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Progenitor Cell Origin Plays a Role in Fate Choices of Mature B Cells

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B cells, the Ab-producing cells of the immune system, develop from hematopoietic stem cells (HSCs) through well-defined stages during which Ig genes are rearranged to generate a clonal BCR. Signaling through the BCR plays a role in the subsequent cell fate decisions leading to the generation of three distinct types of B cells: B1, marginal zone, and follicular B cells. Common lymphoid progenitors (CLPs) are descended from HSCs, and although recent observations suggest that CLPs may not be physiological T cell precursors, it is generally accepted that CLPs are obligate progenitors for B cells. In addition, a CLP-like progenitor of unknown significance that lacks expression of c-kit (kit−/− CLP) was recently identified in the mouse model. In this study, we show that CLPs, kiti− CLPs and a population within the lin− Sca1+kit+flt3− IL7R+ (CLPs; lin− Sca1lokitloflt3+IL7R+), which are predominantly lymphoid committed (6), is a progenitor origin as B cells derived from CLPs and kiti− CLPs express more Sca1 than those derived from lin− Sca1+kit+flt3− cells. These observations indicate a role for progenitor origin in B cell fate choices and suggest the existence of CLP-independent B cell development. The Journal of Immunology, 2010, 184: 000–000.

Several major types of mature B cells are distinguished. B1 cells occur mainly in the pleural and peritoneal cavities and, in addition to producing Abs in response to infection, also produce “natural” IgM (23, 24). B2 cells reside in the spleen, the blood, and the lymph nodes. The spleen contains two types of B2 cells: marginal zone (MZ) and follicular (FO) B cells (25). MZ B cells (IgDhi IgMloCD21hiCD23lo) reside in the region demarcating the white and red pulp, respond to type 2 thymus-independent Ags, such as multivalent polysaccharides, are recruited rapidly into Ab responses to blood-borne pathogens and play a critical role in their clearance. In contrast, FO B cells (IgDlo IgMhiCD21loCD23hi) inhabit the follicles, circulate in the blood, and produce high-affinity Abs for which they require T cell help.

The mechanisms underlying the development of these different types of B cells are unclear. Studies in knockout mice where signaling through the B cell receptor (BCR, the clonal Ig expressed on the surface) was either enhanced or decreased suggested that BCR signal strength determines cell fate choices of transitional B cells, AA4.1+ CD21− CD23− IgMlo cells derived from AA4.1+ IgM+ immature B (iB) cells that migrate from the BM to the spleen, and subsequently develop into mature splenic B cells (19, 20, 26–30). According to these, low BCR signal strength results in MZ B cells, whereas intermediate signal strength results in FO B cells. High signal strength leads to the development of B1 B cells (26, 29–32). In addition to BCR signal strength, BCR specificity has also been shown to play a role, as positive selection by autoantigens is required for the generation of MZ and B1 B cells in transgenic models (33–35). Additional mechanisms must play a role, however. Lymphopenia and impaired B cell development favor the generation or maintenance of MZ and B1 cells, likely through their enhanced capacity of homeostatic proliferation (25, 29, 36, 37). Furthermore, plasticity exists among mature B cells as small resting lymph node B cells, the equivalent of recirculating FO B cells, can adopt a MZ phenotype after transfer into a lymphopenic host (38). In the spleen, evidence suggests a distinct differentiation pathway for MZ B cells. MZ B cells develop from AA4.1+ CD21− CD23− IgMlo T1, into AA4.1+ CD21− CD23− IgMlo T2 and finally through a AA4.1+CD21loCD23+ IgMhi MZ precursor stage into mature MZ B cells (39). Development of these processes is not yet well understood and is the subject of ongoing research.

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Abbreviations used in this paper: BM, bone marrow; CLP, common lymphoid progenitor; FO, follicular; HSC, hematopoietic stem cell; iB, immature B; LSKF, lin− Sca1+kit+flt3−; MHC II, MHC class II; MPP, multipotential progenitor; MZ, marginal zone; PC, peritoneal cavity; wt, wild type.

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of MZ B cells requires LFA1 and α4β1 integrins (40), as well as Notch2 (41), which interacts with DL1 expressed on MZ endothelial cells, an interaction that is enhanced by Fringe glycosyltransferases (42). Finally, it has been argued that B1 cells represent a distinct B cell lineage (43, 44). A lin B220−CD19+IgM− B1-specified pro-B cell has recently been identified, suggesting that B1 development branches off from B2 development at an early stage (45). B1 precursors have also been reported to be CD138− (46).

