Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138$^-$ and CD138$^+$ plasma cells

Anouk Caraux,[1] Bernard Klein,[1-3] Bruno Paiva,[4,5] Caroline Bret,[1-3] Alexander Schmitz,[6] Gwenny M. Fuhler,[7] Nico A. Bos,[7] Hans E Johnsen,[6] Alberto Orfao,[5,8] and Martin Perez-Andres[5,8] for the Myeloma Stem Cell Network (MSCNET)

[1]INSERM, U847, Montpellier, France; [2]CHU Montpellier, Institute of Research in Biotherapy, France; [3]Université Montpellier1, France; [4]Service of Hematology, Hospital Universitario de Salamanca, Salamanca, Spain; [5]Centro de Investigación del Cáncer, University of Salamanca-CSIC, Salamanca, Spain; [6]Service of Hematology, Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark; [7]University Medical Center, Groningen, Netherlands, and [8]Service of Cytometry, Department of Medicine, University of Salamanca, Salamanca, Spain

ABSTRACT

Generation of B and plasma cells involves several organs with a necessary cell trafficking between them. A detailed phenotypic characterization of four circulating B-cell subsets (immature-, naïve-, memory- B-lymphocytes and plasma cells) of 106 healthy adults was realized by multiparametric flow cytometry. We show that CD10, CD27 and CD38 is the minimal combination of subsetting markers allowing unequivocal identification of immature (CD10$^+$CD27$^-$CD38$^+$, 6±6 cells/μL), naïve (CD10$^-$CD27$^-$CD38$^-$, 125±90 cells/μL), memory B lymphocytes (CD10$^-$CD27$^+$CD38$^-$, 58±42 cells/μL), and plasma cells (CD10$^-$CD27^{++}CD38^{++}, 2.1±2.1 cells/μL) within circulating CD19$^+$ cells. From these four subsets, only memory B lymphocytes and plasma cells decreased with age, both in relative and absolute counts. Circulating plasma cells split into CD138$^-$ (57±12%) and CD138$^+$ (43±12%) cells, the latter displaying a more mature phenotypic profile: absence of surface immunoglobulin, lower CD45 positivity and higher amounts of cytoplasmic immunoglobulin, CD38 and CD27. Unlike B lymphocytes, both populations of plasma cells are KI-67$^+$ and show weak CXCR4 expression.

Key words: plasma cells, healthy donors, bone marrow, peripheral blood, dendritic cells.

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Introduction

Human B-cell biology has been extensively documented.[1] Circulating human B cells comprise two-thirds of CD27$^-$CD20$^+$CD19$^+$CD38$^-$ naïve B lymphocytes and one third of CD27$^+$CD20$^+$CD19$^+$CD38$^-$ memory B cells. Very low numbers of plasma cells (2/μL) are found in peripheral blood of healthy donors. Because of their low count, only few studies have been devoted to characterizing their phenotype, most of them dealing with newly generated plasma cells after *in vivo* immunization.[2] Steady-state circulating plasma cells lack CD20, express CD19 and CD38^{high}. It has been recently reported that steady-state circulating plasma cells are mainly of mucosal origin, the majority of them secreting IgA (84%), expressing CCR10 (56%) and β7 integrin (32%).[3] Steady-state circulating plasma cells are generally termed plasmablasts because only half express CD138, a proteoglycan that is a hallmark of plasma cells,[4] while they are CD45$^+$ and HLA-class II$^+$. Plasmablasts are generated in the lymph nodes, and induced to circulate for a short period until they will reach a niche in bone marrow, spleen, mucosa associated lymphoid tissues (MALT) or lymph nodes.[5] These niches will provide circulating early plasma cells with those factors required to survive and to further differentiate into long-living mature plasma cells.[1] In murine bone marrow, plasma cell niche involves SDF-1 producing cells and is shared with hematopoietic stem cells

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Correspondence: Prof. Bernard Klein, INSERM U847, Institute for Research In Biotherapy, CHU Montpellier, Hospital St Eloi, Av Augustin Fliche, 34295 Montpellier, France. Phone: international +33.467.330455. Fax: international +33.467.330459. E-mail:bernard.klein@inserm.fr/Prof. Alberto Orfao, Centro de Investigación del Cáncer Avda. Universidad de Coimbra S/N, Campus Miguel de Unamuno, 37007-Salamanca, Spain. Phone: international +34.923.294811.Fax: international +34.923.294795. E-mail:orfao@usal.es
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and pro-pre B cells.[1] The rarity of this niche is a matter of regulation of normal Ig production.[6] In particular, newborn plasmablasts, generated after *in vivo* Ag immunization, have to compete with old plasma cells for binding to a niche, inducing the old plasma cells to recirculate.[7]

