

Reduced production of B-1–specified common lymphoid progenitors results in diminished potential of adult marrow to generate B-1 cells

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Edited by Leonard A. Herzenberg, Stanford University, Stanford, CA, and approved July 13, 2011 (received for review May 5, 2011)

B-1 B cells have been proposed to be preferentially generated from fetal progenitors, but this view is challenged by studies concluding that B-1 production is sustained throughout adult life. To address this controversy, we compared the efficiency with which hematopoietic stem cells (HSCs) and common lymphoid progenitors (CLPs) from neonates and adults generated B-1 cells in vivo and developed a clonal in vitro assay to quantify B-1 progenitor production from CLPs. Adult HSCs and CLPs generated fewer B-1 cells in vivo compared with their neonatal counterparts, a finding corroborated by the clonal studies that showed that the CLP compartment includes B-1– and B-2–specified subpopulations and that the former cells decrease in number after birth. Together, these data indicate that B-1 lymphopoiesis is not sustained at constant levels throughout life and define a heretofore unappreciated developmental heterogeneity within the CLP compartment.

bone marrow | clonal analysis | hematopoiesis

Immune system development has been hypothesized to be a layered process in which lymphoid populations of increased complexity are produced in successive waves in the fetus, neonate, and adult. The initial wave of fetal lymphopoiesis has been proposed to generate lymphocytes involved in innate immunity, whereas waves appearing later produce cells involved in adaptive immune responses (1). A corollary of the layered immune system hypothesis is that, after peaking in the fetus/neonate, the initial wave(s) of lymphopoiesis wanes as the adult wave establishes.

The layered immune system hypothesis arose from studies aimed at defining the origin of two types of B lymphocytes, termed B-1 and B-2 cells (2). B-1 cells are innate effectors distinguished by their preferential localization in serous cavities and an unusual sIgM^{high} CD11b⁺ CD5⁺ B-1a and sIgM^{high} CD11b⁺ CD5[−] B-1b phenotype (3, 4). B-1a cells spontaneously secrete IgM natural antibodies, whereas production of Ig by B-1b cells can be induced by antigen exposure, and the latter cells exhibit immunologic memory (5, 6). Human B-1 cells with properties similar to those described in mice have recently been described (7). In contrast, conventional B cells, referred to as B-2 cells, are mediators of adaptive immune responses, predominate in the spleen and lymph nodes, and undergo somatic hypermutation after antigen encounter (8, 9).

Studies showing that cells from fetal liver are more efficient than cells from adult bone marrow at reconstituting B-1 cells in irradiated recipients initially suggested that innate B cells arise from progenitors that appear during a fetal wave of development (10, 11). CD5⁺ B-1a cells, in particular, were preferentially generated from fetal sources (2, 12, 13). These early findings are supported by more recent studies showing the existence of lineage negative (Lin[−]) CD45R^{−/low} CD19⁺ B-1–specified progenitors that arise in the embryonic yolk sac, peak in number in the fetal liver, and then decline in the adult (14–17). Taken together with data showing that selected $\gamma\delta$ T cells are produced more efficiently from fetal than adult progenitors (18) and recent reports that distinct types of B and T cells are generated during

fetal and adult life in humans (19, 20), a model postulating that the adult immune system consists of lymphocytes, particularly those lymphocytes involved in innate immunity, that have emerged in distinct waves of development has evolved.

However, multiple studies have shown the generation of B-1 cells from adult bone marrow (21). Of particular note are recent reports showing the development of B-1 cells from adult hematopoietic stem cells (HSCs) and common lymphoid progenitors (CLPs) (22–25). The concomitant production of B-1 and B-2 cells from adult CLPs, defined as B lineage-specified progenitors from which surface IgM⁺ B cells derive (26), also links the development of these two B-cell populations. In view of these observations, it has been suggested that de novo B-1 production is sustained throughout life (22, 25), which challenges the premise that progenitors for B-1 cells are primarily generated during a fetal/neonatal wave of development.

