Developmental switch of mouse hematopoietic stem cells from fetal to adult type occurs in bone marrow after birth

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Hematopoiesis originated by hematopoietic stem cells (HSCs) is distinguishable between fetal and adult mice. However, it is not clear whether the altered mode of differentiation is due to the change of properties of HSCs or different microenvironments in fetuses and adults. Here we show that fetal HSCs are fully capable of giving rise to all classes of B cells in the adult microenvironment. HSCs that are derived from fetal liver but not adult bone marrow (BM) of IL-7 receptor α chain (IL-7R α)-deficient mice can also differentiate into B cells, suggesting that both IL-7 and thymic stromal-derived lymphopoietin (TSLP) are dispensable for fetal B cell development, because IL-7R α is commonly used as a subunit of functional receptor complexes for IL-7 and TSLP. Similar IL-7/TSLP independent B cell potential is maintained by BM HSCs until 1 week after birth. In contrast, BM HSCs in mice older than 2 weeks of age absolutely requires IL-7R α for B lymphopoiesis. These results demonstrate that fetal HSCs acquired adult characteristics between 1 and 2 weeks after birth in mouse BM.

B cell development | fetal liver | IL-7 | early B cell factor

Hematopoietic stem cells (HSCs) play an indispensable role in maintenance of blood cell homeostasis by their life-long self-renewal activity and multipotent differentiation potential into all classes of hematopoietic cells (1). Although hematopoiesis occurs mainly in the bone marrow (BM) of adult mice, the site of hematopoiesis is changed several times during ontogeny in mouse fetuses (2). The first wave of hematopoiesis is observed in the yolk sac and the aorta—gonad—mesonephros region at 7–9 days postconception (dpc), which lasts until 13 dpc (3–6). HSC activity is also detected in placenta after 11 dpc (7, 8). The next wave of hematopoiesis begins in fetal liver (FL) at 12 dpc and in spleen at 15 dpc (9, 10). HSC activity is subsequently detected in BM as early as 17.5 dpc (11) and is sustained throughout the life of an animal. This transition of hematopoiesis is due to the migration and relocation of HSCs that are likely regulated by chemokines (11–16) as well as adhesion molecules (17, 18).

In addition to the different sites of hematopoiesis, it is well known that HSCs acquire different intrinsic properties and differentiation potentials throughout ontogeny. For instance, red blood cells derived from HSCs in the yolk sac are the primitive nucleated erythrocytes with embryonic hemoglobins, whereas erythrocytes derived from FL and adult BM are non-nucleated definitive erythrocytes containing only adult hemoglobin (19–21). HSCs in FL (FL-HSCs) have greater repopulating capacity than HSCs derived from adult BM (BM-HSCs) in irradiated recipients (22). Moreover, only FL-HSCs can generate V γ 5⁺ γ δ T cells [denoted as V γ 3⁺ in the original publication (23)] in fetal thymuses, whereas adult BM-HSCs cannot (24, 25).

Similar to the T cell compartment, various aspects of the fetal and adult B cell developmental program are also different. There are two different types of B cells, namely B-1 and B-2 B cells, that are distinguishable based on the function, localization, and phenotype. B-1 B cells, which are potent autoreactive B cells, are predominantly localized in the peritoneum and pleural cavities and are subdivided into CD5⁺ B-1a B cells and CD5[−] B-1b B

cells (26). B-2 B cells are the majority of the B cells in secondary lymphoid organs and are subdivided into CD21^{hi}CD23^{−/lo} marginal zone B cells and CD21^{int}CD23^{hi} follicular B cells (27, 28). As in V γ 5⁺ γ δ T cells, B-1a B cell development has been suggested as a fetal-specific event (29). Furthermore, it has been shown that, whereas IL-7 is required for adult B cell development, it is dispensable during fetal hematopoiesis (29, 30). However, it is not clear whether this difference in cytokine requirement is due to the different microenvironments between FL and BM or to different intrinsic properties between FL- and BM-HSCs. In this study, we found that both FL- and adult BM-HSCs can give rise to all classes of B cells, including B-1a B cells in adult mice. In addition, FL-HSCs but not adult BM-HSCs derived from IL-7R α ^{−/−} mice differentiate into B cells, suggesting that different requirement of IL-7 in FL and BM B cell development is due to cell autonomous difference between FL-HSCs and BM-HSCs. This developmental switch occurs in BM-HSCs between 1 and 2 weeks after birth.

