

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

Running title: Blood B-lymphocytes and plasma cells in adults

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Abbreviations used in this paper:

PC, plasma cells; HDs, healthy donors; BM, bone marrow; PB, peripheral blood; DC, dendritic cells; HSC, hematopoietic stem cells; sIg, surface Ig; Igλ, Ig light chain lambda; Igκ, Ig light chain kappa; MFI, mean fluorescence intensity; cyIg, cytoplasmic Ig; SI, staining index.

ABSTRACT

Generation of B and plasma cells (PC) 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 PC – 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/ μ l), naïve ($CD10^-CD27^-CD38^-$, 125 ± 90 cells/ μ l), memory B-lymphocytes ($CD10^-CD27^+CD38^-$, 58 ± 42 cells/ μ l), and PC ($CD10^-CD27^{++}CD38^{++}$, 2.1 ± 2.1 cells/ μ l) within circulating $CD19^+$ cells. From these four subsets, only memory B-lymphocytes and PC decreased with age, both in relative and absolute counts. Circulating PC split into $CD138^-$ ($57\%\pm12\%$) and $CD138^+$ ($43\%\pm12\%$) 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 PC are $KI-67^+$ and show weak CXCR4 expression.

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 (PC, 2/ μ L) are found in peripheral blood (PB) of healthy donors (HDs). Because of their low count, only few studies were devoted characterizing their phenotype, most of them dealing with newly generated PC after *in vivo* immunization.⁽²⁾ Steady-state circulating PC lack CD20, express CD19 and CD38^{high}. It has been recently reported that steady-state circulating PC are mainly of mucosal origin, the majority of them secreting IgA (84%), expressing CCR10 (56%) and β 7 integrin (32%).⁽³⁾ Steady-state circulating PC are generally termed plasmablasts because only half express CD138, a proteoglycan that is a hallmark of PC⁽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 BM, spleen, mucosa associated lymphoid tissues (MALT) or lymph nodes.⁽⁵⁾ These niches will provide to circulating early PCs those factors required to survive and to further differentiate into long-living mature PC.⁽¹⁾ In murine BM, PC niche involves SDF-1 producing cells and is shared with hematopoietic stem cells (HSC) and pro-pre B-cells.⁽¹⁾ The rarity of this niche is a matter of regulation of normal Ig production.⁽⁶⁾ In particular, new-born plasmablasts, generated after *in vivo* Ag immunization, have to compete with old PC for binding to a niche, inducing the old PC to recirculate.⁽⁷⁾

Another minor population of circulating B-cells which accounts for 2-4% of all PB 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 PB after HSC 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 BM niches able to support B-cell generation and PC survival, due to the progressive replacement of hematopoietic BM by fat cells; ii) a defect in germinal centers due to a decreased follicular DC 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, with the aim 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 steady-state circulating PC as regards to their activation status and homing phenotype.

DESIGN AND METHODS

Detailed methodologies are described in the Supplementary Appendix.

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 PC involves sequential maturation steps in different organs and tissues⁽¹⁴⁾ and a necessary cell traffic between these organs through PB.⁽¹⁵⁾ Varying strategies have been applied for their identification and no study has comparatively analyzed these four B-cell subsets in large cohorts of healthy donors (HDs). Here, we show that four B-cell subsets were systematically identified in PB of 106 HDs 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 PC ($CD10^-CD19^+CD20^-CD27^{++}CD38^{++}$)⁽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 PC 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 BM B-cell precursors (*i.e.* $CD5$, $CD10$, and $CD38^{++}$) (Supplementary Figure S1), supporting they are immature B-lymphocytes leaving the BM prior to full maturation.⁽⁸⁾ Transition from naïve to memory B-lymphocytes was characterized by increased $CD24$, $CD25$, $CD27$ and $CD53$ expression, and a down-regulation of $CD5$ and $CD23$ (Table 1). A fourth discrete $CD19^+$ B-cell subset with higher light-scatter characteristics and a PC 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 HDs analyzed.

Fluctuation of peripheral blood B-cell subsets according to age

In our large cohort of 106 HDs, naïve and memory B-lymphocytes were highly represented, while immature B-lymphocytes and PC 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 2A). In contrast, statistically significant inverse correlations were found between age and both the percentage and absolute count of circulating memory B-lymphocytes (n=106; $R^2 \leq -0.22$, $P \leq .05$) and PC (n=106; $R^2 \leq -0.27$, $P \leq .02$). This also holds true when Ig heavy chain isotype-specific subsets of PC (IgG⁺, IgA⁺ and IgM⁺) and memory B-lymphocytes (only for IgG⁺ and IgM⁺) were considered separately (Figure 2B). Our results indicate that production of immature and naïve B-lymphocytes is not significantly affected by aging, in contrast to what has been previously suggested by others.^(12, 16) On the contrary, differentiation of naïve B-lymphocytes into memory B-lymphocytes and then PC, is clearly reduced. These findings would confirm and extend on 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 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⁺ PC 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 PC and their serum antibody counterpart (Supplementary Figure S2).