As at least two distinct lymphoid-specified progenitors at a similar stage of differentiation exist, CLPs and kit+ CLPs, we explored the possibility that the origin of B cells within the hematopoietic stem and progenitor compartment might contribute to their cell fate choice in the mature B cell compartment. Therefore, we analyzed the B cell potential of CLPs and kit+ CLPs in more detail, hypothesizing that both progenitors might show a differential bias toward distinct types of mature B cells. We observed, however, that the LSKF− compartment, which is highly enriched in repopulating HSCs, also contains progenitors for a FO-biased B cell development program that is independent from CLPs or kit− CLPs, which show a higher propensity to generate MZ B cells. Furthermore, B cells generated from CLPs and kit+ CLPs expressed more Sca1 than those derived directly from the LSKF− compartment. CLPs and kit+ CLPs differ in their B1 potential, however, as kit+ CLPs produce relatively more B1 cells than CLPs. Taken together, our observations indicate that CLPs are not obligate B cell progenitors and that progenitor origin plays a role in the regulation of cell fate choices in the B cell lineage.

## Materials and Methods

### Mice

The 4- to 8-wk-old C57BL/6 (CD45.2) mice and B6.Ly5.2 (B6.Ly5.2) mice were purchased from the National Cancer Institute Animal Facility (Frederick, MD). CD45.1+CD45.2+ C57BL/6 mice were generated at the Mount Sinai Animal Facility (New York, NY) by crossing C57BL/6 and B6.Ly5.2 (B6.Ly5.2) mice. Rag1−/− mice (B6.129.2-Rag1tm1Mom/J) (CD45.2) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Experiments and animal care were performed in accordance with the Mount Sinai Animal Institutional Care and Use Committee.

### Antibodies

FITC-conjugated anti-TER119, B220−Mac1−Gr1−CD4−CD2−CD3e−CD8a−CD19−CD21−APC-conjugated anti-CD25, biotin-conjugated anti-Sca1, and anti-CD86; APC-Cy7-conjugated anti-CD19, PerCP-conjugated streptavidin and PerCP-Cy5.5-conjugated CD45.2 were purchased from BD Pharmingen (San Diego, CA). PE-conjugated anti-Flt3−CD21−Mac1−PE-Cy7-conjugated anti-IL7Rα, streptavidin−CD23−, and CD45.1−APC-conjugated anti-AAA4.1; PECy5-conjugated anti-IgM, as well as Alexa Fluor 750-conjugated anti-CD45.1 were from eBioscience (San Diego, CA). PE-conjugated anti-IgD and FITC-conjugated anti-CD45.1 were from Southern Biotechnology Associates (Birmingham, AL). Pacific blue-conjugated anti-Sca1 and anti-1-A/E were purchased from Biolegend (San Diego, CA).

### Preparation of hematopoietic cells

BM cells were prepared by flushing the femur and tibia of mice with cold DMEM (Mediatech, Herndon, VA), containing 2% FBS and 100 ng/ml penicillin/streptomycin. Spleen cell suspensions were prepared by mincing the organs through nylon mesh. RBCs were lysed in 2 ml 1× RBC lysis buffer (eBioscience). BM cells were washed and resuspended in an appropriate volume of PBS or DMEM for staining. Peritoneal cells were pressed by B1 B cells, VH11 and VH9 were assessed through a nested PCR. DNA amplification was carried out using Advantage cDNA polymerase (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cdna was prepared using SuperScript III Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The two gene segments predominantly expressed by B1 B cells, Vη11 and Vη4Q2 were assessed through a nested PCR. CD19 and CD22 PCR. DNA amplification was carried out using Advantage cDNA polymerase (BD Biosciences). On this instrument, all phycorerythrin-containing antibodies are excited by green 530-nm laser. Data were analyzed using FlowJo software. Biexponential transformation as implemented in Flowjo was used when >8% or dots fell off the axis. Lineage markers used for the exclusion of lineage-positive cells were CD3, CD4, CD8a, CD21, CD22, Gr1, Mac1, and Ter119. Subpopulations were defined as follows: HSC, lin−Sca1+Kit+Rag1−/−; MPP, lin−Sca1+Kit+Rag1−; CLP, lin−Sca1+Kit+Rag1−/−IL7Rα; kit−CLP, lin−Sca1+Kit+Rag1−/−IL7Rα/FITC+; pre-B cells, AA4.1−B220−CD19+IgM−CD25+; pro-B cells, AA4.1−B220−CD19+IgM−CD25−; B2 cell, AA4.1−B220−CD19+IgM−CD25−; B1 cell, AA4.1−B220−CD19+IgM−CD25−; MC cell, AA4.1−B220−CD19+IgM−CD25−; B1 cell, B220+Mac1−; and B2 cell, B220+Mac1−.