To address this controversy, we compared both the frequency and total number of B-1 cells generated by neonatal and young adult HSCs and CLPs in vivo. In addition, we developed a clonal in vitro assay that allowed B-1 progenitor production from CLPs to be quantified. The results of the in vivo transplantation experiments revealed that the number of B-1 cells generated by adult HSCs and CLPs is reduced compared with their neonatal counterparts. These results were corroborated by the clonal analyses, which showed that the CLP compartment includes B-1– and B-2–specified subpopulations and that the former cells decrease in number between neonatal and adult life. Taken together, these data show that B-1 production is not sustained at constant levels after birth and define an unappreciated developmental heterogeneity in the common lymphoid progenitor population.

Results

Adult HSCs Have Attenuated B-1 Potential. To determine if B-1 potential is sustained at a constant level in postnatal bone marrow, we isolated Lin[−] CD117 (c-kit)^{high} Sca-1^{high} CD150⁺ HSCs (27) from the bone marrow of 2.5-wk-old neonatal and 15-wk-old adult mice (Fig. 1A) and transplanted equivalent numbers into CD45.1 Rag2^{−/−} recipients. The production of donor B-1 cells in the peritoneal cavity, which normally includes B-1a, B-1b, and B-2 cells (Fig. 1B), was examined 6 wk later based on preliminary studies indicating that this time was the earliest time that we could detect them in reliable numbers. Because B-1 cells can expand and mask initial input numbers (11, 12, 28), we did not examine reconstitution at later time points.

Author contributions: C.L.B., E.M.-R., and K.D. designed research; C.L.B. performed research; C.L.B., E.M.-R., and K.D. analyzed data; and C.L.B. and K.D. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1107172108/-DCSupplemental.

tiation of B-lymphoid progenitors (29). Consistent with data showing that B-1 progenitors are CLP progeny (23), Lin⁻ CD45R^{-low} CD19⁺ B-1 progenitors were easily detected in these cultures after 4 d (Fig. 3A). As expected, Lin⁻ CD45R⁺ CD19⁻ B-2 prepro-B cells, which are known to be CLP progeny (8, 9), were also observed. These data indicate that both B-1 and B-2 progenitors can simultaneously emerge in these cultures and that one type of progenitor does not suppress the growth of the other. To establish that this culture system detects progenitors with normal developmental potential, replicate wells seeded with 50 CLPs were analyzed at 9 d after initiation of the cultures. As shown in Fig. 3B, both CD19⁺ CD45R^{low/-} B-1 and CD19⁻ CD45R⁺ B-2 progenitors acquired a B220⁺ CD19⁺ phenotype.

We then determined if the cultures would support the differentiation of single CLPs into B-1 and/or B-2 progenitors. We were able to show that this was the case and that CLPs from both neonatal and adult bone marrow could generate B-1 and B-2 progenitors after 4–5 d in culture (Fig. 3C). Over the course of four separate experiments in which over 1,000 individual CLPs were examined, B-1 and B-2 progenitors were never detected together in a single well. Instead, wells contained either B-1 or B-2 progenitors (Fig. 3C). This result indicates that B-1 vs. B-2 fate decisions are already made before the CLP stage of development and reveals an unappreciated developmental heterogeneity within this compartment.

B-1 Progenitor Production Is Attenuated in the Adult. We next used the above clonal assay to quantify the number of B-1 and B-2 progenitors generated from single neonatal and adult CLPs. CLPs from neonatal and adult bone marrow generated B-2

progenitors at similar levels (Fig. 4A), reflecting the fact that B-2 development, which initiates in the fetus, is sustained throughout young adult life. However, this finding was not the case with B-1 progenitors. Phenotypic analysis of cells harvested 4–6 d after initiation of cultures with a single CLP showed a clear differentiation bias of neonatal CLPs to the B-1 lineage; of 384 neonatal CLPs tested, 54 (14%) generated B-1 progenitors (Fig. 4A). In contrast, adult CLPs showed a significant reduction in their capacity to differentiate into B-1 cells; of 772 adult CLPs tested, 24 (3%) produced B-1 progenitors (Fig. 4A).

