increased proliferative capacity of cells expressing the exon 2 mutation. Our finding of bimodal FOXP3 expression in the carrier differs from the only other published report of a carrier with an exon 2 mutation (c.227delT) who expressed only one population of $CD4^+CD25^+CD127^{lo}$ cells, all expressing WT FOXP3.[7] Our results are the first to confirm the natural ability of isoforms lacking exon 2 to promote their own transcription, resulting in expression of a functional isoform of FOXP3 that can support Treg cell development.

In conclusion, we report a family with autoimmunity across 3 generations, including 2 affected male subjects. We have shown that a milder IPEX phenotype was due to selective deletion of FOXP3 exon 2 expression, resulting in loss of FOXP3fl but retained expression of FOXP3Δ2. This report confirms that FOXP3Δ2 can support Treg cell development *in vivo* and mitigate at least some of the clinical features of complete FOXP3 deficiency, as observed in patients with classic IPEX syndrome. These findings provide powerful patient-derived evidence for the functional capabilities of the FOXP3Δ2 isoform. The variable penetrance is important because it suggests that future patients might be identified in populations with milder autoimmunity not reaching the criteria for IPEX syndrome.

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Distribution of subsets of blood monocytic cells throughout life

To the Editor:

Currently, it is well established that monocytes are a heterogeneous type of cell consisting of phenotypically and functionally distinct subpopulations found to be numerically altered in blood in patients with a wide variety of disease conditions, such as infection, autoimmunity, respiratory and cardiovascular diseases, and inflammatory disorders.[1,2] Thus, 3 subpopulations of circulating monocytic cells have been identified based on expression of the CD14 LPS receptor and the CD16 low-affinity Fc IgG receptor: (1) $CD14^{hi}CD16^-$ classical monocytes (cMos), (2) $CD14^{hi}CD16^+$ intermediate monocytes (iMos), and (3) $CD14^{-/lo}CD16^+$ nonclassical monocytes (ncMos).[3] Monocytes circulate in blood for up to 3 days until recruited to virtually any human tissue, where they differentiate into either tissue macrophages or myeloid dendritic cells.[4] Then, tissue macrophages can migrate from their tissue location through the lymph system[5] before they potentially die outside the circulation.[6]

Despite our knowledge of the biology of monocytes increasing in recent years, normal reference ranges for the distinct monocyte subsets in blood throughout life (eg, from cord blood [CB] and newborns to elderly subjects) have never been systematically defined. Moreover, the precise maturational and functional relationship between the distinct populations of blood monocytes and their tissue distribution profiles remains unknown.

To provide a frame of reference for future identification of disease-associated altered profiles, we investigated the distribution of distinct monocytic cell subsets in normal blood versus secondary lymphoid tissues, such as bone marrow (BM), lymph node, and spleen. Our aim was to shed light on 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 male and 75 female