### Reconstitution of Rag1−/− and C57BL/6 mice

Donor-derived CLPs were injected in the tail vein of sublethally irradiated (700 cGy) C57BL/6 mice. Donor-derived cells were distinguished by expression of CD45.1. For the competitive transplants, sorted LSKF− cells from CD45.1+CD45.2+ C57BL/6 mice were mixed with sorted MPPs, CLPs, and kit− CLPs from BM of CD45.1+ C57BL/6 mice and then were injected in the tail vein of sublethally irradiated (700 cGy) Rag1−/− mice. For assessment of lymphocyte-replete hosts, 5 × 106 B cells from CD45.1+ C57BL/6 mice were injected in the tail vein of unconditioned CD45.2+ C57BL/6 hosts.

### Immunohistochemistry

Spleens were removed and snap frozen in OCT compound (Tissue Tek, Sakura Finetek, Torrance, CA) and stored at −80°C. Cryostat sections (7–8 μm) were air-dried, fixed in 100% acetone (5 min at room temperature), and washed with PBS. Sections were stained with anti-IgM-Cy3 (Chemicon International, Temecula, CA), MOMA-1-FITC (Serotec, Oxford, U.K.), and biotinylated anti-Sca1 (BD Biosciences). Cy5-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA), B biotin, and biotinylated Ab. Slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Images were acquired with a Leica DMRA2 fluorescence microscope (Leica, Wetzlar, Germany) and a digital Hamamatsu charge-coupled device camera and were analyzed with Openlab software (Improvision, Perkin Elmer, Waltham, MA).

### IgH rearrangement in B1 and B cells

Donor-derived B1 B cells were sorted 4 wk after transplantation of 5000 LSKF− CLPs, and kit− CLPs into sublethally irradiated Rag1−/− mice. mRNA was extracted using RNeasy microkit (Qiagen) following the manufacturer’s instructions. cdna was prepared using SuperScript III Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The two gene segments predominantly expressed by B1 B cells, Vη11 and Vη4Q2 were assessed through a nested PCR. DNA amplification was carried out using Advantage cdna polymerase mix (BD Clontech, Palo Alto, CA), following the protocol described by Montecino-Rodriguez et al. (45).

### Proliferation assay and expression of activation markers CD86 and MHC class II

Donor-derived splenic B cells were sorted 3 wk after transplantation of 3000 LSKF− cells, CLPs, and kit− CLPs into sublethally irradiated Rag1−/− mice. MZ and FO B cells were plated in 96-well plates containing RPMI 1640 medium (Mediatech, Manassas, VA), supplemented with 50 μM 2-ME, 10% FBS, 2 mM glutamine (Life Technologies, Grand Island, NY), and 10 mM HEPES (Mediatech). The cells were stimulated with 10 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) for 48 h. pulsed with 1 μCi/well [3H] thymidine, and harvested into a 96-well filter plate. The [3H]thymidine incorporation was measured as counts per minute using the liquid scintillator and luminescence counter 1450 Microbeta TRILUX (PerkinElmer, Wellesley, MA). Unstimulated cells were used as a control. Each population was plated in triplicate. For the expression of activation markers, cells were stimulated with 10 μg/ml LPS or Fab′ (10), for 24 h and stained for CD86 and MHC class II (MHC II), immediately after being harvested from the recipient mice (40), and after stimulation in vitro.

