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Distribution of subsets of blood monocytic cells throughout life

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Title: Distribution of subsets of blood monocytic cells throughout life

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Conflict of interest disclosure: J. J. M. van Dongen, and A. Orfao report being the inventors on patent PCT/NL2012/050132 (Methods and means for monitoring disruption of tissue homeostasis in the total body). J. J. M. van Dongen and A. Orfao report to be chairmen of the EuroFlow scientific foundation, which receives royalties from licensed patents, which are collectively owned by the participants of the EuroFlow Foundation. In addition, J.J.M. van Dongen and A. Orfao report an Educational Services Agreement between BD Biosciences and their universities. The rest of the authors declare that they have no relevant conflicts of interest.

Capsule summary. *This study provides a novel and detailed frame of reference of normal counts of different monocytic cell subsets in blood throughout life and highlights their differential distribution among distinct lymphoid tissues.*

Key words. Monocytes [D009000]; Macrophages [D008264]; Phagocytes [D010586]; Flow Cytometry [D005434]; Age groups [D009273]; Spleen [D013154]; Lymph Nodes [D008198].

To the Editor:

Currently, it is well established that monocytes are a heterogeneous cell that consists of phenotypically and functionally distinct subpopulations, found to be numerically altered in blood in a wide variety of disease conditions, such as infection, autoimmunity, respiratory and cardiovascular diseases, and inflammatory disorders.^{1, 2} Thus, three subpopulations of circulating monocytic cells have been identified based on the expression of the CD14 lipopolysaccharide receptor and the CD16 low-affinity Fc IgG receptor: (a) classical CD14^{hi}CD16⁻ monocytes (cMo); (b) intermediate CD14^{hi}CD16⁺ monocytes (iMo); and (c) non-classical CD14^{-/lo}CD16⁺ monocytes (ncMo).³ Monocytes circulate in blood for up to three days, until recruited to virtually any human tissue, where they differentiate into either tissue macrophages (TiMas) or myeloid dendritic cells.⁴ Then, TiMas might migrate from their tissue location -via the lymph system⁵-, before they potentially die outside the circulation.⁶ Despite our knowledge about the biology of monocytes has increased in recent years, normal reference ranges for the distinct monocyte subsets in blood throughout life - e.g. from cord blood (CB) and newborns to elderly individuals- have never been systematically defined. Moreover, the precise maturational and functional relationship between the distinct populations of blood monocytes and their tissue distribution profiles, remain unknown. In order to provide a frame of reference for future identification of disease-associated altered profiles, here we investigated the distribution of distinct monocytic cell subsets in normal blood vs secondary lymphoid tissues, such as BM, lymph nodes and spleen; our aim was to shed light on the changes in the distribution of these monocytic cell subsets in blood and other lymphoid tissues. For this purpose, a total of 188 EDTA-anticoagulated blood samples were studied: 11 CB specimens from full-term neonates and 177 blood samples from 164 healthy subjects and 13 solid organ donors (102 males and 75 females) with a median age (range) of 26 years (y) (4 days-92y), distributed by age-groups as shown in Supplemental Table 1. In addition, 9 BM samples -6 males and 3 females; median

(range) age: 49y (21y-83y)- from 9 of the above referred healthy donors, together with 13 lymph node and 13 spleen paired samples -9 males and 4 females; median age (range): 69y (49y-81y), collected in parallel with blood specimens from solid organ donors, were studied. All organ donors were in brain death at the moment of tissue collection (performed within the first hour after heart failure), the organs being maintained viable throughout the procedure.

The identification of the different monocytic cell subsets was performed using high-sensitive 10-color flow-cytometry. The immunophenotypic criteria used for the identification of monocytic subpopulations, as well as the precise flow-cytometry protocols, panels and reagents used, are detailed in the Supplemental Materials. Five distinct subsets of monocytes were systematically identified in every sample analyzed: 1) CD62L⁺ and 2) CD62L⁻ cMo; 3) iMo; and both 4) SLAN⁻ and 5) SLAN⁺ ncMo. An additional population of FcεRI⁺ CD14^{hi}CD16⁻ monocytes, was also identified in a subset of samples stained with anti-FcεRI (Supplemental Figure 1).