Another minor population of circulating B cells which accounts for 2-4% of all peripheral blood B cells has been documented:[8] transitional or immature B cells. These cells have an immature phenotype ($CD10^+$, $CD24^{high}$, $CD38^{high}$), unmutated Ig genes and a reduced ability to be activated *in vitro*.[8,9] Notably, these immature B cells appear first in peripheral blood after hematopoietic stem cell allograft[8] and their frequency is highly increased in cord blood.[8,9] Recently, additional heterogeneity has been reported for human transitional B cells with a more differentiated stage expressing ABCB1 transporter and intermediate density of CD10 and CD38.[10,11]

There is a progressive defect to mount high affinity humoral immune responses in elderly people.[12] This defect implies several mechanisms: i) a decrease in bone marrow niches able to support B-cell generation and plasma cell survival, due to the progressive replacement of hematopoietic bone marrow by fat cells; ii) a defect in germinal centers due to a decreased follicular dendritic cell function and T-cell senescence; iii) a defect of B cells to undergo Ig class switch recombination and somatic mutation due to reduced E47 and AID gene expressions.[13]

In this study we have first characterized the above listed populations of circulating B cells using multi-parameter flow cytometry to define the best combination of markers to identify them, and to study their fluctuation with age. In addition, we have characterized in detail the activation status and homing phenotype of steady-state circulating plasma cells.

Design and Methods

Detailed methodologies are fully described in the Online Supplementary Appendix. Briefly, peripheral blood from 106 adult healthy donors was analyzed after informed consent was given. Erythrocyte-lysed whole peripheral blood samples or mononuclear cells obtained by Ficoll-hypaque density gradient centrifugation were labeled with Abs conjugated with different fluorochromes (Abs are listed in the Online Supplementary Appendix). For intracellular staining of Ig or KI-67, cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences). B-cell subpopulations were identified using a combination of 7-8 fluorochrome-conjugated Abs. The fluorescence was acquired with a FACSCanto II or a FACSAria flow cytometer and analyzed with the Infinicyt 1.3 software (Cytognos SL, Salamanca, Spain). $CD20^-CD38^{++}CD138^-$ cells and $CD20^-CD38^{++}CD138^+$ cells were sorted with a FACSAria flow cytometer to perform cytospins. Cells were stained with May-Grünwald-Giemsa. Mean values and their SD, median and range were calculated for continuous variables with SPSS statistical software pack-