### Ca2+ flux

Intracellular Ca2+ levels were measured using the Ca2+ indicator Indo-1 (Invitrogen). Splenic B cells were obtained 4 wk

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after transplantation of 3000 LSKF−/− MPPs, CLPs, and kit−/− CLPs CD45.1 cells into sublethally irradiated Rag1−/− mice. Before staining, 109 CD45.2+ wt spleen cells were added, which served as internal control and were distinguished from donorderived cells by the expression of the CD45 allelic variants. Cells were resuspended in 500 μl HBSS (Life Technologies) containing 10% FBS and incubated with 5 μl of 1 μM Indo-1 at 32°C for 40 min. Cells were then washed with HBSS and incubated with anti-CD16/CD32 for 5 min and with Abs against AA4.1, CD21, CD23, CD19, CD45.1, and CD45.2 for 15 min at 4°C. Cells were washed with HBSS and warmed to 37°C just before the analysis. Indo-1–loaded cells were then examined with a FACS Vantage SE flow cytometer equipped with an ultraviolet laser and appropriate filters for the 405- and 488-nm wavelengths. After the establishment of a stable baseline, the cells were simulated with 10 μg/ml F(ab′)2 goat anti-mouse IgM μ-chain (The Jackson Laboratory) and monitored for another 5 min. The change in intracellular Ca2+ levels was determined through the ratio of emission signals of Indo-1 at 405 and 488 nm, representing the ratio of free to bound Ca2+. The kinetic analysis was performed using FlowJo software.

**Statistical analysis**

For multiple comparisons, one-way ANOVA, as implemented in SPSS software, was used. If p < 0.5 pairwise comparison of groups using unpaired t test was performed. Error bars represent SEM.

**Results**

**Different lymphoid progenitors generate MZ and FO B cell in different ratios**

To analyze their B cell potential, we injected 3000 CLPs or kit−/− CLPs purified from CD45.1+ congenic C57BL/6 mice (see Fig. 1A for representative sort gates) into sublethally irradiated CD45.2+ C57BL/6 mice. Previous studies have shown that both IL7Rα and IL7Rα+ lin Sca1+kit− cells have similar B cell potential (21). In the current study, we defined kit−/− CLPs as lin− Sca1−kit− cells with similar Flt3 and IL7Rα expression as CLPs but lacking expression of c-kit. As in preliminary experiments, differentiation into distinguishable MZ and FO B cells occurred between 2 and 3 wk after transfer (see Fig. 1B for representative analysis gates), we measured the output of mature B cells in the spleen after 3 wk. Similar data as those presented below were obtained 4 wk after transfer, however. Because we assumed that both LSKF−/− cells (containing short-term and long-term HSCs) and MPPs (1–5) would be universal B cell precursors that fully reconstitute the mature B cell compartment, we used these populations as controls (see Fig. 1A for representative sort gates). As AA4.1 expression decreases progressively during the maturation of B cells in the spleen (20, 27, 39), we distinguished mature B cells from transitional B cells using this marker. The different expression profiles of CD21/CD23 on what we label AA4.1+ (with distinguishable FO and MZ fractions; Fig. 1B) versus AA4.1− (with T1 and T2, and no MZ fraction; Fig. 1B) indicates that this gating is accurate.

Among donor-derived splenic B cells, kit−/− CLPs and CLPs had generated a larger fraction of mature B cells (defined as donor-derived CD45.1+ AA4.1− cells expressing IgM and/or IgD; Fig. 1B) and a smaller fraction of transitional B cells (defined as CD45.1+ AA4.1+ IgMhi cells, Fig. 1B) than MPPs and, in particular, than LSKF−/− cells (Fig. 1C). This is a reflection of the developmental hierarchy of the injected populations, as earlier progenitors, LSKF−/− cells and MPPs, take longer to generate fully mature progeny (1–5, 11). Nevertheless, up to 80% of LSKF−/−–derived B cells had a mature AA4.1+ phenotype 3 wk after transfer (Fig. 1C). There was a striking difference in ratio of mature MZ to FO B cells generated by these progenitors (see Fig. 1B for representative analysis gates). The MZ/FO ratio in the progeny of kit−/− CLPs and CLPs was 3-fold higher than in the progeny of LSKF−/− cells, whereas MPPs were between these two extremes (Fig. 1D). Thus, CLPs, kit−/− CLPs on one hand and LSKF−/− cells on the other hand have qualitatively different potentials with the respect to the generation of mature B cells: CLPs and kit−/− CLPs have a relatively stronger propensity to generate MZ B cells than LSKF−/− cells, which are highly FO biased. An MZ fate is favored in conditions of lymphopenia (25, 36–38), suggesting that the observed difference in B cell potential might be due to different degrees of lymphopenia in the host. However, as the injected populations competed with endogenous B cell development, their progeny encountered the same average degree of lymphopenia upon arrival in the spleen, thus eliminating bias introduced by varying levels of B cell reconstitution. Furthermore, splenic B cell number was similar in recipients of CLPs, kit−/− CLPs, or LSKF−/− cells (Fig. 1E). Therefore, the difference in MZ/FO ratio among B cells derived from CLPs, kit−/− CLPs, and LSKF−/− cells cannot be explained by different degrees of lymphopenia to which the cells are exposed. Nonetheless, it cannot be excluded that the lower MZ/FO ratio of MPP- derived B cells might be due to the fact that, because of the high proliferative capacity of MPPs, recipients of MPPs had the highest number splenic B cells. Thus, our observations suggest that distinct progenitor populations display cell-intrinsic qualitative differences in their capacity to generate mature B cell subtypes.