Overall, our results show that the distribution of monocyte subsets in blood varies substantially during life, particularly during the first 6 months of life, when all monocyte subsets peaked (Figure 1); however, while cMo reached their highest numbers in CB samples, iMo and ncMo peaked in newborns. These homeostatic changes most likely reflect the high production rate and release of recently generated CD62L⁺ classical BM monocytes into blood,⁷ and thereby the early-in-life requirement for sufficient numbers of monocytes to fill the distinct tissues. Afterward, the three major monocyte subsets decreased until the age of 8y-13y, subsequently increased again at adolescence (particularly CD62L⁺ cMo), and remained high in younger adults, decreasing thereafter until the age of 30y-50y. From 50y onwards, all above monocyte subsets increased in blood, which might reflect an increased tissue turnover, apoptosis, and/or "immunosenescence".⁸ Based on the overall pattern of distribution of monocytic subsets in blood, with sequential peaks for cMo, iMo and ncMo, particularly during the earliest periods of life and after the age of 50y, our results would support the notion that iMo and ncMo might correspond to monocytic cell subsets at more advanced stages of maturation than cMo, also in

accordance with previous reports.⁹ Whether such maturation occurs in blood or outside the bloodstream is still a subject of debate, although some reports support the notion that the differentiation steps of cMo into iMo and ncMo more likely occur outside the blood compartment, rather than inside blood.^{6,9}

To gain insight into the potential tissue relationship between cMo and both iMo and ncMo, we further investigated the distribution of the distinct subsets of monocytes in normal BM, lymph node and spleen. Our results showed that their relative distribution varies substantially in BM, lymph node and spleen, compared to blood (Figure 2). Thus, while CD62L⁺ cMo were by far the most abundant subset of cMo in blood and BM, they were outnumbered by CD62L⁻ cMo in lymph node and spleen. Moreover, higher percentages of iMo were found in lymph node and spleen vs both blood and BM, where this cell subset represented a minor monocytic population. In contrast, ncMo were particularly represented in blood and spleen samples, while found at very low percentages in BM and lymph node; interestingly, no (or very small numbers) of SLAN⁺ ncMo were found in lymph node samples. The predominance of CD62L⁻ cMo in lymph node and spleen would most likely reflect the more mature nature of cMo in these lymphoid tissues (vs BM and blood). Altogether, these findings suggest that, consistently with *in vivo* 6,6-²H₂ glucose monocyte tracking studies,^{6,9} cMo might lose CD62L and differentiate into iMo in lymphoid tissues; iMo might then recirculate via the lymph system² and sequentially give rise to SLAN⁻ and SLAN⁺ ncMo.

In summary, our results show that the number of circulating blood monocytes and their subsets varies significantly throughout life, and provide a frame of reference for further studies in distinct disease conditions. The differential distribution of the distinct monocyte subsets evaluated in different human tissues, might reflect distinct functional features and kinetics of monocytic cell subsets throughout the body. Further studies are required to confirm this hypothesis.

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FIGURE LEGENDS

Figure 1. Distribution of different subsets of circulating monocytes in cord blood and peripheral blood samples from healthy subjects through life.

*: P-value <0.05 vs CB. #: P-value <0.05 vs subjects aged ≥ 30 y<50y. +: P-value <0.05 vs NB.

cMo: CD14^{hi}CD16⁻ classical monocytes; iMo: CD14^{hi}CD16⁺ intermediate monocytes; ncMo: CD14^{-/lo}CD16⁺ non-classical monocytes; N.: number; CB: cord blood; NB: newborns; m: month(s); y: year(s).

Figure 2. Relative distribution of different subsets of monocytes in adult blood vs paired bone marrow (BM), and paired lymph node and spleen samples.

Panels A-E: *: P-value <0.05 vs blood. #: P-value <0.05 vs lymph node. Panels F-J: multidimensional representations of the distinct monocyte cell subsets in the different tissues. P-value >0.05 for the comparisons of the two blood sample groups for all monocytic subsets.