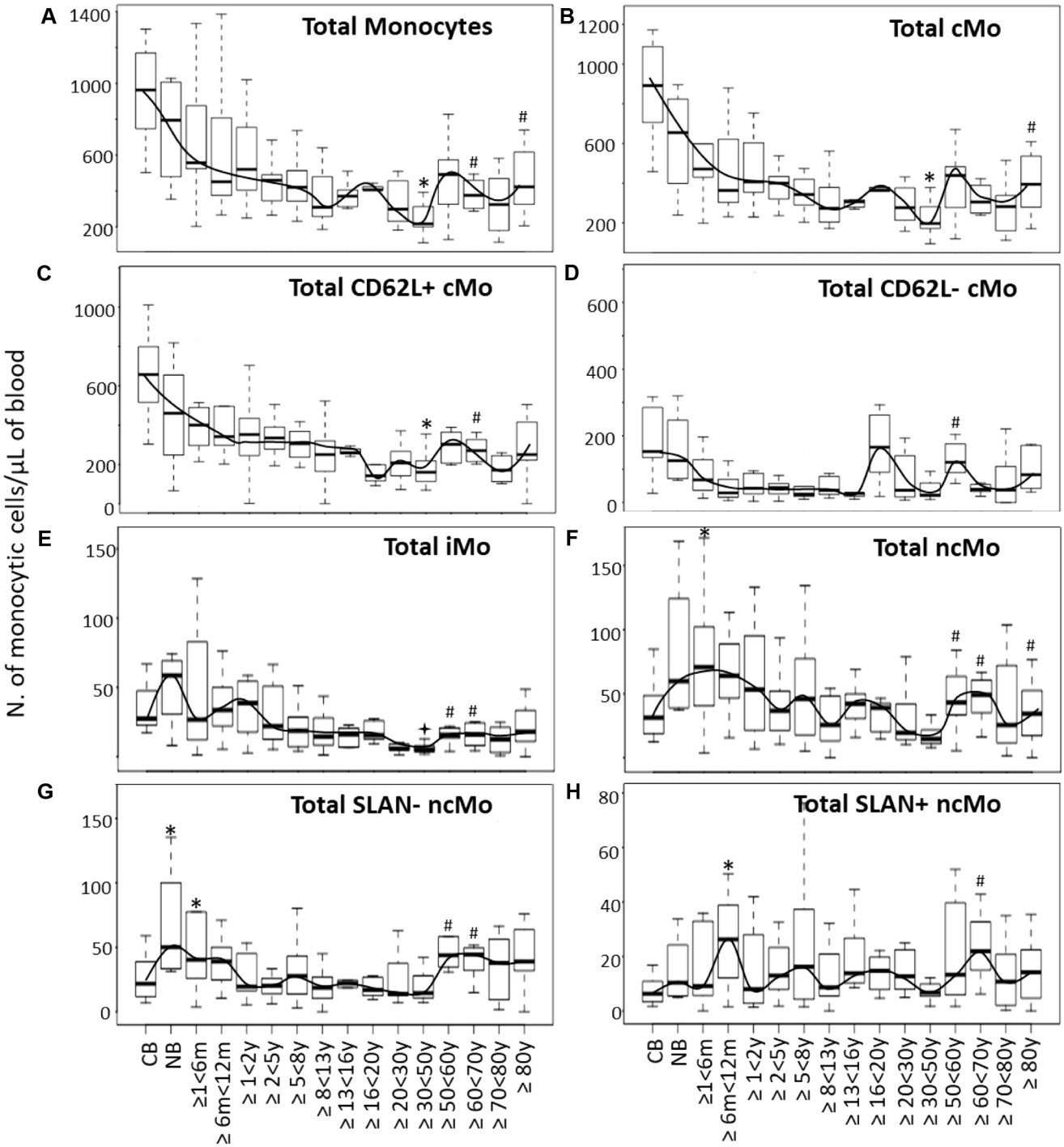

FIG 1. Distribution of different subsets of circulating monocytes in CB and peripheral blood samples from healthy subjects through life. *$P < .05$ versus CB. #$P < .05$ versus subjects aged 30 or more to less than 50 years. +$P < .05$ versus newborns. *NB,* Newborns.

subjects) with a median age of 26 years (range, 4 days to 92 years) distributed by age group, as shown in Table E1 in this article's Online Repository at www.jacionline.org. In addition, 9 BM samples (6 from male and 3 from female subjects; median age, 49 years [age range, 21-83 years]) from 9 of the above referred healthy donors, together with 13 lymph node and 13 spleen paired samples (9 from male and 4 from female subjects; median age, 69 years [age range, 49-81 years]) 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), with the organs maintained as viable throughout the procedure.