Results

Adult BM-HSCs, as Well as FL-HSCs, Can Give Rise to B-1a B Cells in Adult Mice. To clarify the mechanistic difference in fetal and adult B cell development, we first investigated the contribution of FL-HSCs to different B cell subsets in the adult microenvironment. We purified FL-HSCs from 18.5-dpc fetuses (CD45.2) and intravenously injected them into lethally irradiated adult recipient mice (CD45.1) (Fig. 1*A* and *B*). In this study, we used an HSC enriched, c-Kit^{hi}Lin^{−/lo}Sca-1⁺ (KLS) population from FL and BM as HSCs (see *Material and Methods*). Whole BM (WBM) cells from 8-week-old congenic mice (CD45.1/CD45.2) were also injected as competitors of FL-HSCs. At 6 weeks postinjection, we examined the surface phenotypes of cells from peritoneum cavity and spleen by FACS. We observed three distinct subsets, which are CD45.1⁺CD45.2[−] residual host cells, CD45.1⁺CD45.2⁺ cells derived from competitors, and CD45.1[−]CD45.2⁺ HSC-derived cells (Fig. 1*A* and *B*). FL-HSCs gave rise to all classes of B cells, suggesting that microenvironment of adult mice is sufficient for B cell development, including B-1a B cell development (Fig. 1*A*). Similarly, adult BM-HSCs gave rise to all classes of B cells (Figs. 1*B* and 2), although CD5 expression levels were lower on B-1a B cells derived from adult BM-HSCs compared with B-1a B cells derived from FL-HSCs (Fig. 1*A* and *B*; see also Fig. 7, which is published as supporting

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Abbreviations: BM, bone marrow; FL, fetal liver; HSC, hematopoietic stem cell; FL-HSCs, HSCs in FL; BM-HSCs, BM derived from HSCs; IL-7R α , IL-7 receptor α ; KLS, c-Kit^{hi}Lin^{−/lo}Sca-1⁺; TSLP, thymic stromal-derived lymphopoietin; WBM, whole BM; dpc, days postconception; EBF, early B cell factor.

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Fig. 1. Differentiation of all classes of B cells from FL- and adult BM-HSCs. (A) FL-HSCs were sorted from WT mice (CD45.2) (18.5 dpc) and i.v.-injected into lethally irradiated adult recipients (CD45.1) with WBMs (CD45.1/CD45.2) (see *Material and Methods*). Donor-derived B-1 B cell development in the peritoneum cavity (PerC) and B-2 B cell development in the spleen (Spl) are shown. (B) BM-HSCs were sorted from adult WT mice (8 weeks old) and injected into adult recipients with WBMs. Analyses of donor-derived peritoneal B-1 and splenic B-2 B cells are shown. In all experiments, more than three mice were analyzed, and representative plots are shown.

information on the PNAS web site). In the same reconstituted mice, we observed both B-1 and B-2 B cells from CD45.1⁺CD45.2⁺ WBM (data not shown). This suggests that adult hematopoietic progenitors are fully potent to differentiate into any types of B cells, including B-1a B cells. A previous study suggests that B-1a B cells are efficiently generated from fetal but not adult HSCs/progenitors (29, 31). This discrepancy could be due to the different number of HSCs injected in the two studies. Although much less efficient than FL cells, adult BM cells do give rise to B-1a and B-1b cells in the study by Herzenberg's group (32). In this current study, we injected 5×10^3 purified HSCs, approximately five times more HSCs than with the number of HSCs in 2×10^6 BM cells (containing $\approx 1 \times 10^3$ KLS cells) injected in the previous study (32).

Requirement of IL-7 in B Cell Development Is Differently Programmed in FL- and Adult BM-HSCs. It has been shown that IL-7 is indispensable for adult but not for fetal B cell development (30, 33).

Fig. 2. BM- and FL-HSCs are comparable in generating all classes of B cells. Open and closed bars indicate the chimerism of FL-HSC-derived cells and adult BM-HSC-derived cells in each B cell lineage, respectively. Recipient mice analyzed in Fig. 1 A and B were used to calculate the chimerism. The mean of chimerism $\pm$ SD, which was calculated as described in *Material and Methods*, is shown. A significant difference was not detected in a comparison between the chimerism of FL- and adult BM-HSC-derived cells in all classes of B cells by Student's *t* test.