Figure 1

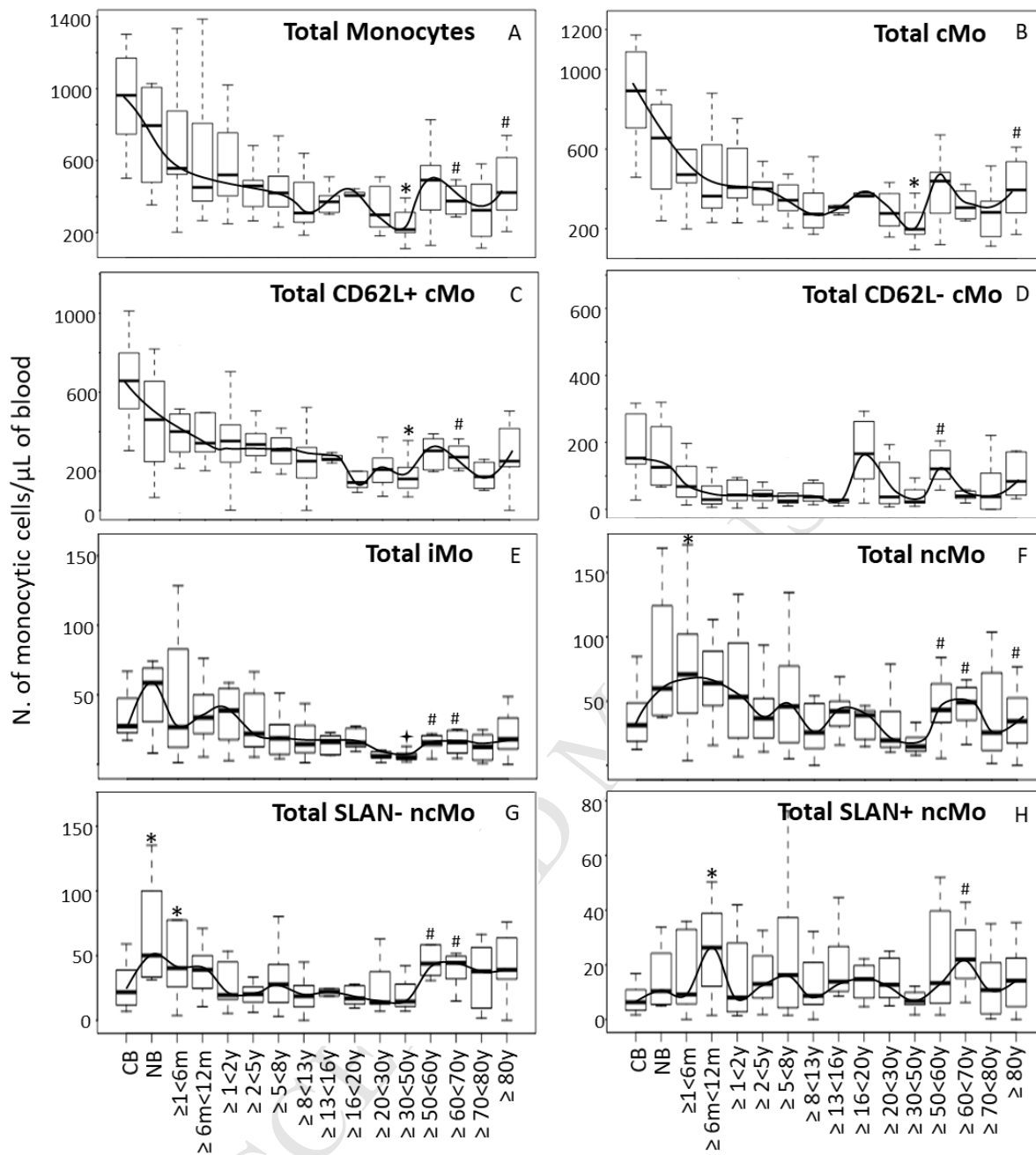