Identification of different monocytic cell subsets was performed by using highly sensitive 10-color flow cytometry. The immunophenotypic criteria used for identification of monocytic subpopulations, as well as the precise flow cytometry

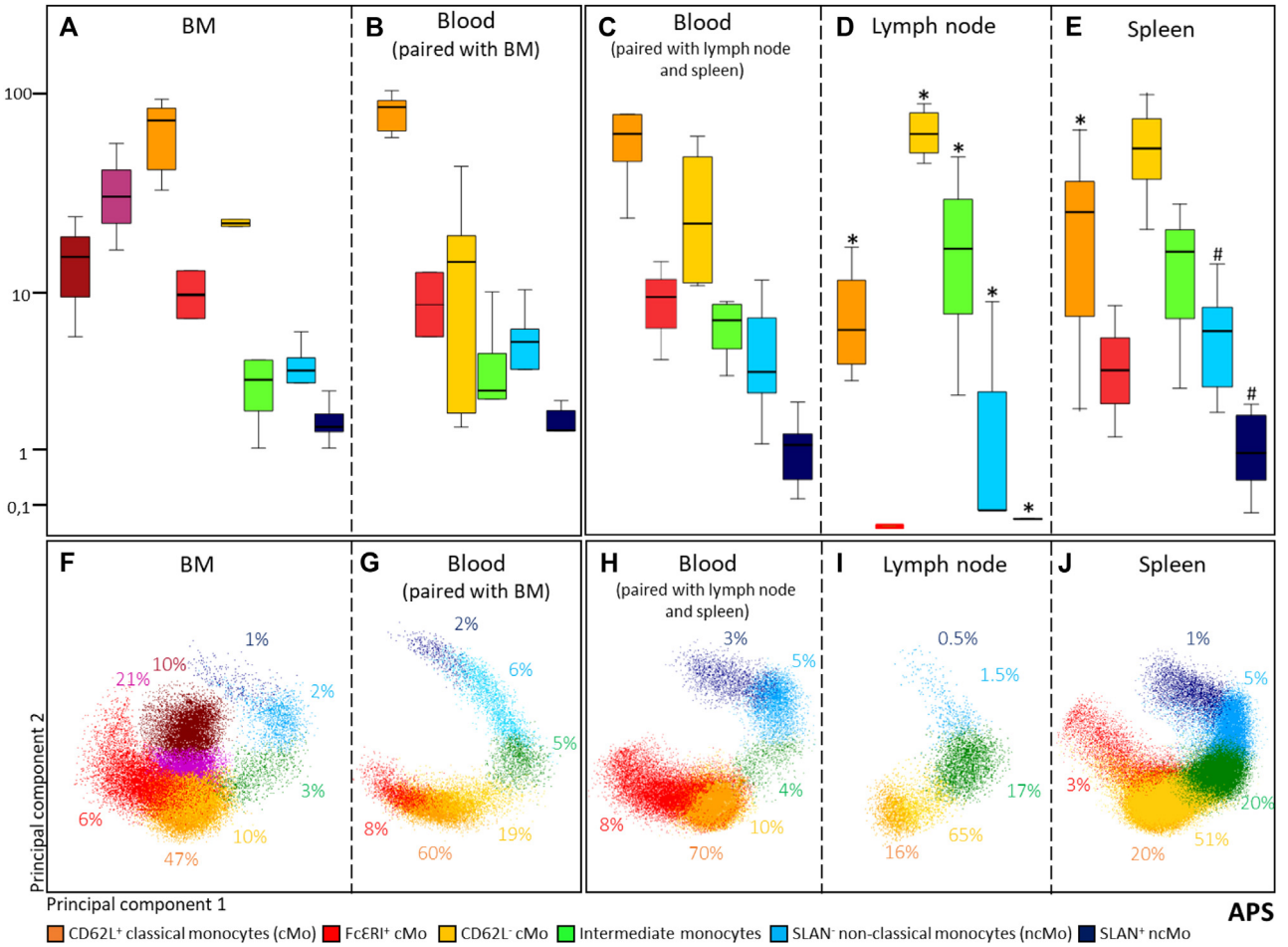

FIG 2. Relative distribution of different subsets of monocytes in adult blood versus paired BM and paired lymph node and spleen samples. **A-E,** $*P < .05$ versus blood. $\#P < .05$ versus lymph nodes. **F-J,** Multidimensional representations of distinct monocyte cell subsets in different tissues. $P > .05$ for comparison of the 2 blood sample groups for all monocyte subsets.