To find further support for this notion, we injected 1500 CD45.1+ MPPs, CLPs, kit−/− CLPs, or LSKF−/− cells into sublethally irradiated Rag1−/− hosts (CD45.2+), which have no T or B cells, and therefore provide an extremely lymphopenic environment to developing B cells (47). As shown in the representative example in Fig. 2A, even in such a lymphopenic environment, LSKF−/− cells generated relatively fewer MZ B cells than CLPs and kit−/− CLPs, whereas MPPs were in between these two extremes, confirming the higher MZ/FO differentiation ratio of CLPs and kit−/− CLPs compared with cells in the LSKF−/− population. To determine whether different progenitor populations exhibit cell-intrinsic differences in their ability to generate specific mature B cells, we performed competitive transplants, in which 1500 CD45.1+ MPPs, CLPs, or kit−/− CLPs were injected together with 1500 CD45.1+CD45.2+ LSKF−/− cells in sublethally irradiated Rag1−/− mice (CD45.2; see Fig. 2B for a representative example of analysis gates of donor populations in the spleen). Because competing populations were exposed to the same environment, any difference in B cell development potential will be intrinsic to the respective progenitor populations. After 3 wk, MPPs had generated 10-fold more B cells than any of the other cell populations, so that recipients of MPPs + LSKF−/− cells were significantly less lymphopenic than recipients of either CLPs + LSKF−/− cells or kit−/− CLPs + LSKF−/− cells (Fig. 2C). The MZ/FO ratio among mature AA4.1− splenic B cells was 4- and 6-fold higher in CLP and kit−/− CLP-derived cells, respectively, compared with competing LSKF−/−–derived cells (Fig. 2D). Despite varying degrees of lymphopenia in host mice (Fig. 2C), the MZ/FO ratio of LSKF−/−–derived cells was equally low (Fig. 2D). Therefore, the differences in MZ/FO ratio between CLPs or kit−/− CLPs on one hand and LSKF−/− cells on the other hand must be cell intrinsic and cannot be explained by varying degrees of host lymphopenia. We observed several differences using Rag1−/− compared with C57BL/6 mice as hosts, however. In all progenitor populations the MZ/FO ratio was higher in Rag1−/− hosts than in C57BL/6 hosts (Figs. 1D, 2D). Furthermore, in contrast to experiments where sublethally irradiated C57BL/6 mice were used as recipients (Fig. 1), the MZ/FO ratio was slightly, although significantly (p = 0.017, paired t test), higher in the progeny of kit−/− CLPs than of CLPs in Rag1−/− hosts (Fig. 2D). Furthermore, the MZ/FO ratio of kit−/− CLPs and CLPs in Rag1−/− hosts was 9-fold higher than in C57BL/6 hosts. In contrast, the MZ/FO ratio of LSKF−/− cells was only 5-fold higher than in C57BL/6 hosts. These observations indicate that lymphopenia affects the propensity to generate MZ B cells more strongly in CLPs and, in particular, in kit−/− CLPs than in LSKF−/− cells.
Collectively, these results demonstrate that cells within the LSKF<sup>−</sup> population can give rise to a B cell development program that is less biased to the development of MZ B cells and is less affected by lymphopenia than the program that emanates from CLPs and kit<sup>−</sup>CLPs.