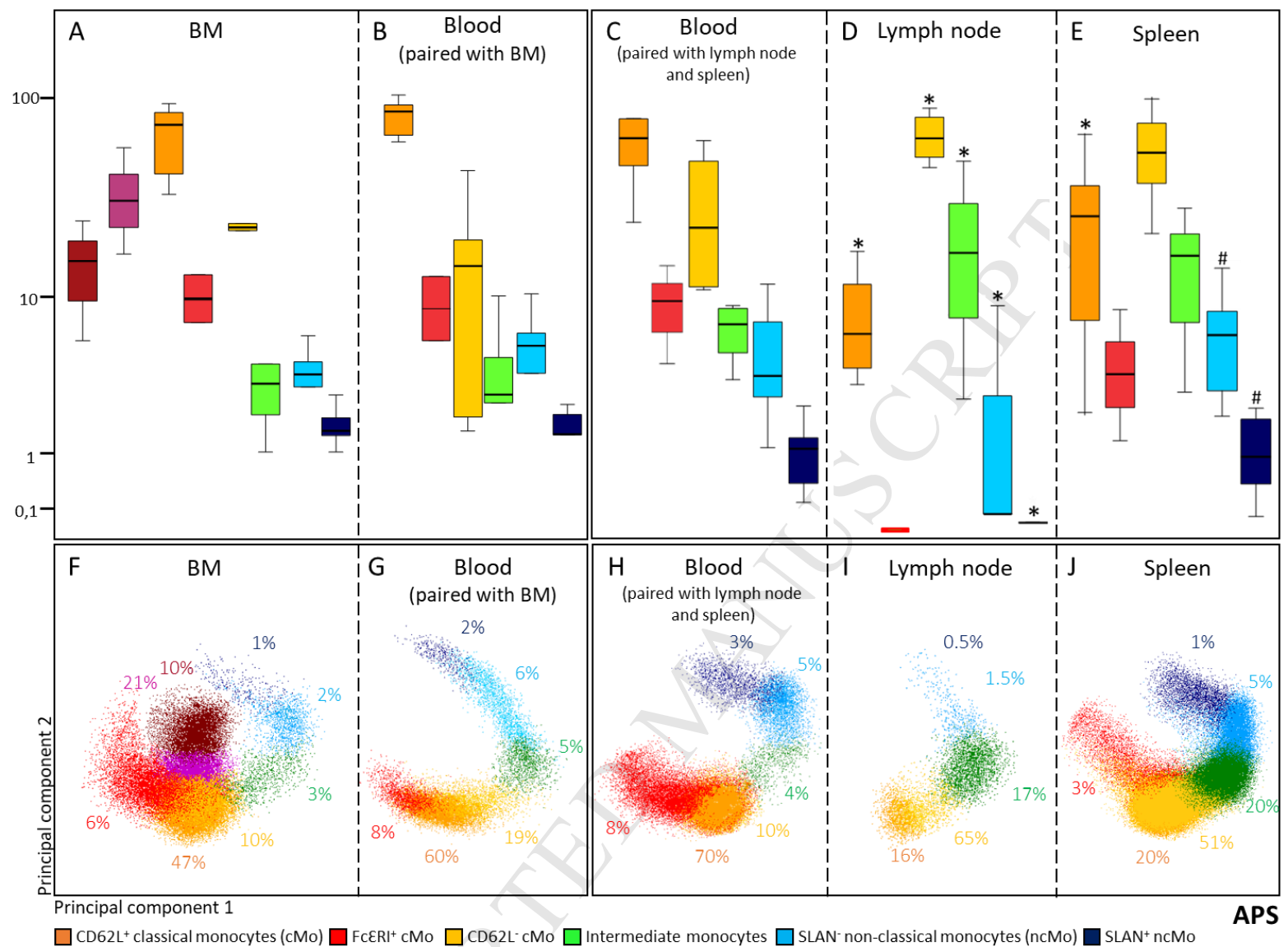