In addition to the lower efficiency with which they were produced, the quality of the B-1–specified CLPs in the adult was also reduced, because neonatal CLPs generated a higher number of B-1 progenitors per clone compared with their adult counterparts (Fig. 4B). Thus, in addition to the reduced efficiency with which adult CLPs generate B-1 progenitors, the progenitors that are produced exhibit diminished proliferative potential. Together, these declines likely account for the reduction in the frequency of B-1 progenitors in adult compared with fetal bone marrow (Fig. 4C).

Discussion

The results of this study show that B-1 developmental potential is not sustained at a constant level after birth. Instead, the data clearly show that, by young adulthood, the potential of HSCs and CLPs to generate B-1 cells is significantly attenuated. These findings are in agreement with a report by Lam and Stall (30), which showed that fetal B-cell developmental programs, although detectable in postnatal bone marrow, are highly reduced, and a recent study from Ghosh et al. (16), which reported that B-1 progenitors, phenotypically defined as described herein, are present at high numbers in neonatal spleen and significantly decline in number in the adult. This reduced B-1 potential of adult HSCs and CLPs is not part of the general decline in B

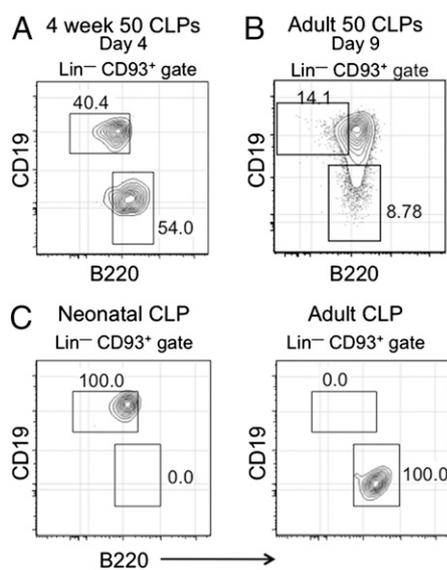


Fig. 3. Development of a clonal assay to quantify B-1 progenitor production. (A) Fifty CLPs from bone marrow of 4-wk-old mice were seeded into wells, and 4 d later, cells were harvested and phenotyped. The FACS plot shows that the culture conditions support the development of both B-1 and B-2 progenitors in the same well. The CD45R^{-low} CD19⁺ B-1 and CD45R⁺ CD19^{low/-} B-2 progenitors harvested from the in vitro cultures 5 d later were resolved based on labeling of comparable populations in fresh bone marrow samples shown in Fig. 4C. (B) Replicate wells seeded with 50 CLPs were analyzed at 9 d after initiation of the cultures. CD19⁺ CD45R^{low/-} B-1 progenitors matured into CD19⁺ CD45R^{dim} cells, and the CD19⁻ CD45R⁺ B-2 progenitors acquired a B220⁺ CD19⁺ phenotype. (C) Single CLPs from bone marrow of neonatal (2.5 wk) or adult (15 wk) mice were seeded into wells, and the phenotype of the cells was determined 4–6 d later. Values on the plots indicate the frequency of the different progenitor cell populations in the cultures.