**B cells derived from LSKF<sup>−</sup> cells and from CLPs or kit<sup>−</sup>CLPs differ in Sca1 expression**

Next, we looked for markers that might distinguish B cells derived from LSKF<sup>−</sup> cells, CLPs, and kit<sup>−</sup>CLPs. Both in competitive transplants in Rag<sup>−/−</sup> hosts and in noncompetitive transplants in sublethally irradiated C57BL/6 mice, we observed that B cells generated from kit<sup>−</sup>CLPs and CLPs expressed more Sca1, a marker of hematopoietic stem and progenitor cells and of activated T cells (48), than those derived from LSKF<sup>−</sup> cells. In wild-type (wt) mice (data not shown) and in donor-derived B-lineage cells (Fig. 3A), Sca1 is expressed at low levels in pro-B cells (CD19<sup>+</sup>IgM<sup>+</sup>AA4.1<sup>+</sup>CD25<sup>+</sup>) is mostly absent in pre-B cells (CD19<sup>+</sup>IgM<sup>+</sup>AA4.1<sup>+</sup>CD25<sup>+</sup>) and reappears in a fraction of iB cells after they lost CD25 expression. Mature splenic B cells express a wide range of Sca1 and Sca1 expression was higher on MZ than on FO B cells, possibly a reflection of the activated state of MZ B cells (Fig. 3B). By fluorescence microscopy, Sca1 marked the MZ, although Sca1<sup>+</sup> B cells could be observed in the follicles (Fig. 3C). For reasons that are unclear, however, the difference in Sca1 expression between MZ and FO B cells appeared more pronounced when analyzed by fluorescence microscopy than by flow cytometry.

In noncompetitively transplanted C57BL/6 hosts, we calculated the ratio between the geometric mean of the Sca1 fluorescence between cells derived from kit<sup>−</sup>CLPs, CLPs, MPPs, or LSKF<sup>−</sup> cells and the geometric mean of the Sca1 fluorescence of endogenous cells of the same phenotype in each recipient. The expression of Sca1 on pre- and pro-B cells was similar in all donor-derived populations (Fig. 3D). However, iB derived from kit<sup>−</sup>CLPs and T1 cells derived from both CLPs and kit<sup>−</sup>CLPs expressed ~2-fold more Sca1 than those derived from LSKF<sup>−</sup> cells (Fig. 3D). Similar observations were made in donor-derived mature B cells. Likely because of the higher baseline expression of Sca1 on mature B cells (Fig. 3B), these differences were less pronounced, although they were still statistically significant (Fig. 3D). Sca1 expression on MPP-derived cells was variable, and in-between that of cells generated from CLPs and kit<sup>−</sup>CLPs, and from LSKF<sup>−</sup> cells, respectively (Fig. 3D). No progenitor-dependent differences were observed in the expression of CD21, CD23, IgM, or IgD in any of the donor-derived populations in the spleen (data not shown).

In competitively repopulated Rag<sup>−/−</sup> hosts, we compared Sca1 expression by calculating the ratio between the geometric mean of the Sca1 fluorescence between cells derived from kit<sup>−</sup>CLPs, CLPs, MPPs, or LSKF<sup>−</sup> cells and the geometric mean of the Sca1 fluorescence of endogenous cells of the same phenotype in each recipient. The expression of Sca1 on pre- and pro-B cells was similar in all donor-derived populations (Fig. 3D). However, iB derived from kit<sup>−</sup>CLPs and T1 cells derived from both CLPs and kit<sup>−</sup>CLPs expressed ~2-fold more Sca1 than those derived from LSKF<sup>−</sup> cells. Sca1 expression on MPP-derived cells was variable, and in-between that of cells generated from CLPs and kit<sup>−</sup>CLPs, and from LSKF<sup>−</sup> cells, respectively (Fig. 3D). No progenitor-dependent differences were observed in the expression of CD21, CD23, IgM, or IgD in any of the donor-derived populations in the spleen (data not shown).
irrespective of their progenitor cell origin (Fig. 3E). However, both CLPs and kit CLPs produced iB and T1 cells that expressed 10-fold more Sca1 than LSKF− cells. FO and MZ B cells originating from CLPs and kit CLPs expressed 3-fold and 50% more Sca1 than those derived from LSKF− cells, respectively. MPP-derived cells expressed Sca1 at levels slightly, although not statistically significantly higher than that of LSKF− cells (Figs. 3E, 4). There were no progenitor-dependent differences in the expression of CD21, CD23, IgM, or IgD in any of the donor-derived populations in the spleen (data not shown). It is interesting to note that lymphopenia differentially affected Sca1 expression depending on the progenitor origin of the cells, because the difference in Sca1 expression on iB, T1, MZ, and FO cells derived from kit CLPs and CLPs on one hand and LSKF− cells on the other hand was more pronounced in Rag1−/− mice than C57BL/6 hosts (Fig. 3D, 3E). Furthermore, although kit− CLPs generated iB cells with elevated Sca1 expression in both C57BL/6 and Rag1−/− host, CLPs only generated such iB cells in lymphopenic Rag1−/− hosts. These data indicate that, not only in terms of MZ/FO ratio, but also in terms of Sca1 expression, the B cell development program that emmates from CLPs and kit− CLPs and is more responsive to lymphopenia than that originating from LSKF− cells.