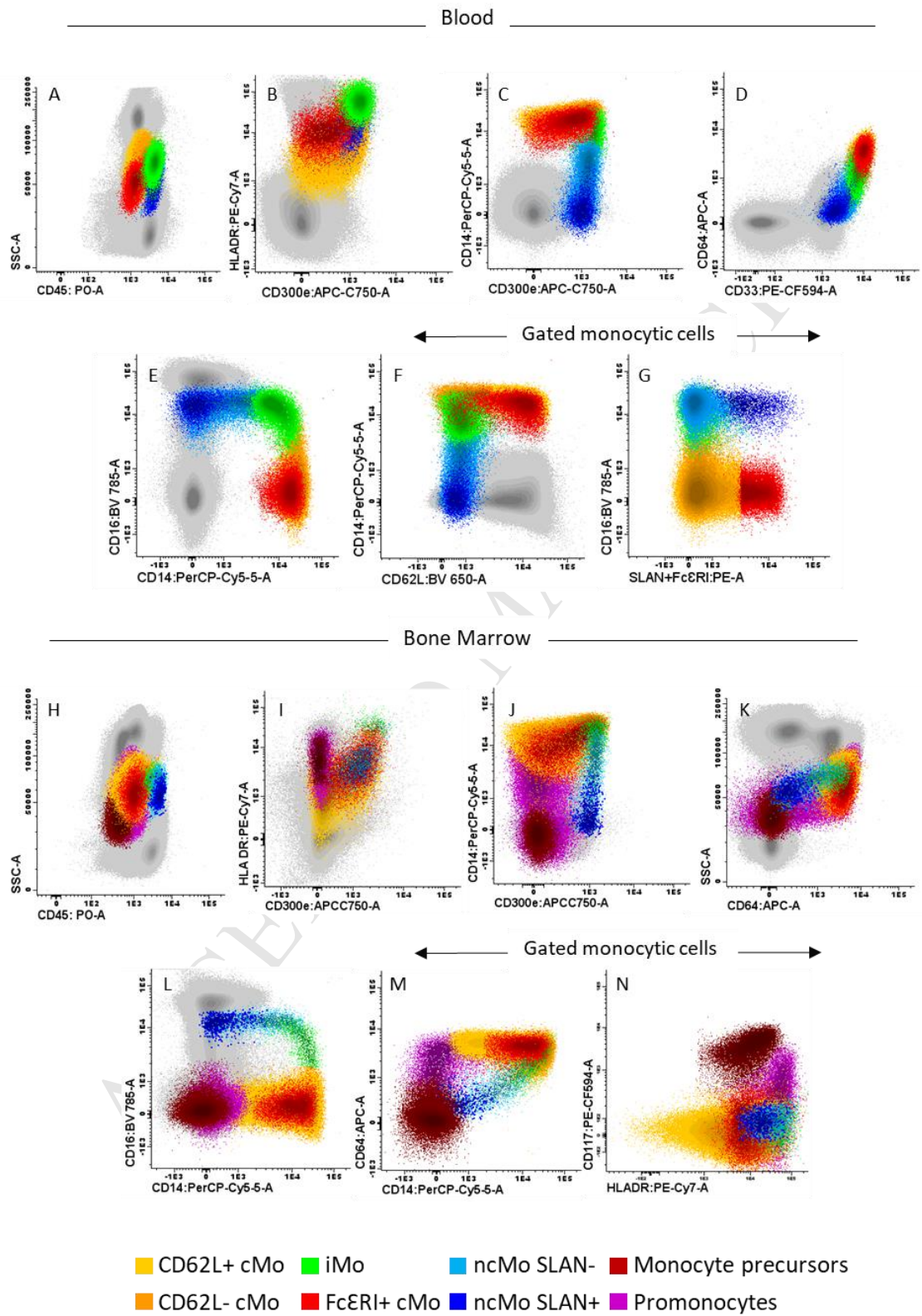


