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LETTER TO THE EDITOR

Immunophenotypic analysis and quantification of B-1 and B-2 B cells during human fetal hematopoietic development

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Mouse B-1 cells were first described in 1983 as a rare population of CD5+ splenic B cells that spontaneously secrete immunoglobulin (Ig)-M and their frequency is increased in autoimmune-prone mice, thereby linking B-1 cells to autoimmunity.[1,2] Shortly after description of B-1 cells, controversy regarding their origin and developmental relationship with B-2 lymphocytes arose.[3–5] The 'selection model' proposed that the decision of an Ig-expressing B cell to become B-1 or B-2 is the result of a response to a particular antigen.[3] In contrast, the 'layered model' proposed that B-1 and conventional B-2 cells belong to separate lineages originating from distinct hematopoietic stem/progenitor cells (HSPCs) arising at different times during development.[6] The origin of the 'layered immune system' model is that B-1 cells are detectable at E10 in the aorta-gonad-mesonephros (AGM) even before the first HSCs emerge, and B-1 cells successfully develop in HSC-deficient mouse embryos.[7,8] This suggests that B-1 cells may derive very early in development in the yolk sac (YS) or para-aortic splanchnopleura (P-sP) hemogenic endothelial cells independent of any HSC contribution.[8,9] B-1 cells are believed to participate in microbial defense and immune homeostasis, and their dysfunction is associated with systemic illness. This has led to an increasing interest in the physiological and clinical contribution of human B-1 cells, which, however, have been difficult to address owing to the absence of bona fide markers for the human B-1 cell population.[10] In 2011, Rothstein and colleagues reported a previously unrecognized population of CD20+ B cells within the CD27+ compartment, which is distinguished from memory B cells by expression of CD43.[11] This CD20+CD27+CD43+ subset was functionally proposed to be human B-1 cells in both cord blood (CB) and peripheral blood (PB). Controversy over the frequency of human B-1 cells in normal individuals rapidly arose.[12,13] Griffin *et al*. reported that the proportion of CD43+ cells among the CD20+CD27+ B cells averaged 20–50%, with the frequency declining with age (from CB to PB in the elderly). Although other authors confirmed a decline with age of the overall population of CD20+CD27+CD43+ circulating human B-1 cells, they reported a much lower frequency of ~2% of total CD20+ B cells.[12,13] These differences seem to be attributed to different protocols for reliable flow cytometric characterization/enumeration of human B-1 cells.[10]

No information is available about the existence/frequency of B-1 cells during human fetal hematopoiesis. Here we performed a flow cytometric phenotypic characterization and quantification of B-1 cells, as well as naive and memory B cells, throughout the human fetal development, analyzing human fetal liver (FL) at 10–11 and 20–21 weeks of gestation, human fetal bone marrow (FBM) at 20–21 weeks of gestation and CB at birth. Flow cytometry acquisition and data analysis were performed on a FACSCanto II cytometer using the FACSDiva software (BD Biosiciences, San Jose, CA, USA; Figure 1). To avoid staining artifacts and doublet formation, the following measurements were taken into consideration:[10–13] (i) only fresh samples were used; (ii) high-affinity–high-specificity antibodies coupled to fluorophores with high signal-to-noise ratio were used (see legend to Figure 1); (iii) flow cytometric cell acquisition was performed at a low-speed flow rate (<3.000 events per second); and (iv) fluorescent-minus-one (FMO) controls were applied in multiparametric analysis to set FMO-defining gates in the absence of clear separation between populations.

Phenotypically, naive (CD3 – CD20+CD19+CD27 – CD43 –), memory (CD3 – CD20+CD19+CD27+CD43 –) and B-1 B cells (CD3 – CD20+CD19+CD27+CD43+) were consistently found in FL, FBM and CB (Figures 1a and e–g). B-1 cells displayed a CD38 expression similar to B cells, regardless the tissue analyzed (Figure 1c), and an important fraction spontaneously expressed IgD ($30 \pm 13\%$) and IgM ($21 \pm 6\%$, $20 \pm 6\%$ and $70 \pm 7\%$ in FL-, FBM- and CB-derived B-1 B cells, respectively; Figure 1d). Interestingly, IgM expression in CB B-1 B cells is higher than in FL/FBM B-1 B cells, and similar to IgM expression in B-2 B cells, suggesting that IgM expression in B-1 B cells increases throughout ontogeny (Figure 1d). Quantitatively, naive B cells are less represented in week 10–11 FL ($53 \pm 15\%$ of total B cells) but their