protocols, panels, and reagents used, are detailed in the Methods section in this article's Online Repository at www.jacionline.org (Tables E2 and E3). Five distinct subsets of monocytes were systematically identified in every sample analyzed: (1) $CD62L^+$ and (2) $CD62L^-$ cMos; (3) iMos; and (4) $SLAN^-$ and (5) $SLAN^+$ ncMos. An additional population of $Fc\varepsilon RI^+CD14^{hi}CD16^-$ monocytes was also identified in a subset of samples stained with anti-FcεRI (see Fig E1 in this article's Online Repository at www.jacionline.org).

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 (Fig 1); however, although cMos reached their highest numbers in CB samples, iMo and ncMo numbers 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-life requirement for sufficient numbers of monocytes to fill the distinct tissues. Afterward, the 3 major monocyte subsets decreased until the age of 8 to 13 years, subsequently increased again at adolescence (particularly $CD62L^+$ cMos), and remained high in younger adults, decreasing thereafter until the age of 30 to 50 years. From 50 years onward, all of the 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 cMos, iMos, and ncMos, particularly during the earliest periods of life and after the age of 50 years, our results would support the notion that iMos and ncMos might correspond to monocytic cell subsets at more advanced stages of maturation than cMos, which is 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 cMos into iMos and ncMos more likely occur outside the blood compartment rather than in the blood.[6,9]

To gain insight into the potential tissue relationship between cMos and both iMos and ncMos, we further investigated the distribution of the distinct subsets of monocytes in normal BM, lymph node, and spleen samples. Our results showed that their relative distribution varies substantially in BM, lymph node, and spleen compared with that in blood (Fig 2). Thus, although $CD62L^+$ cMos were by far the most abundant subset of cMos in blood and BM, they were outnumbered by $CD62L^-$ cMos in lymph node and spleen. Moreover, higher percentages of iMos were found in lymph node and spleen versus both blood and BM, in which this cell subset represented a minor monocytic

population. In contrast, ncMos were particularly represented in blood and spleen samples, although they were found at very low percentages in BM and lymph node; interestingly, no (or very small numbers of) $SLAN^+$ ncMos were found in lymph node samples. The predominance of $CD62L^-$ cMos in lymph node and spleen would most likely reflect the more mature nature of cMos in these lymphoid tissues (vs BM and blood). Altogether, these findings suggest that, consistent with *in vivo* 6,6-$^2H_2$ glucose monocyte tracking studies,[6,9] cMos might lose CD62L and differentiate into iMos in lymphoid tissues. iMos might then recirculate through the lymph system[2] and sequentially give rise to $SLAN^-$ and $SLAN^+$ ncMos. Despite this, there were no statistically significant differences between the phenotypic profile of the different monocytic subsets in blood versus lymph node and spleen (see Fig E2 in this article's Online Repository at www.jacionline.org).

In summary, our results show that the number of circulating blood monocytes and their subsets varies significantly throughout life, which provides 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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Autonomous regulation of IgE-mediated mast cell degranulation and immediate hypersensitivity reaction by an inhibitory receptor CD300a

To the Editor:

Although phosphatidylserine (PS) confined to the inner leaflet of plasma membrane is exposed on the cell surface when cells undergo apoptosis, viable cells, including mast cells (MCs), also externalize PS in certain cellular states.[1,2,E1-E3] However, the pathophysiological significance of PS exposure on viable cells remains elusive. To address the role of PS externalization on the cell surface of viable MCs, we monitored PS surface exposure on bone marrow–derived cultured MCs (BMMCs) by confocal microscopy after stimulation with trinitrophenyl (TNP)-specific IgE and TNP-ovalbumin (OVA) in the presence of PSVue 643, a fluorescent dye with rapid binding capacity for PS. The dye began to accumulate on the cell surface of live BMMCs within 600 seconds after FcεRI stimulation, whereas the nonstimulated BMMCs remained negative for the staining (Fig 1, *A*; see Video E1 in this article's Online Repository at www.jacionline.org), indicating that PS is externalized within 10 minutes after activation.