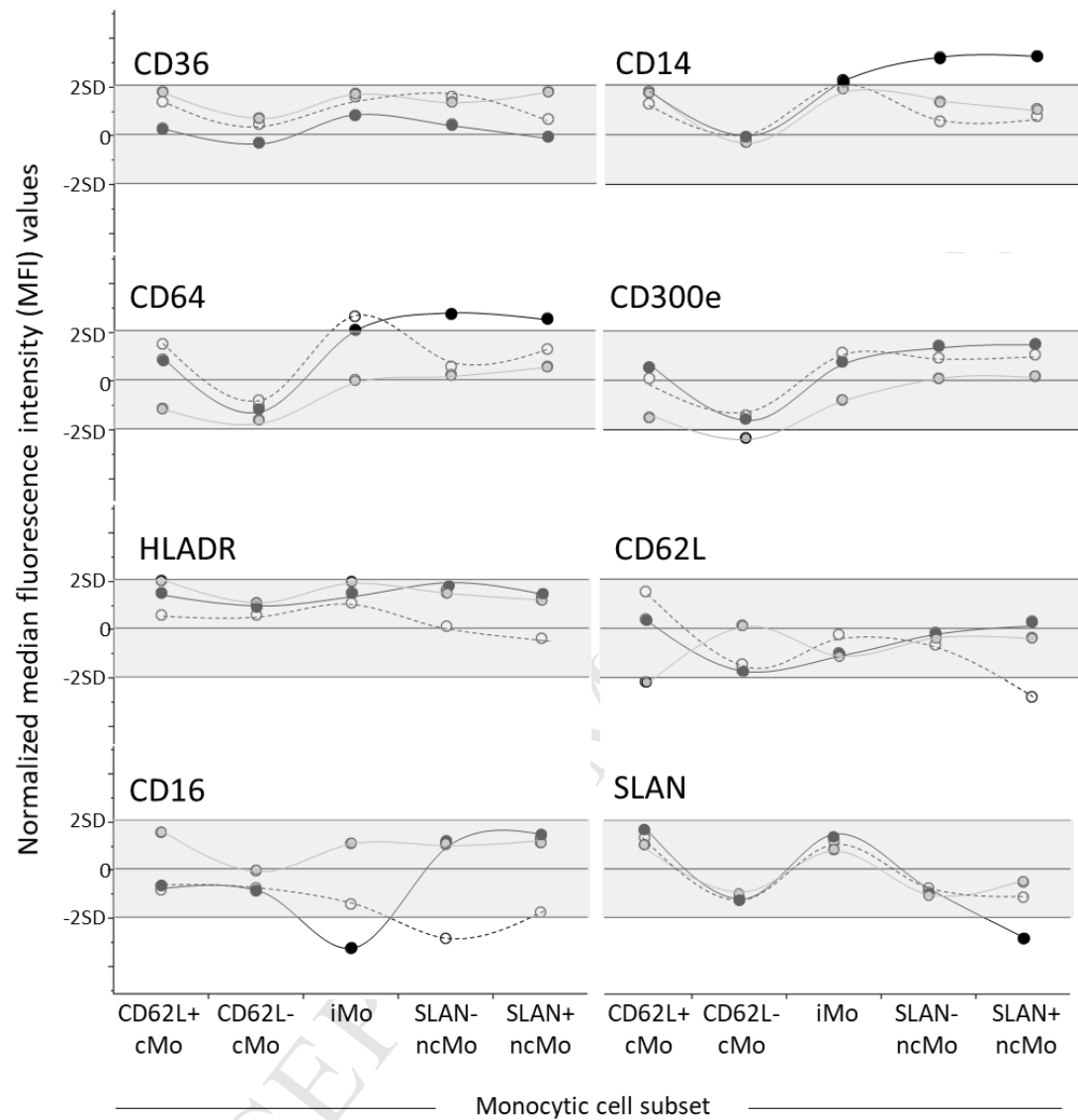
Figure 2



SUPPLEMENTAL FIGURE 1: Identification of different subsets of circulating monocytes in representative peripheral blood and bone marrow samples from a healthy donor.



SUPPLEMENTAL FIGURE 2: Comparison between the phenotypic profile of the different subsets of monocytic cells in bone marrow (BM) lymph node and spleen vs blood.



SUPPLEMENTAL MATERIAL

- Supplemental figure legends-

SUPPLEMENTAL FIGURE 1: Identification of different subsets of circulating monocytes in representative peripheral blood and bone marrow samples from a healthy donor.

Colored events in Panels A-G correspond to the distinct blood monocytic cell subsets identified in this study, after they had been discriminated from all other PB and BM leucocytes (gray dots), based on their typical SSC/CD45 profile (Panel A), and their unique positivity for CD300e, HLADR, CD33, CD64 and CD14 (Panels B-D). Within total monocytes, the different subsets of monocytic cells were identified based on their expression profile for CD14 and CD16 (Panel E) plus Fc ϵ RI (Panel G): CD14^{hi}CD16⁻ cMo, CD14^{hi}CD16⁺ iMo, and CD14^{-/lo}CD16⁺ ncMo and CD14⁺CD16⁻ Fc ϵ RI⁺ cells. Within cMo, the CD62L⁺ and CD62L⁻ subsets were further identified (Panel F), while ncMo were further subsetted into SLAN⁻ and SLAN⁺ ncMo (Panel G). Additionally, monocyte precursors and promonocytes were identified in bone marrow, based on their phenotypic profile for CD117 and HLADR (Panels I and N), and expression of CD64 (Panel K).

cMo: CD14^{hi}CD16⁻ classical monocytes; iMo: CD14^{hi}CD16⁺ intermediate monocytes; ncMo: CD14^{-/lo}CD16⁺ non-classical monocytes.

SUPPLEMENTAL FIGURE 2: Comparison between the phenotypic profile of the different subsets of monocytic cells in bone marrow (BM) lymph node and spleen vs blood.

Normalized PB phenotypes were used as reference and they are represented as a gray rectangle ranging from -2 SD to +2 SD (y-axis) of median fluorescence intensity (MFI) values normalized for each marker and each cell population (ordered in the "x" axis from CD62L⁺ cMo to SLAN⁺ ncMo), and compared to other tissues: circles correspond to (normalized) MFI values for each marker in BM (gray), lymph node (black) and spleen (white).

cMo: classical monocytes; iMo: intermediate monocytes; ncMo: non-classical monocytes; SD: standard deviation.