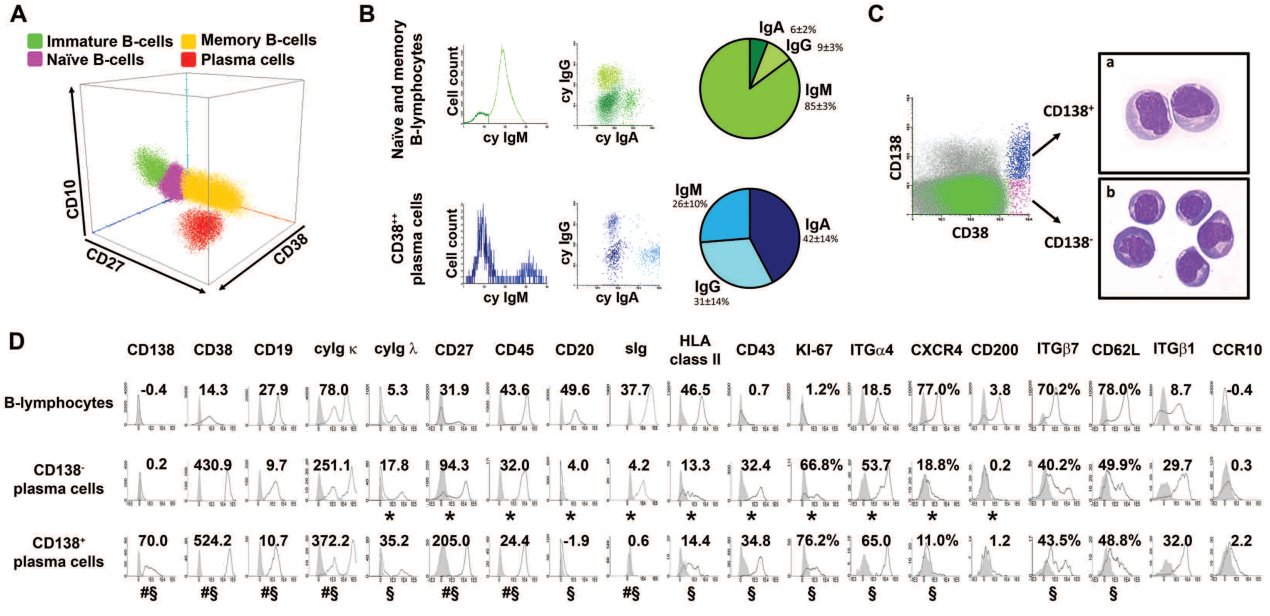

Figure 1. Phenotype of peripheral blood B-cell subsets, and focus on circulating plasma cells. (A) Distribution of immature (green), naïve (pink), memory B lymphocytes (orange), and plasma cells (red), according to the expression of CD10, CD27 and CD38. (B) Cell phenotype was analyzed by gating on $CD19^+CD20^+CD38^{-/+}$ - naïve and memory - B lymphocytes (green), and $CD19^+CD20^-CD38^{++}$ plasma cells (blue). Histograms and dotplots show FACS labelings of cytoplasmic Ig (cylg). The contribution of each Ig isotype is represented in the pie charts and numbers are the mean percentages ± one SD for 13 healthy individuals. (C) $CD20^-CD38^{++}CD138^+$ (a) and $CD20^-CD38^{++}CD138^-$ (b) peripheral blood plasma cells were FACS sorted and stained with May-Grünwald-Giemsa (x1000 magnification). (D) Open histograms show FACS labelings with indicated mAbs. Gray histograms display the corresponding negative control mAbs. Cell phenotype was analyzed by gating on $CD19^+CD20^+$ B lymphocytes, and both $CD38^{++}CD138^-$ and $CD38^{++}CD138^+$ plasma cells. Data from one representative experiment is shown. Numbers in panels indicate mean values of the staining indexes for each specific mAb used or the percentage of positive cells, determined on between 3 to 30 different healthy donors.

age (SPSS 10.1 Inc., Chicago, IL). *P* values less than 0.05 were considered statistically significant.

Results and Discussion

Immunophenotypic characteristics of human peripheral blood B-cell subsets

Circulating B cells in a given individual are important indicators of the state of B-cell production because generation of B lymphocytes and plasma cells involves sequential maturation steps in different organs and tissues[14] and a necessary cell traffic between these organs through peripheral blood.[15] Various strategies have been applied for their identification and no study has comparatively analyzed these four B-cell subsets in large cohorts of healthy donors. Here, we show that four B-cell subsets were systematically identified in peripheral blood of 106 healthy donors showing phenotypic profiles of immature ($CD10^+CD19^+CD20^+CD27^-CD38^{++}$)[8], naïve ($CD10^-CD19^+CD20^+CD27^-CD38^-$)[1], and memory ($CD10^-CD19^+CD20^+CD27^+CD38^-$) B lymphocytes,[1] in addition to plasma cells ($CD10^-CD19^+CD20^-CD27^{++}CD38^{++}$).[1] Principal component analysis showed that CD10, CD27, and CD38 are the minimal marker combination for unequivocal identification of immature, naïve, and memory B lymphocytes, as well as plasma cells among $CD19^+$ B cells (Figure 1A). Compared to naïve B lymphocytes, circulating immature B lymphocytes, previously described as phenotypically similar to transitional murine B cells,[8] retain a phenotype of late bone marrow B-cell precursors (i.e. CD5, CD10, and $CD38^{++}$) (*Online Supplementary Figure S1*), supporting the hypothesis that they are immature B lymphocytes leaving the bone marrow prior to full maturation.[8] Transition from naïve to memory B lymphocytes was characterized by increased CD24, CD25, CD27 and CD53 expression, and a downregulation of CD5 and CD23 (*Online Supplementary Table S1*). A fourth discrete $CD19^+$ B-cell subset with higher light-scatter characteristics and a plasma cell phenotype ($CD20^-$, $CD38^{++}$, $CD27^{++}$ and cytoplasmic Ig $(cyIg)^+$, with heterogeneous positivity for CD138 (57±12% $CD138^-$ cells and 43±12% $CD138^+$ cells) was detected in all 106 healthy donors analyzed.