MCs abundantly express CD300a, an inhibitory immunoreceptor for PS.[3-5] By imaging flow cytometry analyses, we found that both PS and CD300a showed a polarization and

METHODS

Subjects and samples

A total of 188 EDTA-anticoagulated blood samples were studied. Eleven CB specimens from full-term neonates of healthy mothers and 177 blood samples from 164 healthy subjects and 13 solid organ donors (102 male and 75 female subjects; median age, 26 years [age range, 4 days to 92 years]) were acquired with the following age distribution: newborns (median, 4.5 days [range, 4-10 days]), 4 cases; children 1 or more to less than 6 months of age, 14 subjects; children 6 or more to less than 12 months of age, 10 subjects; children 1 year or more to less than 2 years, 13 subjects; children 2 or more years to less than 5 years, 18 subjects; children 5 or more years to less than 8 years, 18 subjects; children 8 or more years to less than 13 years, 17 subjects; children 13 or more years to less than 16 years, 6 subjects; children 16 or more years to less than 20 years, 5 subjects; adults 20 or more years to less than 30 years, 13 subjects; adults 30 or more years to less than 50 years, 15 subjects; adults 50 or more years to less than 60 years, 13 subjects; adults 60 or more years to less than 70 years, 10 subjects; adults 70 or more years to less than 80 years, 9 subjects; and adults 80 or more years, 12 subjects (Table E1). CB, childhood, and adult samples were collected at the Erasmus University Medical Center; the University Hospital of Salamanca, Salamanca; and the corresponding primary health care centers of Salamanca, respectively.

In addition, 9 normal BM samples (6 from male and 3 from female subjects; median age, 49 years [age range, 21-83 years]) collected in parallel to blood samples from 9 healthy donors (undergoing orthopedic surgery) and 13 lymph node plus 13 spleen paired samples (9 from male and 4 from female subjects; median age, 69 years [age range, 49-81 years]) 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), with the organs maintained as viable throughout the procedure, according to the standard transplantation protocol.

All study subjects had no medical history of either immunologic or oncohematologic disorders. All participants, except for the anonymized solid organ donors, were enrolled in the study after informed consent was provided by each subject, their corresponding legal representative, or both. 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 cytometric immunophenotypic studies

All samples (fresh whole CB, blood, BM, lymph node, and spleen specimens) were stained and processed within less than 1 to 24 hours after collection. For CB, blood, and BM samples, the EuroFlow bulk-lyse standard operating procedure[E1] was used to lyse nonnucleated red cells before staining. Lymph node and spleen samples were mechanically dissociated into single-cell suspensions before staining.[E2] Afterward, 5×10^6 leukocytes per sample aliquot were stained with a single 10-color combination of the following mAb reagents (mAb [clone]–fluorochrome; see Tables E2 and E3): CD45 (HI30)–Pacific Orange, CD62L (DREG-56)–Brilliant Violet 650, CD16 (3G8)–Brilliant Violet 786, CD36 (CLB-IVC7)–fluorescein isothiocyanate, CD14 (MφP9)–peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5), anti-SLAN (DD-1)–phycoerythrin (PE), CD33 (WM53)–PE-CF594, anti–HLA-DR (G46-6)–PE–cyanine 7, CD64 (10.1)– allophycocyanin, and CD300e (UP-H2)–allophycocyanin–cyanine 750. 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 (Tables E2 and E3) because the monocyte subsets expressing each of these 2 markers were mutually exclusive (data not shown). In BM samples additional markers were also included to further identify uncommitted monocytic precursors: CD34 (P67.6)–PerCP-Cy5.5 and CD117 (YB5.B8)–PE-CF594 (Tables E2 and E3). Reagents were purchased from BD (San Jose, Calif), except CD45 (Thermo Fisher Scientifics, Waltham, Mass), CD36 (Cytognos S.L., Salamanca, Spain), anti-SLAN (Milteny Biotec, Cologne, Germany), and both CD300e and anti-FcεRI (IMMUNOSTEP, Salamanca, Spain). Sample acquisition was performed immediately after immunostaining with LSR Fortessa X-20 flow cytometers (BD) using FACSDiva software (BD). For instrument setup, calibration, and monitoring, the EuroFlow instrument setup and compensation protocol (www.EuroFlow.org) for 12-color measurements was used. For data analysis, the Infinicyt software (Cytognos S.L) was used.