Detailed characterization of circulating CD138⁻ and CD138⁺ plasma cells

FACS-sorted CD138⁻CD20⁻CD38⁺⁺ and CD138⁺CD20⁻CD38⁺⁺ cells showed a typical PC cytology with no obvious morphological differences (Figure 1C). CD138⁺ PC showed a greater staining index (SI) than CD138⁻ PC 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⁻ PC, unlike CD138⁺ PC, 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⁺ PC. After cell permeabilization, 42%±14% cyIgA⁺, 31%±14% cyIgG⁺ and 26%±10% cyIgM⁺ PC were identified (Figure 1B), with no differences in isotype distribution between CD138⁻ and CD138⁺ PC. The lower frequency of sIgG⁺ vs. cyIgG⁺ PC 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 PC. Although HLA-class II (including HLA-DR) expression was heterogeneous and lower in circulating PC compared to PB B-lymphocytes (n=6, $P\leq 0.001$), a similar expression was found in CD138⁻ vs. CD138⁺ PC (Figure 1D). Unlike B-

lymphocytes, both CD138⁻ and CD138⁺ PC were CD43⁺. Regarding homing receptors, both CD138⁻ and CD138⁺ PC showed higher levels of α 4 integrin (n=6, $P \leq .03$), heterogeneously lower amounts of CXCR4 ($18.8 \pm 9.1\%$ and $11.0 \pm 6.1\%$, respectively; n=6, $P \leq .0001$), and negativity for CD200 as compared to B-lymphocytes, which were constantly positive for the later two markers (Figure 1D). CD138⁺ PC expressed lower levels of β 7 integrin (n=6, $P = .02$) and L-selectin/CD62L (n=5, $P = .03$) and higher levels of β 1 integrin (n=6, $P = .008$) than B-lymphocytes (Figure 1D). CCR10 was not expressed by circulating B-lymphocytes while it was weakly positive on both CD138⁻ and CD138⁺ PC. 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 (supplementary Figure S3).^(17, 18) No significant difference in CCR10 expression was found among PC subsets showing different Ig heavy chain isotypes (data not shown). Furthermore, both circulating B-lymphocytes and CD138⁻ and CD138⁺ PC were constantly negative for VCAM1 (CD106), α 5 integrin (CD49e), LFA-3 (CD58), and CD70, as well as for the CD56 and CD117 markers, which are aberrantly expressed by malignant PC (data not shown).⁽¹⁹⁾ Based on KI-67 antibody, circulating B-lymphocytes were quiescent ($1.2 \% \pm 0.8\%$ KI-67⁺ cells) while circulating CD138⁻ or CD138⁺ PC displayed a highly-activated phenotype with $66.8\% \pm 29.7\%$ and $76.2\% \pm 12.5\%$ KI-67⁺ cells, respectively (n=11; $P \leq .00003$, Figure 1D). Further staining with Annexin-V showed that both CD138⁻ and CD138⁺ PC 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 PC 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 PB PC were IgA⁺ and CCR10 was very weakly expressed by circulating PC. This discrepancy is not due to a defect of the anti-CCR10 mAb used (Supplementary Figure S3), but could be due to a difference in the gating strategy to define PC 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 PC? Given mainly their HLA-DR and CD45 expressions, they are generally thought to be plasmablasts newly-generated in lymphoid organs. But HLA-DR and CD45 expressions are also characteristics of long-living PC. A large fraction of CD138⁺ BM PC expresses HLA-class II (60%) or CD45 (65%).^(2, 20) PC, which are believed to be long-living based on murine models or by grafting human PC in SCID 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 PC survival factor.⁽²¹⁾ The phenotype of these PC is close to that of circulating CD38⁺⁺CD138⁻ PC we presently reported. In human spleen, these PC are located outside the follicles, express highly CD38, cyIg (45% cyIgM, 40% cyIgG and 15% cyIgA), and, unlike BM PC, weakly sIg, CD20 and did not express CD138⁽¹⁾. They also express HLA-DR and CD45 and have mutated Ig genes.