SUPPLEMENTAL MATERIAL

-Supplemental methods-

Subjects and samples. A total of 188 EDTA-anticoagulated blood samples were studied. Eleven cord blood (CB) specimens from full-term neonates from healthy mothers and 177 blood samples -from 164 healthy subjects and 13 solid organ donors (102 males and 75 females; median age of 26 years (y); range: 4 days-92y)-, with the following age-distribution: newborns (NB) (median: 4.5 days; range: 4-10 days), 4 cases; children $\geq 1 < 6$ months, 14 subjects; $\geq 6 < 12$ months, 10; $\geq 1 < 2$ y, 13; $\geq 2 < 5$ y, 18; $\geq 5 < 8$ y, 18; $\geq 8 < 13$ y, 17; $\geq 13 < 16$ y, 6; $\geq 16 < 20$ y, 5; adults $\geq 20 < 30$ y, 13 cases; $\geq 30 < 50$ y, 15; $\geq 50 < 60$ y, 13; $\geq 60 < 70$ y, 10; $\geq 70 < 80$ y, 9; and adults ≥ 80 y, 12 cases (Supplemental Table 1). CB, childhood, and adult samples were collected at the Erasmus University Medical Center, University Hospital of Salamanca in Spain and the corresponding Primary Health Care Centers of Salamanca, respectively).

In addition, 9 normal BM samples -6 males and 3 females; median age of 49y (range: 21-83y), collected in parallel to blood samples from 9 healthy donors (undergoing orthopedic surgery), and 13 lymph node plus 13 spleen paired samples -9 males and 4 females; median age of 69y (range: 49-81y), collected from solid organ donors (in addition to paired blood samples) at the Surgery Service of the University Hospital of Salamanca-, were studied in parallel. All organ donors were in brain death at the moment of tissue collection (performed within the first hour after heart failure), the organs being maintained viable throughout the procedure, according to the standard transplantation protocol.

All study subjects had no medical history of either immunological or onco-hematological disorders. All participants -except for the anonymized solid organ donors- were enrolled in the study after informed consent was given by each individual and/or their corresponding legal representatives. The study was approved by the Ethics Committee of the University Hospital of

Salamanca/IBSAL, and was conducted in accordance with the Declaration of Helsinki.

Multiparameter flow cytometry (FCM) immunophenotypic studies. All samples (fresh whole CB, blood, BM, lymph node and spleen specimens) were stained and processed within < 1-24h after collection. For CB, blood and BM samples, the EuroFlow bulk-lyse standard operating procedure (SOP)¹ was used to lyse non-nucleated red cells prior to staining. Lymph node and spleen samples were mechanically dissociated into single cell suspensions, prior to staining.² Afterward, 5×10^6 leukocytes/sample aliquot were stained with a single 10-color combination of the following monoclonal antibody (Mab) reagents - Mab (clone)-fluorochrome- (Supplemental Table 2): CD45 (HI30)- Pacific Orange (PacO), CD62L (DREG-56)-Brilliant Violet 650 (BV650), CD16 (3G8)-BV786, CD36 (CLB-IVC7)-fluorescein isothiocyanate (FITC), CD14 (MφP9)-peridinin chlorophyll protein-cyanine 5.5 (PerCPCy5.5), anti-SLAN (DD-1)-phycoerythrin (PE), CD33 (WM53)-PECF594, anti-HLADR (G46-6)- phycoerythrin cyanine 7 (PECy7), CD64 (10.1)- allophycocyanine (APC), and CD300e (UP-H2)- APC cyanine 750 (APCC750). In a subset of 15 specimens (3 blood, 6 BM, 3 lymph node and 3 spleen samples) anti-FcεRI (clone: AER-37) was also added in a PE-conjugate format, in addition to the anti-SLAN-PE reagent (Supplemental Table 2), as the monocyte subsets expressing each of these two markers were mutually exclusive (data not shown). In BM samples, additional markers were also included to further identify uncommitted monocytic precursors: CD34 (P67.6)-PerCPCy5.5 and CD117 (YB5.B8)-PECF594 (Supplemental Table 2). Reagents were purchased from Becton/Dickinson Biosciences (BD, San Jose, CA), except CD45 (Thermo Fisher Scientifics, Carlsbad, CA), CD36 (Cytognos S.L., Salamanca, Spain), anti-SLAN (Milteny Biotech, Cologne, Germany), and both CD300e and anti-FcεRI (Immunostep, Salamanca, Spain). Sample acquisition was performed immediately after immunostained in LSR Fortessa X-20 flow cytometers (BD), using the FACSDiva™ software (BD). For instrument setup, calibration and monitoring, the EuroFlow instrument setup & compensation protocol (www.EuroFlow.org) for 12-color measurements was used. For data analysis, the Infinicyt™ software (Cytognos S.L) was employed. For each blood sample, relative and absolute (double-platform) cell counts were calculated and recorded for both the whole

