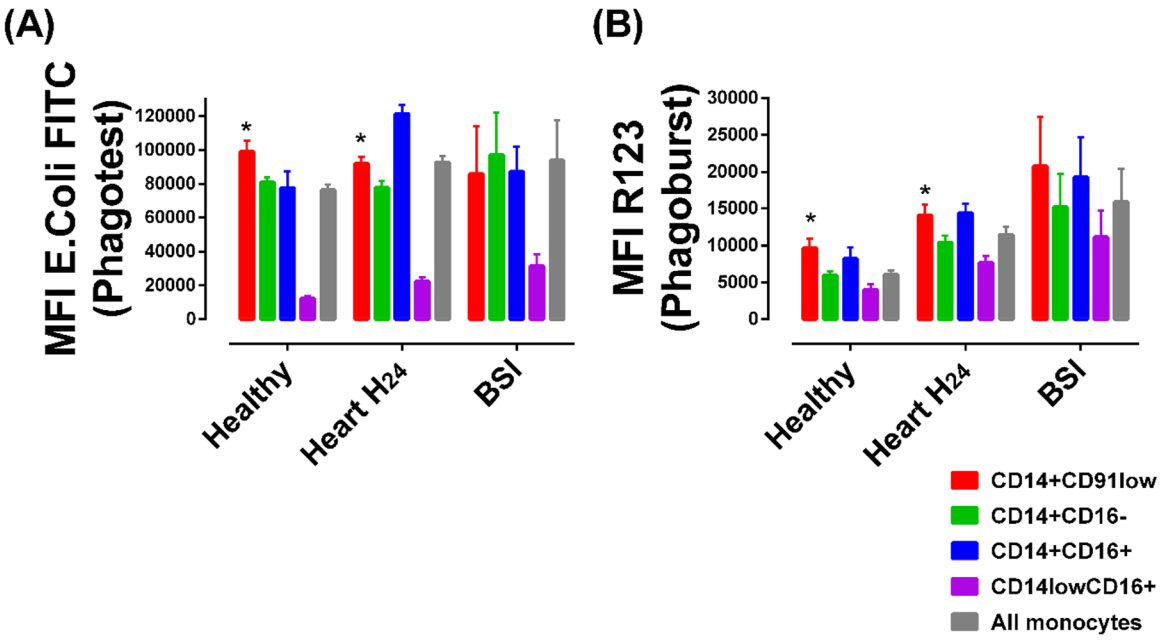

Figure 5: Phagocytic and oxidative burst capacity of monocyte subsets. (A) Phagocytosis and (B) oxidative burst capacity (n=6 healthy controls, n=12 patients 24 h after heart surgery and n=3 BSI patients). *: $p<0.05$, CD91low vs. classical monocytes.

growth factors. Membrane-bound CD91 may be anti-inflammatory by inhibiting NF-kappaB activity [28] and promoting the polarization of macrophages into the anti-inflammatory M2 phenotype [29]. Thus, CD91 expression on monocytes may be involved in tapering excessive inflammation. On the other hand, CD91 is being shed at increased levels as a response to inflammatory mediators such as LPS and IFN-γ *in vitro*[30]. Soluble LRP1 (sLRP1) plasma levels are indeed increased in chronic inflammatory diseases. sLRP1 increases expression of TNF-α, CCL2 and IL-10 in macrophages. How the different effects of membrane-bound and soluble CD91 integrate in acute inflammation following infection or tissue injury remains speculative at this point.

We have identified a subset of blood monocytes belonging to the CD14 positive monocyte subset, and devoid of CD91. We showed that CD91low monocytes are infrequent in healthy subjects but expand substantially in acute inflammation following infection and tissue injury compared to the three previously characterized subsets. The number of CD14+CD91low cells increased 15-fold in BSI, and up to 20-fold after cardiac surgery, compared to healthy controls. In contrast, CD14+CD16+ intermediate monocytes were only 5-fold higher than controls, although they have been considered in previous studies as the prominent inflammatory subset [31, 32]. Thus, the CD91low monocytes seems to be the most specific monocyte fraction expanded in acute inflammation from infectious or non-infectious origin.

CD91low monocytes feature lower expression of CD14, CD300e and HLA-DR than classical ones. We confirmed the monocytic lineage of the subset by showing their expression of CD11b, CD13, CD14, CD33, CD36, CD64, and the absence of CD15 and CD66b. In addition, standard cytology analysis of sorted CD91low cell cytospins confirmed their monocyte morphology, although the nuclei tended to have a more regular shape compared to the other subsets. These cytological properties together with the low expression of CD300 suggest that they represent immature monocytes released from the bone marrow in response to inflammation. Raggi et al. identified CD300a as a new hypoxia-inducible gene in human monocytes and macrophages [33]. Whether a similar mechanism accounts for the downregulation of CD91 levels during inflammation and BSI, warrants further studies.