Figure 1. Flow cytometric analysis and quantification of B-1, naive and memory B cells in human FL, FBM and in neonatal CB. (**a**–**d**) Flow cytometric strategy for the identification of the distinct B-cell subsets. Mononuclear cells (MNCs) were isolated by density gradient centrifugation using Ficoll-Hypaque from independent human FL (n = 22; 10 of 10–11 weeks and 12 of 20–21 weeks of gestation), FBM (n = 11 of 20–21 weeks of gestation) and CB (n = 14). After lysing red cells, MNCs were immunofluorescently stained for CD20 (Horizon V450 antibody, clone 2H7), CD19 (Alexa Fluor antibody, BD Biosiciences, clone HIB-19), CD3 (PE antibody, BD Biosiciences, clone UCHT1), CD27 (APC antibody, BD Biosiciences, clone M-T271) and CD43 (FITC antibody, BD Biosiciences, clone 1G10). In several cases, FL-, FBM- and CB-derived MNCs were also stained with CD38 (PE-Cy7 antibody, BD Biosiciences, clone HIT2), IgM (PerCP-Cy5.5 antibody, BD Biosiciences, clone G20-127) and IgD (PE antibody, clone IAG-2). Flow cytometry acquisition and data analysis were performed on a FACSCanto II cytometer using the FACSDiva software. (**a**) Gating strategy showing how MNCs were analyzed first within a large forward side scatter area (FSC-A) vs side scatter area (SSC-A) lymphoid gate followed by a FSC-A vs forward side scatter height (FSC-H) doublet discrimination gate. Then, cells were gated based on CD20 positivity and CD3 negativity (top right panel). On the basis of CD27 and CD43 expression three CD3 – CD20+ subpopulations were immunophenotypically identified in early-gestation FL, mid-gestation FL and FBM, and neonatal CB: CD3 – CD20+CD27 – CD43 – (naive B cells), CD3 – CD20+CD27+CD43 – (memory B cells) and CD3 – CD20+CD27+CD43+ (B-1 cells; bottom panels). (**b**) All FL/FBM CD20+ B cells were CD19+ (light gray histogram represents the isotype-matched control). (**c**) CD38 expression is identical in B-1 cells and CD19+CD20+CD3 – B cells, regardless the tissue. (**d**) B-1 cells spontaneously expressed both IgD (left panels) and IgM (right panels) in cell surface. Light gray histograms represent isotype-matched controls. (**e**–**g**) Frequency of CD3 – CD20+CD27+CD43+ B-1 cells (**e**), CD3 – CD20+CD27 – CD43 – naive B cells (**f**) and CD3 – CD20+CD27+CD43 – memory B cells (**g**) in 10–11- and 20–21-week-old FL, 20–21-week-old FBM and neonatal CB.