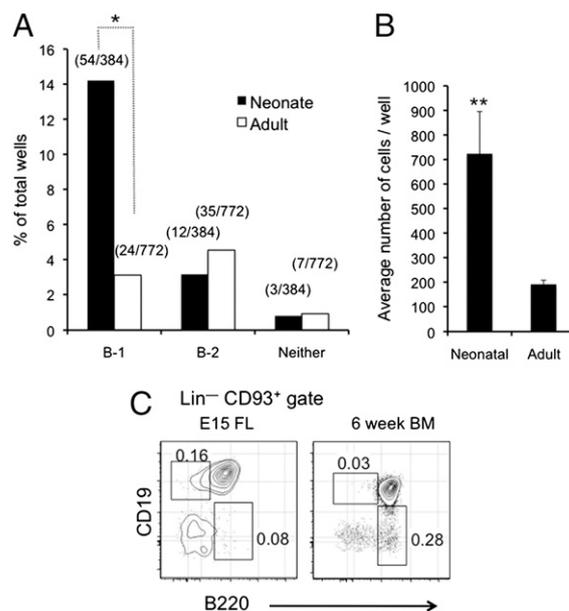


Fig. 4. Clonal analysis of CLP developmental potential. (A) Frequency of wells seeded with single neonatal or adult CLPs in which B-1 and B-2 progenitors were observed. The number of positive wells over the number of wells seeded is indicated. (B) Single CLPs from bone marrow of 2.5- and 15-wk-old mice were seeded into wells. The number of B-1 progenitors per well produced from the different aged CLPs was determined 6 d later. (C) Frequency of Lin⁻ CD45R^{-low} CD19⁺ B-1 progenitors among Lin⁻ CD93⁺ cells in E15 fetal liver and bone marrow of 6-wk-old WT mice. * $P \leq 0.05$; ** $P \leq 0.01$.

lymphopoiesis that accompanies aging (31–33); B-2 development was normal in the adult 15-wk-old mice analyzed, and age-related reductions in B lymphopoiesis are not observed at that age in the strains that we used (32).

In addition to providing quantitative data showing that B-1 progenitor production is significantly attenuated in the adult, the *in vitro* clonal assay system allowed us to distinguish between two alternative explanations for this occurrence (Fig. 5). One possibility was that CLPs are bipotential cells that can generate both B-1 and B-2 progenitors, but their B-1 potential declines after neonatal life (Fig. 5, model 1). Alternatively, the CLP compartment could have included both B-1- and B-2-specified progenitors, with the number of the former cells declining in the young adult (Fig. 5, model 2). The data support the latter view. Regardless of age, an individual CLP generated either B-1 or B-2 progenitors, indicating that B-1 vs. B-2 fate decisions are made before the CLP stage of development, thus providing additional support for the hypothesis that B-1 and B-2 cells represent distinct lineages (28).

To determine whether B-1- and B-2-specified HSCs or CLPs could be isolated by phenotype, we examined specific cell surface markers expressed on these populations. Fetal/neonatal HSCs express CD11b (34). In view of the preferential generation of B-1 progenitors from young stem cells, we tested the possibility that CD11b⁺ HSCs would generate B-1 cells more efficiently than CD11b⁻ HSCs. However, this was not the case (Fig. S1). We also examined the CLP compartment for determinants that would segregate B-1-specified CLPs, like CD138 (17), CD11b (Mac-1) (35), and MHC class II antigens (17, 36). However, neither CD138 nor CD11b selectively identified B-1- or B-2-specified CLPs (Figs. S2 and S3), and MHC class II antigens were not expressed on CLPs (Fig. S4). The stage of development at which B-1 vs. B-2 commitment takes place also remains undetermined. Attempts to determine whether B-1 and B-2 biased HSCs existed were made using the clonal assay system, but these efforts were not successful.

In addition to the reduced production of B-1 progenitors in the adult, the number of B-1 progenitors produced by a single neonatal CLP was higher than the number generated from a single adult CLP. This observation is consistent with reports that neonatal HSCs proliferate more vigorously than their adult counterparts (37, 38). Thus, the reduced ability of adult bone marrow to generate B-1 cells together with the reduced proliferative potential of the progenitors that are produced likely

combine and account for the attenuated B-1 potential of young adult bone marrow stem and progenitor cells.

The degree to which B-1 production occurs in the adult has remained a lingering issue. Our data are in agreement with multiple studies showing that adult bone marrow stem and progenitor cells can produce B-1 cells. However, they also clearly show that, when production efficiency is taken into consideration, B-1 lymphopoiesis declines significantly by young adulthood. These results, combined with our previous data showing that the production of B-1 progenitors is most robust in the fetus (14), are consistent with the tenets of the layered immune system hypothesis (1).

Experimental Procedures

Mouse. C57BL/6J (CD45.2) mice were obtained from Jackson Laboratory. 129Sv/SvEvTac-*Rag2*^{tm1Fwa} (*Rag2*^{-/-}; CD45.1) mice were obtained from Taconic Farms. Mice were housed in the Division of Laboratory Animal Medicine, and experiments were conducted according to University of California at Los Angeles Institutional Animal Care and Use Committee guidelines.