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 counts were derived. The gating strategy used for identification of each cell subset is shown in Fig E1.

Statistical analyses

For continuous variables, medians, means, and SDs, as well as ranges and 10th, 25th, 75th, and 90th percentiles, were calculated; for categorical variables, frequencies were used. The statistical significance of differences observed between 2 or more groups was assessed by using nonparametric Mann–Whitney U and Kruskal–Wallis tests for independent variables, respectively. For comparisons between 2 or more groups of paired samples, nonparametric Friedman and Wilcoxon tests were used, respectively. Spearman rank correlation (R software package, www.R-project.org) was used to explore the degree of association between 2 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 of .05 or less were considered to be associated with statistical significance.

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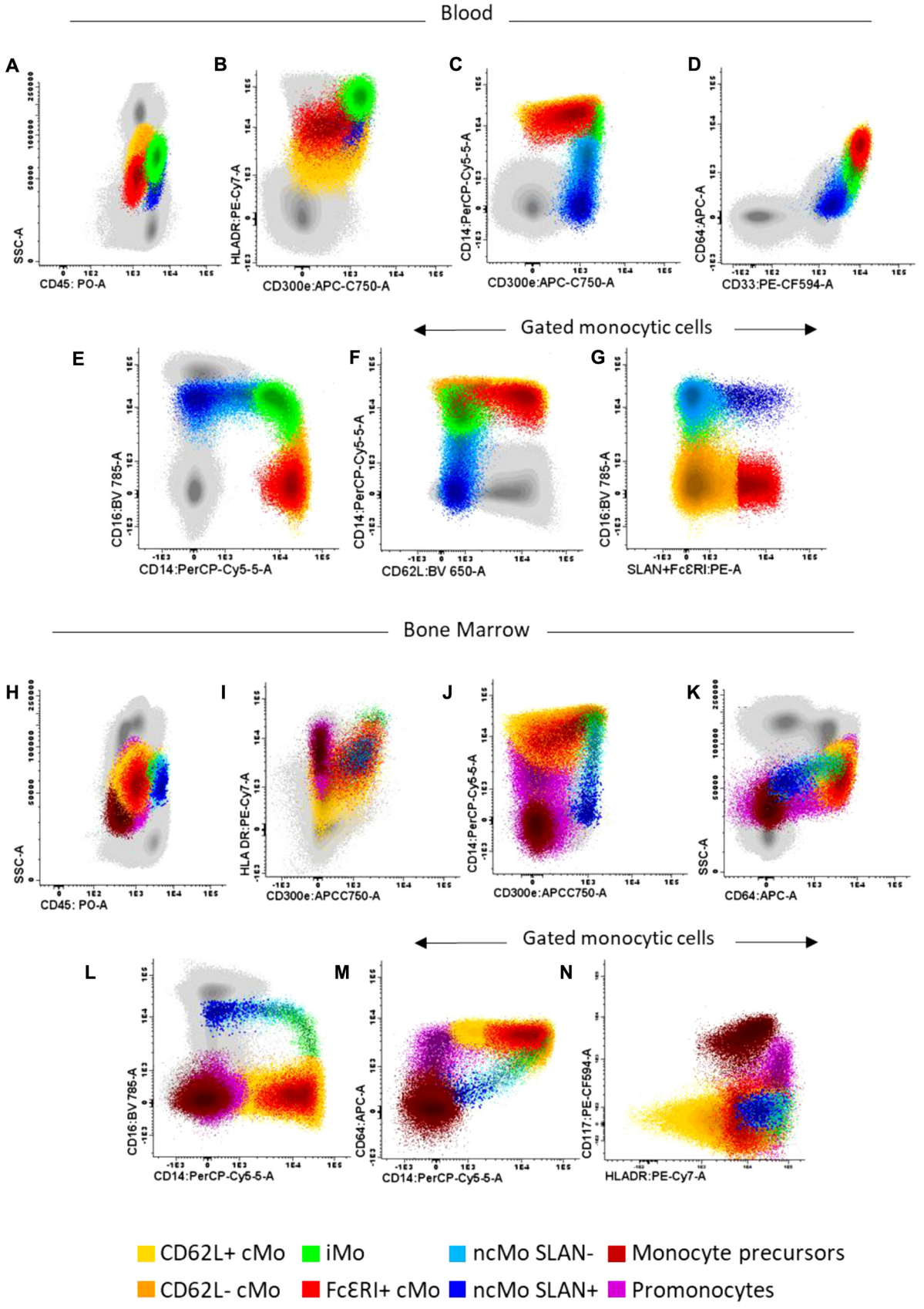