To examine whether this difference is due to the intrinsic difference of FL- and adult BM-HSCs, we assessed B cell development from FL- and adult BM-HSCs derived from IL-7R $\alpha^{-/-}$ mice, as we did in Fig. 1. In recipients injected with IL-7R $\alpha^{-/-}$ FL-HSCs, we observed B-1a (17.4%) and B-1b (68.8%) B cells (Fig. 3A Upper). We also observed marginal zone (19.3%) and follicular (39.8%) B cells (Fig. 3A Lower). The percentage of B-1 B cells in the peritoneum cavity (7.5%) and B-2 B cells in spleen (3.6%) was severely reduced compared with the mice injected with WT FL-HSCs (Figs. 1A and 3A), suggesting the role of IL-7R α in expansion of the B cell pool.

In contrast to the mice that were transplanted with IL-7R $\alpha^{-/-}$ FL-HSCs, we hardly detected B-1 B cells (0.3%) and B-2 B cells (0.8%) in the recipients injected with IL-7R $\alpha^{-/-}$ adult BM-HSCs (Fig. 3B). Hematopoietic cells were efficiently reconstituted in the recipient mice injected with either WT or IL-7R $\alpha^{-/-}$ HSCs, judging by the myeloid chimerism in BM of the host mice (Fig. 8, which is published as supporting information on the PNAS web site). Therefore, these data indicate that IL-7 dependency in B cell development is programmed differently in FL- and adult BM-HSCs.

BM-HSCs Change Their Properties from the Fetal to Adult Type Between 1 and 2 Weeks of Age. Previous studies showed that adult IL-7R $\alpha^{-/-}$ mice harbor peripheral B cells, although the cell number of each population is severely reduced (34, 35). In fact, we observed all classes of B cells, such as B-1a and B-1b B cells in the peritoneal cavity, and marginal zone and follicular B cells in the spleen of 8-week-old IL-7R $\alpha^{-/-}$ mice (Fig. 9, which is published as supporting information on the PNAS web site). In addition, B220⁺IgM⁺ cells were present in BM of adult IL-7R $\alpha^{-/-}$ mice (Fig. 4B). Therefore, it is possible that minor or leaky B cell development continues in BM of adult IL-7R $\alpha^{-/-}$ mice, which may contribute to the formation of B cells observed in IL-7R $\alpha^{-/-}$ mice (Fig. 9). However, FACS analysis of the B220⁺IgM⁺ cells in adult BM of IL-7R $\alpha^{-/-}$ mice showed that this population has the same phenotype as recirculating mature B cells (26) in WT mice (Fig. 4A and B). Thus, these data strongly suggest that B cell development in adult IL-7R $\alpha^{-/-}$ mice at 8 weeks of age or older is completely blocked, as we reported (36). Consistent with this notion, we observed B220⁺IgM⁺IgD^{-lo} immature B cells in BM of IL-7R $\alpha^{-/-}$

Fig. 3. B cell development from FL- but not from adult BM-HSCs is IL-7R α -independent. (A) FL-HSCs were sorted from IL-7R $\alpha^{-/-}$ mice (18.5 dpc) (CD45.2) and injected into lethally irradiated adult recipients with WBMs from 8-week-old mice (CD45.1/CD45.2). Analyses of donor-derived peritoneal B-1 and splenic B-2 B cells are shown. (B) BM-HSCs were sorted from adult IL-7R $\alpha^{-/-}$ mice (8 weeks old) and injected into adult recipients with WBMs. Donor-derived B cells were few, and B-1a, B-1b, marginal zone, and follicular B cells were not detected (ND). The same gates were used as in Fig. 1 A and B. In all experiments, more than three mice were analyzed, and representative plots are shown.

mice only between 2 and 3 weeks but not 4 weeks after birth (Fig. 4C).

Because HSCs are thought to migrate from FL or other organs to BM and initiate hematopoiesis in BM at 17.5 dpc (11), observed immature B cells in 2- and 3-week-old IL-7R $\alpha^{-/-}$ mice are most likely derived from HSCs in BM but not from HSCs in FL. Thus, we hypothesized that BM-HSCs in IL-7R $\alpha^{-/-}$ mice younger than 2 weeks of age still maintain IL-7R α -independent B cell potential, which we observed in FL-HSCs. To clarify this issue, we purified FL- or BM-HSCs (KLS cells) from WT and IL-7R $\alpha^{-/-}$ mice whose ages vary from 18.5 dpc to 4 weeks after birth and examined B cell potential in irradiated adult mice (Fig. 5 A and B). The production of B-1 B cells in the peritoneum cavity and B-2 B cells in spleen from WT HSCs were comparable among various ages (Fig. 5 A and B). BM-HSCs derived from 1-week-old IL-7R $\alpha^{-/-}$ mice could give rise to both B-1 and B-2 B cells, yet the frequency was slightly lower than IL-7R $\alpha^{-/-}$