Transplantation. Donor CD45.2 HSCs (500–1,000) or CLPs (5×10^3 – 5×10^4) isolated from the bone marrow of 2.5-wk-old neonates and 15-wk-old young adults were injected *i.v.* into 4- to 8-wk-old CD45.1 *Rag2*^{-/-} mice (39) that were preconditioned with 500 R from a ¹³⁷Cs irradiator (120 R/min, Mark I-68A; JL Sheppard and Associates) 24 h earlier. Donor (CD45.2) levels of reconstitution in the peritoneal cavity of recipients were assessed at 6 wk posttransplantation.

Flow Cytometry. Isolation and analysis of HSCs, CLPs, B-1 and B-2 progenitors from bone marrow, and B-1a, B-1b, and B-2 cells from the peritoneal cavity were performed on a FACSaria or LSRII (Becton Dickinson), respectively, using previously described protocols (14, 40). The MHC class II antibody (clone AF6-120.1) was obtained from eBiosciences.

Cell Culture and Clonal Analysis. Single CLPs from the bone marrow of 2.5- or 15-wk-old mice were deposited into wells of 96-well microtiter plates by the automatic cell deposition unit on a FACSaria (Becton-Dickinson). Cells were cultured in RPMI 1640, 10% FCS, 5×10^{-5} M 2- β -mercaptoethanol, 1 mM L-glutamine, 100 U/mL streptomycin, 100 μ g/mL penicillin, 50 μ g/mL gentamycin, 20 ng/mL IL-3, 20 ng/mL IL-6, 20 ng/mL Stem Cell Factor (SCF) 20 ng/mL Flt-3 ligand, and 20 ng/mL IL-7 (Biosource International) for 4–6 d in a humidified incubator at 37 °C and 5% CO₂/air. Cells were then harvested and tested for their B-1 and B-2 progenitor phenotype by immunostaining (14) and analysis with the high-throughput sampler 96-well plate adapter for the BD LSR II flow cytometer. Only wells in which 20 or more cells had been generated were considered for analysis.

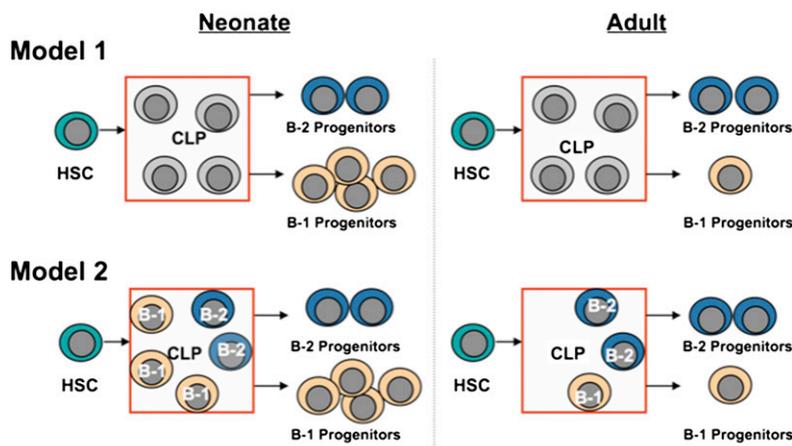


Fig. 5. Two models to explain the attenuated potential of adult CLPs to generate B-1 progenitors. In model 1, both neonatal and adult HSCs generate CLPs that can differentiate into B-1 or B-2 progenitors, but B-1 potential declines within the first few weeks after birth. In model 2, neonatal and adult HSCs generate B-1- or B-2-specified CLPs, with the production of the B-1-specified CLPs predominating during neonatal life and declining in the adult.

Statistics. Data are expressed as mean \pm SEM. Differences between groups were tested by two-tailed, unpaired Student *t* tests ($\alpha = 0.05$). Comparisons of absolute cell numbers were tested by one-tailed, unpaired Student *t* tests ($\alpha = 0.05$).

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