Fluctuation of peripheral blood B-cell subsets according to age

In our large cohort of 106 healthy donors, naïve and memory B lymphocytes were highly represented, while immature B lymphocytes and plasma cells were minor populations (Table 1). No correlation was found between age and percentages or absolute counts of total circulating B cells, immature B lymphocytes or naïve B lymphocytes (Figure 2). In contrast, statistically significant inverse correlations were found between age and both the percentage and absolute count of circulating memory B lymphocytes (n=106; $R^2 \leq -0.22$, $P \leq 0.05$) and plasma cells (n=106; $R^2 \leq -0.27$, $P \leq 0.02$). This also holds true when Ig heavy chain isotype-specific subsets of plasma cells (IgG^+, IgA^+ and IgM^+) and memory B lymphocytes (only for IgG^+ and IgM^+) were considered separately (*Online Supplementary Figure S2A*). Our results indicate that production of immature and naïve B-lymphocytes is not significantly affected by aging, in contrast to previous suggestions by others.[12,16] On the contrary, differentiation of naïve B lymphocytes into memory B lymphocytes and then plasma cells, is clearly reduced. These findings would confirm and extend previous observations describing alterations on B cells consisting of a more restricted diversity.[12,16] In principle, this could not be attributed to a lower ability for Ig class switch since we have reported here decreased numbers of both non-switched IgM^+/IgD^+, and switched IgG^+ memory B lymphocytes with aging.[13] This age-related decrease in memory B lymphocyte counts could potentially be due

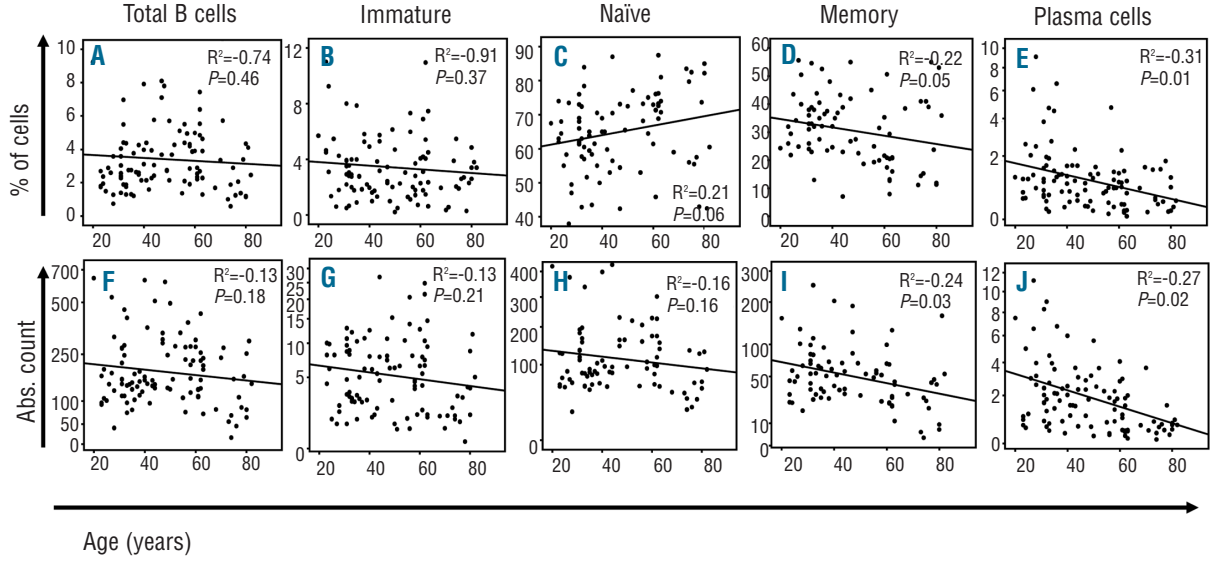

Figure 2. Age-related changes in circulating B-cell subsets. (A) Data plotted in each diagram represent correlation between the age of each individual healthy donor and the percentage and absolute counts of total B cells (panels A and F, respectively), immature (panels B and G), naïve (panels C and H), and memory (panels D and I) B lymphocytes, as well as plasma cells (panels E and J).