FIG E1. Identification of different subsets of circulating monocytes in representative peripheral blood and BM samples from a healthy donor.

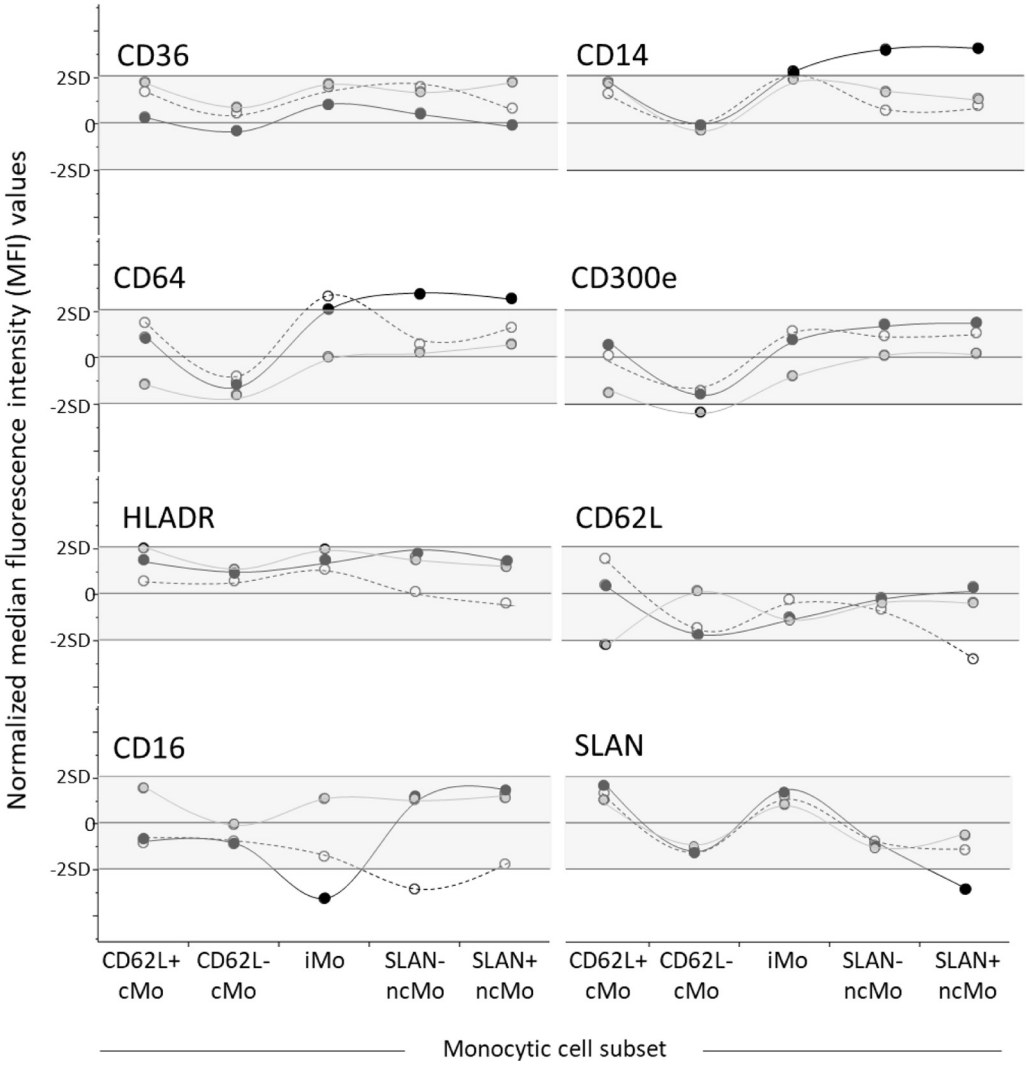

FIG E2. Comparison between the phenotypic profile of the different subsets of monocytic cells in BM, lymph node, and spleen versus blood.

TABLE E1. Age and sex distribution of 177 subjects whose blood samples were analyzed for the distribution of circulating monocyte/macrophage cell subsets

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

NB, Newborns.

TABLE E2. Ten-color combinations of fluorochrome-conjugated mAbs used for identification of monocytic cells and their subsets in the different samples studied: Panel used for immunophenotyping CB, peripheral blood, lymph node, and spleen samples

	PacO	BV650	BV785	FITC	PerCP-Cy5.5	PE	PE-CF594	PE-Cy7	APC	APC-C750
Panel 1	CD45	CD62L	CD16	CD36	CD14	Anti-SLAN + anti-FcεRI*	CD33	HLADR	CD64	CD300e
Reagent source	Thermo Fisher	BD	BD	Cytognos	BD	Miltenyi Biotec + IMMUNOSTEP	BD	BD	BD	IMMUNOSTEP