FL-HSCs (Fig. 5 A and B). However, BM-HSCs in 2-week-old IL-7R $\alpha^{-/-}$ mice gave rise to neither B-1 nor B-2 B cells (Fig. 5 A and B). To further confirm this change of HSC property between 1 and 2 weeks of age, we purified Flt3 $^{-}$ KLS cells [which are exclusively composed of long- and short-term HSCs (37)] from BM in IL-7R $\alpha^{-/-}$ mice and examined the B cell potential of IL-7R $\alpha^{-/-}$ HSCs at the different age. Similar to the results with KLS, which include HSCs and more mature multipotent progenitors (37, 38), Flt3 $^{-}$ KLS cells from 1- but not 2-week-old IL-7R $\alpha^{-/-}$ mice gave rise to B-1 and B-2 B cells (Fig. 10, which is published as supporting information on the PNAS web site). These data clearly demonstrate that the developmental switch occurs in BM-HSCs from the fetal to adult type in mice between 1 and 2 weeks of age.

IL-7R α Is Dispensable for Early B Cell Factor (EBF) Expression in PreproB Cells Derived from Fetal and Neonatal HSCs. We and others previously showed that IL-7R α signaling is essential to up-regulate EBF expression in the early stages of B cell development in adult mice (36, 39). Enforced expression of EBF in adult IL-7R $\alpha^{-/-}$ HSCs could rescue impaired B cell development in the absence of IL-7 signaling; however, the total B cell number was profoundly low in the absence of IL-7R α . Thus, we concluded that EBF expression is enough to restore B cell differentiation but cannot induce expansion of B cell progenitors without IL-7 (36). Recently, we found that preproB to proB transition is IL-7-independent if preproB cells express EBF at least at the levels in freshly isolated preproB cells from WT mice (Fig. 6, dotted line; K.K. and M.K., unpublished results). Based on these observations, we hypothesized that EBF expression may be regulated independently by IL-7 in fetal B cell progenitors. To test this possibility, we compared the EBF expression level in fetal and adult B cell progenitors in IL-7R $\alpha^{-/-}$ mice. EBF expression levels in IL-7R $\alpha^{-/-}$ preproB cells derived from FL (18.5 dpc) and neonatal (1-week-old) BM were higher than those of adult WT preproB cells (Fig. 6). However, the EBF expression was sharply reduced after 2 weeks of age in preproB cells without IL-7R α (Fig. 6). These results suggest that B cell progenitors derived from HSCs in fetuses and neonates ($\approx$ 1 week after birth) can express EBF in an IL-7-independent fashion, whereas B cell progenitors from BM-HSCs 2 weeks of age and older strictly require IL-7 stimulation to express enough EBF for stage transition from the preproB to proB cell stages.

Discussion

Analysis of IL-7 $^{-/-}$ mice initially showed that fetal B cell development can occur without IL-7, although adult B cell development essentially requires IL-7 (30). This difference has been explained by thymic stromal-derived lymphopoietin (TSLP), which may substitute the function of IL-7 in B cell development in fetuses but not in adults (30, 33). In this study, we showed that IL-7R $\alpha^{-/-}$ FL-HSCs can give rise to B cells. Because IL-7R α is also used as a component for TSLP receptor complexes, both TSLP and IL-7 are dispensable for B cell differentiation from FL-HSCs (Fig. 3A). However, B cell numbers obtained from IL-7R $\alpha^{-/-}$ FL-HSCs were much lower than from WT FL-HSCs (Figs. 1A and 3A), suggesting that both IL-7 and TSLP may play an important role in the expansion of B cell progenitors. However, no obvious phenotypic change was reported in B cells in TSLP receptor-deficient mice (40). Hence, IL-7 may be a major growth factor in developing B cells *in vivo*.

The ability of FL-HSCs to produce B cells in the absence of IL-7R α may rely on the higher EBF expression in their progenies than B cell progenitors derived from adult BM-HSCs (Fig. 6). Flt3 signaling is essential for the residual B cell development observed in fetal and adult IL-7R $\alpha^{-/-}$ mice, because no B cells are detectable in IL-7R $\alpha^{-/-}$ mice on the Flt3 $^{-/-}$ background (33, 34). We previously showed that constitutive active Stat5 can

Fig. 5. Switch from fetal- to adult-type properties in BM-HSCs. HSCs were sorted from various ages of WT or IL-7Rα^{-/-} mice and injected into lethally irradiated adult recipients with WBMs (see *Materials and Methods*). The age of mice when HSCs were purified is indicated on top of each plot. All plots are pregated on donor-derived cells. (A) B-1 B cell development was analyzed in spleen at 6 weeks postinjection. (B) B-2 B cell development was analyzed in the spleen at 6 weeks postinjection. At least three recipient mice were analyzed at each time point, and representative plots are shown.