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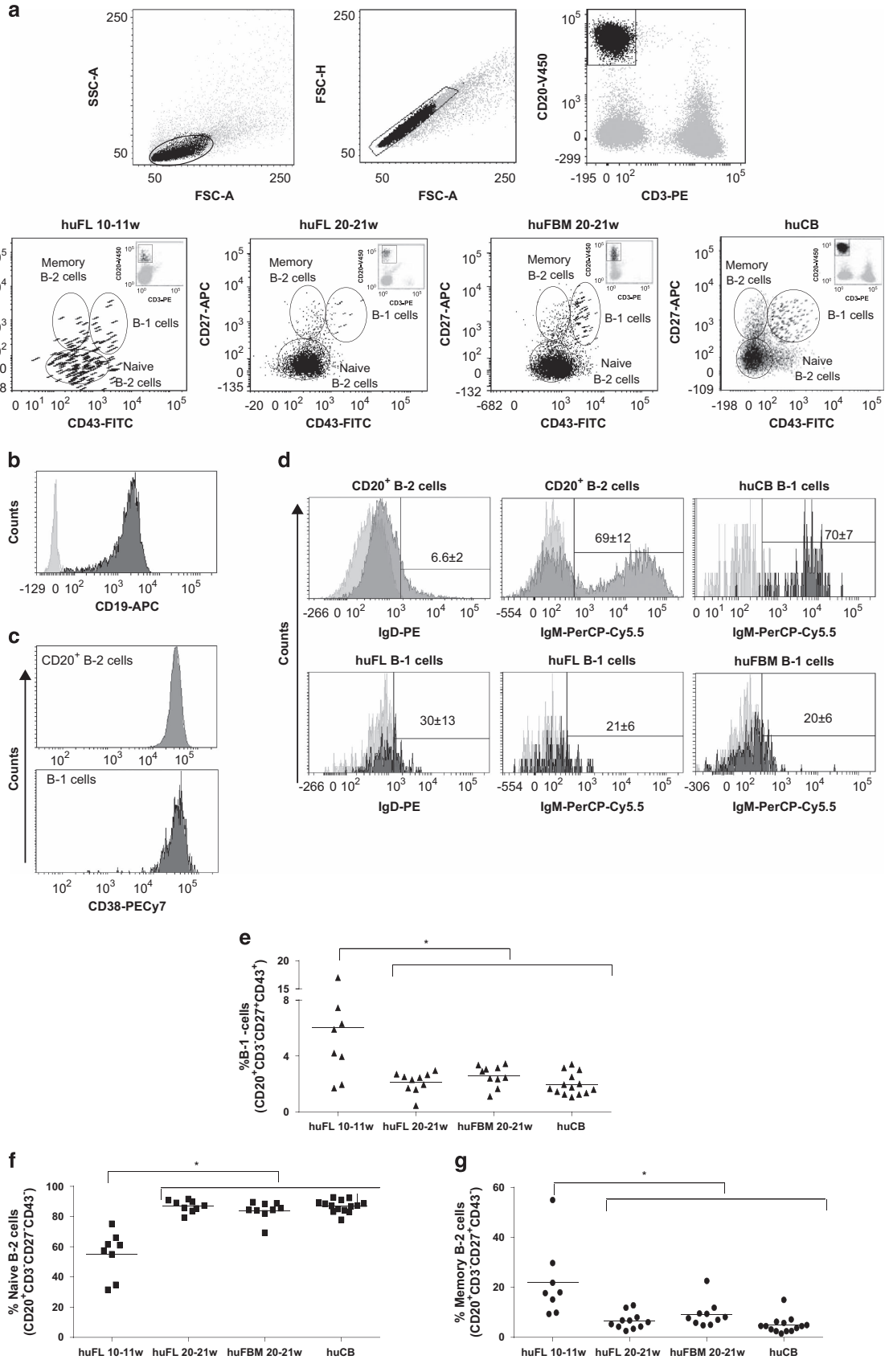

frequency increases up to 87 ± 14% by week 20–21 FL and FBM, and remains stable all the way up to CB (Figure 1f). In contrast, memory B cells (CD27+CD43 −) represent 22 ± 15% of total B cells in early FL and their frequency decreases to 7 ± 3% from week 20–21 FL/FBM to neonatal tissue (CB; Figure 1g).[14] The frequency of B-1 B cells declines during human fetal hematopoiesis (Figure 1g). The highest frequency of B-1 cells is found in early FL (6.1 ± 4% of CD27+CD43+ cells within CD20+CD19+CD3 − population), when the hematopoiesis still mainly takes place in FL before colonizing the BM by week 10.5–11.[15] By mid-gestation (week 20–21) the frequency of B-1 B cells decreases, being very similar in FL (2.1 ± 0.7%) and FBM (2.6 ± 0.7%), and is maintained at birth in CB (1.9 ± 0.8%; Figure 1e). After birth, the proportion of B-1 B cells continues declining with age as it has been reported from CB to PB in the elderly.[11–13] From a developmental standpoint, the fact that B-1 B cells are more abundant in very early phases of human hematopoietic development (before FBM colonization by week 11) supports the 'layered immune system model' proposed in the mouse for the origin of B-1 cells. This layered model proposes that B-1 and B-2 cells belong to separate lineages originating from distinct HSCs arising at different times during development, as B-1 cells in the mouse are already detectable at E10 in the AGM even before the first HSCs emerge, and successfully develop in HSC-deficient mouse embryos.[6] Interestingly, and similar to B-2 B cells, one-third of the fetal B-1 cells spontaneously expressed IgM and IgD. Whether human FL/FBM CD3 − CD20+CD19+CD27+CD43+ IgM/IgD+ or IgM/IgD − B cells are activated cells on their way to plasma cell differentiation or an innate-like subset with B-1 functional properties remains to be functionally addressed. These data provide the means to explore the underlying molecular, cellular and developmental mechanisms regulating this unique B-1 B-cell population in health and disease, especially in regard to infectious and autoimmunity diseases and lymphoid malignancies. For instance, infant B-cell acute lymphoblastic leukemia (B-ALL) has a prenatal origin but the nature of the cell of origin/ target cell for transformation remains elusive.[16,17] Deciphering the cell of origin is of especial interest in MLL-AF4+ infant B-ALL as this leukemia lacks an *in vitro*/*in vivo* model, has a pro-B/mixed phenotype and MLL-AF4 has been found/expressed in mesenchymal stem cells from the newborn patients,[18] suggesting that a pre-HSC or an early B-cell-derived very early in development (from YS or P-sP), perhaps before HSC emergence, may represent a candidate cell of origin for leukemia initiation. In fact, it has recently been reported that the *BCR-ABL* oncogene transforms murine B-1 B-cell progenitors, promoting high-risk B-ALL initiation more rapidly than when it is expressed in B-2 progenitor cells,[19] thus indicating that B-1 cells might represent the cell of origin for infant B-ALL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CB and PM conceived the study, designed and performed the experiments, analyzed the data and wrote the manuscript. EvR, AM-L, AS-P, PG-C, MJ, AN and RWS performed the experiments and provided key biological samples and reagents.