to a lower exposure to new Ag (associated with a less-exposed lifestyle) leading to a more restricted memory B-cell repertoire or to an exhausted ability of memory B lymphocytes that have been triggered many folds along the lifespan of elderly people, to generate expanded responses. The age-associated decrease in the number of circulating IgA$^+$, IgG$^+$, or IgM$^+$ plasma cells was even more pronounced than that of memory B lymphocytes, suggesting the occurrence of lower humoral response rates in the elderly. In line with this hypothesis, no significant correlation was found in our study between Ig heavy chain isotype-specific circulating plasma cells and their serum antibody counterpart (*Online Supplementary Figure S2B*).

Detailed characterization of circulating CD138$^-$ and CD138$^+$ plasma cells

FACS-sorted CD138$^-$CD20$^-$CD38^{++} and CD138$^+$CD20$^-$CD38^{++} cells showed a typical plasma cell cytology with no obvious morphological differences (Figure 1C). CD138$^+$ plasma cells showed a greater staining index (SI) than CD138$^-$ plasma cells for CD38 (22% increased SI; n=30, P=0.002), cyIg κ and λ light chains (47% and 98% increased SI, respectively; n=6, P=0.04), CD27 (117% increased SI, n=12, P=0.0004), and a lower SI for CD45 (24% decreased SI; n=6, P=0.004) (Figure 1D). In addition, CD138$^-$ plasma cells, unlike CD138$^+$ plasma cells, expressed weakly CD20 and sIg (Figure 1D) consisting of sIgA$^+$ (49±12%), sIgG$^+$(13±11%), sIgM$^+$sIgD$^-$(18±12%) and sIgM$^-$sIgD$^+$ (5±17%), but no sIgM$^+$/sIgD$^+$ plasma cells. After cell permeabilization, 42±14% cyIgA$^+$, 31±14% cyIgG$^+$ and 26±10% cyIgM$^+$ plasma cells were identified (Figure 1B), with no differences in isotype distribution between CD138$^-$ and CD138$^+$ plasma cells. The lower frequency of sIgG$^+$ *vs.* cyIgG$^+$ plasma cells might be due to the weak sIg expression that is not optimally detected by the anti-IgG Ab or a more mature sIgG$^-$ phenotype of circulating plasma cells. Although HLA-class II (including HLA-DR) expression was heterogeneous and lower in circulating plasma cells compared to peripheral blood B lymphocytes (n=6, P≤0.001), a similar expression was found in CD138$^-$ *vs.* CD138$^+$ plasma cells (Figure 1D). Unlike B lymphocytes, both CD138$^-$ and CD138$^+$ plasma cells were CD43$^+$. Regarding homing receptors, both CD138$^-$ and CD138$^+$ plasma cells showed higher levels of α4 integrin (n=6, P≤0.03), heterogeneously lower amounts of CXCR4 (18.8±9.1% and 11.0±6.1%, respectively; n=6, P≤0.0001), and negativity for CD200 as compared to B lymphocytes, which were constantly positive for the latter two markers (Figure 1D). CD138$^+$ plasma cells expressed lower levels of β7 integrin (n=6, P=0.02) and L-selectin/CD62L (n=5, P=0.03) and higher levels of β1 integrin (n=6, P=0.008) than B lymphocytes (Figure 1D). CCR10 was not expressed by circulating B lymphocytes while it was weakly positive on both CD138$^-$ and CD138$^+$ plasma cells. Of note, high CCR10 expression was detected on the XG-1 and XG-10 myeloma cell lines or on *in vitro* generated plasmablasts with the same anti-CCR10 mAb reagent (*Online Supplementary Figure S3*).[17,18] No significant difference in CCR10 expression was found among plasma cell subsets showing different Ig heavy chain isotypes (*data not shown*). Furthermore, both circulating B lymphocytes and CD138$^-$ and CD138$^+$ plasma cells were constantly negative for VCAM1 (CD106), α5 integrin (CD49e), LFA-3 (CD58), and CD70, as well as for the CD56 and CD117 markers, which are aberrantly expressed by malignant plasma cells (*data not shown*).[19] Based on KI-67 antibody, circulating B lymphocytes were quiescent (1.2±0.8% KI-67$^+$ cells) while circulating CD138$^-$ or CD138$^+$ plasma cells displayed a highly-activated phenotype with 66.8±29.7% and 76.2±12.5% KI-67$^+$ cells, respectively (n=11; P≤0.00003, Figure 1D). Further staining with annexin-V showed that both CD138$^-$ and CD138$^+$ plasma cells were fully viable with only 6.8% and 7.7% of these cell subsets showing annexin-V$^+$ staining, respectively.