A series of studies have shown that downregulated HLA-DR expression is associated with acute illness-induced immune suppression and poor outcome [17–19]. Depressed HLA-DR expression by peripheral blood monocytes during BSI was also observed by our laboratory in a recent study [16]. CD14+CD91low monocytes express the lowest levels of HLA-DR on their cell surface compared to all other monocyte subsets. Their expansion in infection and tissue injury may thus contribute to the overall decrease in HLA-DR expression observed in acute inflammation.

In contrast to their significantly low HLA-DR expression, we have evidenced that these monocytes express significantly higher levels of myeloperoxidase and lysozyme than the other three type of monocytes, suggesting high antibacterial activity. In addition, we identified that CD14+CD91low monocytes possess high phagocytic activity while at the same time showing intense oxidative burst activity, suggesting a hyperactivated state. Classical and intermediate monocytes were found to display high phagocytic activity compared to non-classical monocytes, an observation in agreement with previous reports [6].

Expression of CD11b, CD14 and CD33 together with downregulation of HLA-DR in CD91low monocytes is similar to the phenotype of myeloid-derived suppressor cells (*m*-MDSCs) [34]. Indeed, CD14+CD91low were significantly increased in acute inflammation and infection relative to healthy controls and typically exhibited low levels of CD14. However, we have observed the emergence of CD91low HLA-DRlow monocytes as early as 2 h after tissue damage in cardiac surgery patients, while it has been shown that *m*-MDSC arise in chronic states of inflammation or infection.

The heterogeneity of human monocytes has been explored in recent studies by immunophenotyping and transcriptomic analyses. By mass cytometry, Hamers et al. [35] identified a monocyte subset (“subset 6”) in healthy subjects with the lowest expression of HLA-DR among all monocytes, decreased levels of CD4, CD91, CX3CR1, CD300e, and elevated CD62L [35], which could correspond to our CD91low population. Merah-Mourah and colleagues described the “sm14dimCD16neg” monocytes which are displayed on the CD14/CD16 dot plot in the same position as the CD91low monocytes, and are inconsistently detected in normal subjects as CD14low and HLA-DRlow monocytes [36]. Reyes et al. [37] described the “MS1” monocyte subset specifically expanded in sepsis patients and characterized by increased expression of IL2R1 (CD121b) and low HLA-DR. The CD91low monocytes described herein do not match with the MS1 subset since membrane expression of CD121b was low. Nevertheless, the interpretation of gene expression studies must take into account that there is no direct relationship between mRNA and protein levels due to post-translational modifications [38].

CD91low monocytes observed in sepsis and tissue injury are mostly CD16 negative and seem to branch out from the classical subset, rather than being a clearcut novel subset. Although present at very low levels, CD91low monocytes can also be detected in healthy individuals. Interestingly, these “steady-state” CD91low monocytes are similar in phenotype

as those found in BSI and heart-operated patients and they also display increased phagocytic activity compared to classical monocytes. Low CD91 expression may thus represent a functional shift acquired from acute inflammation, leading to overrepresentation of monocytes with increased phagocytic and oxidative burst activity together with impaired antigen presentation (low HLA-DR expression). Whether CD91 downregulation is responsible of these functional changes, or merely associated with them, will require further investigation.

Our study has limitations. First, all postcardiac surgery patients received prophylactic cephalosporin therapy. Evidence suggests also that many antibiotics directly modulate the immune system in addition to their own antimicrobial properties [39]. Thus, prior antibiotic treatment has the potential to influence not only the course of infection in ICU patients but also changes in immunophenotypes in both critical care and cardiac patients.

Also, it is a cross-sectional study with a relatively small sample of BSI patients, which may prevent us from finding differences regarding the association between infection progression and monocyte subsets prospectively. Lactate levels are frequently used to monitor tissue hypoxia which showed a modest correlation (corr=0.32, $p<0.05$) with CD91low monocytes. CD91low monocyte levels were not correlated with severity scores SOFA or SAPS II. We previously showed that the discrimination between infected and non-infected intensive care unit patients cannot be achieved with a single monocyte marker but requires the combination of multiple phenotypic changes in a composite score [16]. Longitudinal observation of cardiac surgery patients as a model of inflammation highlights the early increase in CD14+CD91low monocytes while the rise of CRP is witnessed 24 h later, reflecting their inverse correlation. Indeed, it usually requires 12–24 h for the liver to produce CRP, which reaches its peak level around 48–72 h[40]. Overall, the CD91low subset may be used as an early biomarker of inflammation, representing the egress of immature monocytes from the bone marrow, which is detectable before the rise in CRP.

In conclusion, we have characterized a reactive monocyte subset expressing low levels of CD91, expanded during tissue injury and systemic infection. The relationship of CD91low monocytes with other monocyte fractions described in the context of infection, cancer and autoimmune diseases requires further investigation. The functional activity of these cells remains to be elucidated and will require single cell genomic studies and cytokine secretion assays. Finally, the potential use of this population as a diagnostic and prognostic marker should be tested in different clinical scenarios.

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