HSCs are maintained undifferentiated (7). Similarly, FL may also have a yet-to-be-identified niche for HSCs. If this is the case, IL-7-independent B cell differentiation from FL-HSCs should be transient only upon transfer into the adult host, because FL-HSCs cannot sustain its fetal properties in the adult BM environment. Further studies are required to clarify the mechanisms of how HSCs change their properties at different ages.

Fig. 6. EBF expression level in fetal, neonatal, and adult IL-7Rα^{-/-} preproB cells. PreproB cells were sorted from IL-7Rα^{-/-} mice of various ages (see *Materials and Methods*) from the FL at 18.5 dpc and from BM at other ages. The bar shows the relative EBF expression level in preproB cells derived from 18.5 dpc and 1-, 2-, and 8-week-old IL-7Rα^{-/-} mice (23.5, 26.0, 8.7, and 0.3, respectively). EBF expression in each cell pool was normalized against GAPDH expression. Relative EBF expression level in WBM is arbitrarily set as 1. The dotted line indicates the relative EBF expression level in preproB cells derived from 8-week-old WT mice (17.2). The shown value is the mean $\pm$ SD from three independent samples.

Materials and Methods

Mice. IL-7 receptor α chain-deficient (IL-7Rα^{-/-}) (CD45.2) mice were purchased from the Jackson Laboratory, Bar Harbor, ME. Age-matched C57BL/Ka-Thy1.1 (CD45.2) mice were used as WT. Eight- to 10-wk-old C57BL/Ka-Thy1.1-Ly5.1 (CD45.1) mice were used as recipients. C57BL/Ka-Thy1.1 (CD45.1/CD45.2) mice were generated by crossing C57BL/Ka-Thy1.1-Ly5.1 (CD45.1) and C57BL/Ka-Thy1.1 (CD45.2). To obtain FL, timed pregnancies of C57BL/Ka-Thy1.1 and IL-7Rα^{-/-} mice were used. The day of the vaginal plug observation was determined as 0.5 dpc. All mice were bred in a specific pathogen-free environment at the mouse facility of Duke University Medical Center. All studies and procedures were approved by the Duke University Animal Care and Use Committee.

Flow Cytometry. All antibodies used in this study are listed as follows. FITC-anti-CD21/CD35 (CR1/CR2, CD21a/CD21b) (7G6), FITC-anti-Ly-6C (AL-21), PE-anti-CD43 (S7), PE-Cy7-anti-NK1.1 (PK136), APC-anti-CD19 (1D3), PE-Cy7-, APC-, or APC-Cy7-anti-CD45R (B220) (RA3-6B2), biotin-anti-CD24 (HSA) (30-F1) were purchased from BD Biosciences Pharmingen, San Diego, CA. FITC-anti-CD11b (Mac-1) (M1/70), FITC-anti-B220 (RA3-6B2), FITC-anti-CD90.1 (Thy-1.1) (HIS51), FITC-anti-CD90.2 (Thy-1.2) (53-2.1), PE-anti-CD5 (Ly-1) (53-7.3), PE-anti-CD23 (FcεRII) (B3B4), PE-anti-IgD (11-26), PE-Cy5-anti-B220 (RA3-6B2), PE-Cy5-anti-CD3ε (145-2C11), PE-Cy5-anti-CD4 (L3T4), PE-Cy5-anti-CD8a (Ly-2), PE-Cy5-anti-Ly6G (Gr-1) (RB6-8C5), PE-Cy5-anti-Mac-1 (M1/70), PE-Cy5-anti-TER-119, PE-Cy7-anti-CD45.1 (A20), APC-anti-ClqRp (AA4.1), APC-anti-CD117 (c-Kit) (2B8), and biotin-CD45.2 (104) were purchased from eBioscience, San Diego, CA. Texas red (TxR)-goat anti-mouse Igμ (SouthernBiotech, Birmingham, AL) was used for surface IgM staining. Anti-Sca-1 (E13-161-7) was purified from culture supernatant and conjugated with TxR by a standard procedure in our laboratory.