Mei *et al.* have suggested that the great majority of circulating plasma cells could have a mucosa origin, because they secrete mainly IgA (84%) and partially express CCR10.[3,6] We did not confirm these results, since only 40-50% of all peripheral blood plasma cells were IgA$^+$ and CCR10 was very weakly expressed by circulating plasma cells. This discrepancy is not due to a defect of the anti-CCR10 mAb used (*Online Supplementary Figure S3*), but could be due to a difference in the gating strategy to define plasma cells and avoid contaminating cells. We used a gating on CD19$^+$CD38^{high} cells that comprise all and only cyIgκ or cyIgλ positive cells. Mei *et al.* used two gating strategies, either CD19$^+$CD27^{high} cells or cyIg$^+$ cells.[3]

What is the origin and behavior of these circulating plasma cells? Given mainly their HLA-DR and CD45 expressions, they are generally thought to be plasmablasts newly-generated in lymphoid organs. In agreement with this hypothesis, the phenotype of these plasma cells is close to that of *in vitro* generated CD38^{++}CD138$^-$ and CD38^{++}CD138$^+$ plasma cells which we recently reported.[18] But the possibility that a fraction of circulating plasma cells could be bone marrow and/or lymphoid-tissue-localized long-living plasma cells that are induced to re-circulate from their niche should be considered. HLA-DR and CD45 expressions are also characteristics of long-living plasma cells, since a large fraction of CD138$^+$ bone marrow plasma cells expresses HLA-class II (60%) or CD45 (65%).[2,20] plasma cells, which are believed to be long-living based on murine models or by grafting human plasma cells in severe combined immunodeficiency mice,[6] are also present in the spleen, MALT or lymph nodes. They are located in APRIL-rich niches in the subepithelium,[5] APRIL being an important plasma cell survival factor.[21] The phenotype of these plasma cells is close to that of circulating CD38^{++} CD138$^-$ plasma cells (A Caraux *et al.*, unpublished observations, 2010). In human spleen, these plasma cells are located outside the follicles, express highly CD38, cyIg (45% cyIgM, 40% cyIgG and 15% cyIgA), and, unlike bone marrow plasma cells, weakly sIg, CD20 and did not express CD138.[1] They also express HLA-DR and CD45 and have mutated Ig genes. Such a recirculation of plasma cells was hypothesized to explain the appearance of circulating tetanus toxin-unrelated plasma cells, seven days after immunization of healthy donors with the toxin.[7] If this mechanism occurs in case of tetanus toxin immunization, it may also occur in steady-state conditions with newly-generated circulating plasmablasts competing with long-living plasma cells. The activation status of the circulating plasma cells (KI-67$^+$) could indicate that they have been induced to recirculate by local stimulation. A highly-regulated recirculation mechanism has been demonstrated for murine hematopoietic stem cells with circadian variations.[22] These circulating hematopoietic stem cells can home to non-hematopoietic tissues for a time to exert immune surveillance and

may enter back into the peripheral blood via lymphatics and thoracic duct.[23] As human bone marrow plasma cells use a stromal niche that is similar to that used by hematopoietic stem cells[24] and as the count of circulating CD34+ cells in steady-state conditions is similar to that of circulating plasma cells in healthy donors, similar mechanisms could drive the circulation of hematopoietic stem cells and plasma cells.

Authorship and Disclosures

AC and MPA performed the experiments, designed research, and wrote the paper. BP, CB, AS, GF contributed in performing the experiments. NB, HJ contributed in writing the paper. BK and AO designed research and wrote the paper.

The authors reported no potential conflicts of interest.

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