The possibility that a fraction of circulating PC could be BM and/or lymphoid-tissue-localized long living PC that are induced to re-circulate from their niche should be considered. Such a recirculation of PC was hypothesized to explain the appearance of circulating tetanus toxin-unrelated PC, 7 days after immunization of HDs 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 PC. The activation status of the circulating PC (KI-67⁺) could indicate that they have been induced to recirculate by local stimulation. Such a highly-regulated recirculation mechanism has been demonstrated for murine HSC with circadian variations.⁽²²⁾ These circulating HSC can home to non-hematopoietic tissues for a time to exert immune surveillance and may enter back the PB via lymphatics and thoracic duct.⁽²³⁾ As human BM PC use a stromal niche that is similar to that used by HSC ⁽²⁴⁾ and as the count of circulating CD34⁺ cells in steady-state conditions is similar to that of circulating PC in HDs, similar mechanisms could drive the circulation of HSC and PC.

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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FIGURE LEGENDS**FIGURE 1. Phenotype of peripheral blood B-cell subsets, and focus on circulating plasma cells.**

Peripheral blood cells from healthy adult donors were analyzed by flow cytometry. **(A)** Distribution of immature (green), naïve (pink), memory B-lymphocytes (orange), and plasma cells (red), according to the expression of CD10, CD27 and CD38. Cells were gated according to the strategy defined in the supplementary appendix (design and methods section). **(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 (cyIg) - cyIgA, cyIgG, and cyIgM - of one sample representative of 13. The contribution of each Ig isotype is represented in the pie charts and numbers are the mean percentages \pm one standard deviation for the 13 healthy individuals. **(C)** CD20⁻CD38⁺⁺CD138⁺ (a) and CD20⁻CD38⁺⁺CD138⁻ (b) peripheral blood plasma cells from an adult healthy donor were FACS sorted and stained with May-Grünwald-Giemsa (x1000 magnification). They showed eccentrically nucleus, relatively abundant basophilic cytoplasm, and archoplasm. **(D)** Open histograms show FACS labellings with anti-CD138, CD38, CD19, cytoplasmic Ig kappa or lambda light chains (cyIg κ or cyIg λ), CD27, CD45, CD20, surface Ig heavy chains (sIg), HLA class II, CD43, KI-67, α 4 integrin (ITG α 4), CXCR4, CD200, ITG β 7, CD62L, ITG β 1, and CCR10 mAbs. Gray histograms display the corresponding negative control mAbs. The cell phenotype was analyzed by gating on CD19⁺CD20⁺ B-lymphocytes, and both CD38⁺⁺CD138⁻ and CD38⁺⁺CD138⁺ PC. 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 depending on the antibody used. *, # and § indicate that the values observed

are significantly ($p \leq .05$) different (paired Student t test) between B-lymphocytes and CD138⁻ plasma cells (*), CD138⁻ and CD138⁺ plasma cells (#), and B-lymphocytes and CD138⁺ plasma cells (§).

FIGURE 2. Age-related changes in circulating B-cell subsets and in the immunoglobulin heavy chain isotype subsets of memory B-lymphocytes or plasma cells. (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). (B) Data plotted in each diagram represent correlation between the age of each healthy individual donor and the absolute count of memory B-lymphocytes and plasma cells expressing IgG (panels A and D, respectively), IgM (panels B and E), IgA (panels C and F).

FIGURE S1. Expression of CD10, CD20 and CD38 on immature, naïve B-cells and plasma cells. Distribution of the different subsets: immature (■), naïve (■) B-lymphocytes and plasmablasts/plasma cells (■) in bone marrow and in peripheral blood according to the expression of CD10, CD27 and CD38.

FIGURE S2. Correlation between serum concentration of IgG, IgA and IgM antibodies and the percentage of the corresponding Ig heavy chain isotype-specific memory B-cells. Correlation between the concentration of IgG, IgA and IgM antibodies in the serum and the percentage of the corresponding Ig heavy chain isotype-specific memory B-cells (A, B and C panels) and plasma cells (D, E, and F). Serum IgM, IgG and IgA levels (mg/dL) were determined by nephelometry.

FIGURE S3. Labeling of myeloma cells or *in vitro* generated plasmablasts with anti-human CCR10 or anti-human IgA mAbs. The anti-CCR10 mAb (clone 314305 from R&D systems) used was the same as that used by Mei *et al.* This mAb strongly stained the CCR10⁺ XG1 human myeloma cell line, or *in vitro* generated plasmablasts from healthy donors. The mAb to human IgA was also validated by labelling the IgA⁺ XG-10 myeloma cell line after cell permeabilization.

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