monocyte population and each monocytic subset. In BM, lymph node and spleen samples, only relative cell numbers were derived. The gating strategy used for the identification of each cell subset is shown in Supplemental Figure 1.

Statistical analyses. For continuous variables, median, mean and standard deviation (SD) values, as well as range and the 10th, 25th, 75th, and 90th percentiles, were calculated; for categorical variables, frequencies were used. The statistical significance of differences observed between two or more groups was assessed by the non-parametric Mann–Whitney U and Kruskal–Wallis tests for independent variables, respectively. For comparisons between two or more groups of paired samples, the non-parametric Friedman and Wilcoxon tests were used, respectively. The Spearman rank correlation (R-software package, www.R-project.org) was used to explore the degree of association between two continuous variables. For all statistical analyses (but Spearman rank correlation) the SPSS software program (IBM-SPSS Statistics version 23, IBM, Armonk, NY) was used. P-values ≤ 0.05 were considered to be associated with statistical significance.

Supplemental tables and figures-

SUPPLEMENTAL TABLE 1. Age and gender distribution of 177 subjects whose blood samples were analyzed for the distribution of circulating monocyte/macrophage cell subsets.

Age-group	Male	Female	Total
NB	1	3	4
≥1m to <6m	13	1	14
≥6m to <12m	10	0	10
≥1y to <2y	8	5	13
≥2y to <5y	10	8	18
≥5y to <8y	7	11	18
≥8y to <13y	11	6	17
≥13y to <16y	4	2	6
≥16y to <20y	2	3	5
≥20y to <30y	5	8	13
≥30y to <50y	8	7	15
≥50y to <60y	5	8	13
≥60y to <70y	6	4	10
≥70y to <80y	7	2	9
≥80y	5	7	12
Total	102	75	177

NB: newborn; m: months; y: years

SUPPLEMENTAL TABLE 2. 10-color combinations of fluorochrome-conjugated monoclonal antibodies used for the identification of monocytic cells and their subsets in the different samples studied.

A. Panel used for immunophenotyping cord blood (CB), peripheral blood (PB), lymph node and spleen samples

	PacO	BV650	BV785	FITC	PerCP Cy5.5	PE	PECF594	PECy7	APC	APCC750
						Anti-SLAN				
Panel 1	CD45	CD62L	CD16	CD36	CD14	+	CD33	HLADR	CD64	CD300e
						anti-FcεRI*				
Reagent Source	Thermo -Fisher	BD	BD	Cytognos	BD	Miltenyi + Immunostep	BD	BD	BD	Immunostep

* Included in a subset of 3 PB, 1 lymph node and 1 spleen samples

B. Panel used for immunophenotyping bone marrow (BM) samples

	PacO	BV650	BV785	FITC	PerCP Cy5.5	PE	PECF5 94	PECy7	APC	APCC750
					CD14	Anti-SLAN				
Panel 2	CD45	CD62L	CD16	CD36	+	+	CD117	HLADR	CD64	CD300e
					CD34**	anti-FcεRI*				
Reagent Source	Thermo- Fisher	BD	BD	Cytognos	BD	Miltenyi + Immunostep	BD	BD	BD	Immunostep

* Included in a subset of 2 BM samples. **In a subset of 7 BM samples

PacO: Pacific Orange; BV: Brilliant Violet; FITC: fluorescein isothiocyanate; PerCPCy5.5: peridinin chlorophyll protein-cyanine 5.5; PE: phycoerythrin; PECy7: phycoerythrin-cyanine 7; APC: allophycocyanine; APCC750: allophycocyanine-cyanine 750. BD: Becton/Dickinson Biosciences.

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