APC, Allophycocyanin; *APC-C750*, allophycocyanin–cyanine 750; *BV*, Brilliant Violet; *FITC*, fluorescein isothiocyanate; *PacO*, Pacific Orange; *PE-Cy7*, phycoerythrin–cyanine 7; *PerCP-Cy5.5*, peridinin chlorophyll protein–cyanine 5.5.

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

TABLE E3. Ten-color combinations of fluorochrome-conjugated mAbs used for the identification of monocytic cells and their subsets in the different samples studied: Panel used for immunophenotyping BM samples

	PacO	BV650	BV785	FITC	PerCP-Cy5.5	PE	PE-CF594	PE-Cy7	APC	APC-C750
Panel 2	CD45	CD62L	CD16	CD36	CD14 + CD34†	Anti-SLAN + anti-FcεRI*	CD117	HLA-DR	CD64	CD300e
Reagent source	Thermo Fisher	BD	BD	Cytognos	BD	Miltenyi Biotec + IMMUNOSTEP	BD	BD	BD	IMMUNOSTEP

APC, Allophycocyanin; *APC-C750*, allophycocyanin–cyanine 750; *BV*, Brilliant Violet; *FITC*, fluorescein isothiocyanate; *PacO*, Pacific Orange; *PE-Cy7*, phycoerythrin–cyanine 7; *PerCP-Cy5.5*, peridinin chlorophyll protein–cyanine 5.5.

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

†Included in a subset of 7 BM samples.