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REFERENCES

1 Baumgarth N. The double life of a B-1 cell: self-reactivity selects for protective effector functions. *Nat Rev Immunol* 2011; **11**: 34–46.
2 Hayakawa K, Hardy RR, Parks DR, Herzenberg LA. The 'Ly-1 B' cell subpopulation in normal immunodefective, and autoimmune mice. *J Exp Med* 1983; **157**: 202–218.
3 Berland R, Wortis HH. Origins and functions of B-1 cells with notes on the role of CD5. *Annu Rev Immunol* 2002; **20**: 253–300.
4 Herzenberg LA, Tung JW. B cell lineages: documented at last!. *Nat Immunol* 2006; **7**: 225–226.
5 Kantor AB, Herzenberg LA. Origin of murine B cell lineages. *Annu Rev Immunol* 1993; **11**: 501–538.
6 Herzenberg LA, Herzenberg LA. Toward a layered immune system. *Cell* 1989; **59**: 953–954.
7 Kobayashi M, Shelley WC, Seo W, Vemula S, Lin Y, Liu Y *et al.* Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on Cbfbeta for their development. *Proc Natl Acad Sci U S A* 2014; **111**: 12151–12156.
8 Yoshimoto M. The first wave of B lymphopoiesis develops independently of stem cells in the murine embryo. *Ann N Y Acad Sci* 2015; **1362**: 16–22.
9 Montecino-Rodriguez E, Dorshkind K. B-1 B cell development in the fetus and adult. *Immunity* 2012; **36**: 13–21.
10 Griffin DO, Rothstein TL. Human B-1 cell frequency: isolation and analysis of human B-1 cells. *Front Immunol* 2012; **3**: 122.
11 Griffin DO, Holodick NE, Rothstein TL. Human B-1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* 2011; **208**: 67–80.
12 Descatoire M, Weill JC, Reynaud CA, Weller S. A human equivalent of mouse B-1 cells? *J Exp Med* 2011; **208**: 2563–2564.
13 Perez-Andres M, Grosserichter-Wagener C, Teodosio C, van Dongen JJ, Orfao A, van Zelm MC. The nature of circulating CD27+CD43+ B cells. *J Exp Med* 2011; **208**: 2565–2566.
14 McWilliams L, Su KY, Liang X, Liao D, Floyd S, Amos J *et al.* The human fetal lymphocyte lineage: identification by CD27 and LIN28B expression in B cell progenitors. *J Leukoc Biol* 2013; **94**: 991–1001.
15 Zambidis ET, Oberlin E, Tavian M, Peault B. Blood-forming endothelium in human ontogeny: lessons from in utero development and embryonic stem cell culture. *Trends Cardiovasc Med* 2006; **16**: 95–101.
16 Bueno C, Montes R, Catalina P, Rodriguez R, Menendez P. Insights into the cellular origin and etiology of the infant pro-B acute lymphoblastic leukemia with MLL-AF4 rearrangement. *Leukemia* 2011; **25**: 400–410.
17 Sanjuan-Pla A, Bueno C, Prieto C, Acha P, Stam RW, Marschalek R *et al.* Revisiting the biology of infant t(4;11)/MLL-AF4+ B-cell acute lymphoblastic leukemia. *Blood* 2015; **126**: 2676–2685.
18 Menendez P, Catalina P, Rodriguez R, Melen GJ, Bueno C, Arriero M *et al.* Bone marrow mesenchymal stem cells from infants with MLL-AF4+ acute leukemia harbor and express the MLL-AF4 fusion gene. *J Exp Med* 2009; **206**: 3131–3141.
19 Montecino-Rodriguez E, Li K, Fice M, Dorshkind K. Murine B-1 B cell progenitors initiate B-acute lymphoblastic leukemia with features of high-risk disease. *J Immunol* 2014; **192**: 5171–5178.