As Sca1 is an activation marker on lymphoid cells (48), we hypothesized that Sca1hi B cells derived from CLPs and from kit− CLPs would be more activated that Sca1lo B cells generated from LSKF− cells. We transferred these populations into Rag1−/− mice, isolated donor-derived MZ and FO B cells, and cultured these in the presence of LPS. As expected, MZ B cells proliferated more vigorously than FO B cells (49), but no progenitor cell-dependent differences were observed (Fig. 5A). Similarly, no progenitor-origin–dependent difference in the induction of the activation markers, CD86 and MHC II, were found (data not shown). Finally, we transferred kit− CLPs (data not shown), CLPs (Fig. 5B, upper panel), and LSKF− cells (Fig. 5B, lower panel) into Rag1−/− mice and analyzed anti-IgM-induced Ca2+ flux of donor-derived FO and MZ B cells 3 wk later, using spleen cells from wt C57BL/6 mice added before addition of anti-IgM as an internal control. Whereas Ca2+ flux was consistently higher in MZ than in FO B cells, there were no differences in Ca2+ flux dependent on progenitor origin of the cells (Fig. 5B). These data suggest that differences in Sca1 expression between cells derived from kit− CLPs or CLPs on one hand and from LSKF− cells on the other hand are unlikely to be explained by a difference in activation status.

We conclude that B cell development from either CLPs or kit− CLPs results in B cells that express more Sca1 than those derived directly from LSKF− cells.

Different potential of Sca1+ and Sca1− iB cells

As expression of Sca1 on donor-derived iB cells was associated with increased MZ bias of donor-derived mature B cells, and as a fraction of iB cells (AA4.1×B220×IgM+) in wt mice express Sca1 (Fig. 3A), we tested the hypothesis that high expression of Sca1 on iB cells confers increased propensity to generate MZ B cells. We injected 5×105 Sca1+ and Sca1− iB cells from CD45.1+C57BL/6 donors into unconditioned CD45.2+C57BL/6 recipients (see Fig. 6A for representative sort purities). At least a fraction of iB cells are poised to leave the BM and home to the spleen and could therefore be expected to show some engraftment in the absence of lymphopenia. Up to 5% of injected donor cells were found in the spleen (0.005–0.07% of total spleen population; Fig. 6B). After 4–6 days, the MZ fraction of the donor-derived mature B cells was 2-fold higher after transfer of Sca1+ iB cells than of Sca1− iB cells (Fig. 6C, 6D). Furthermore, Sca1+ iB cells generated FO and MZ B cells that expressed more Sca1 than endogenous FO and MZ B cells, whereas FO and MZ B cells generated from Sca1− iB cells expressed less Sca1 than their host counterparts (Fig. 6E). We conclude that higher Sca1 expression in iB cells is associated with the generation of B cells with a higher expression of Sca1 and with increased tendency to generate MZ B cells, even in unconditioned hosts. These results are consistent with the fact the CLPs and kit− CLPs generate iB cells that express more Sca1 than iB cells generated from LSKF− cells and that mature B cells derived from these progenitors express more Sca1 and display a higher MZ/FO differentiation ratio than those derived from LSKF− cells.
CLPs, kit−CLPs, and LSKF− cells differ in B1 potential

CLPs and kit−CLPs behaved very similarly with respect to their potential to generate MZ and FO B cells. Both populations differ, however, as kit−CLPs have lower myeloid potential, TdT, and Rag expression than CLPs (21, 22). In the peritoneal cavity (PC), both B2 (B220+,Mac1−) and B1 (B220+,Mac1+) cells occur (Fig. 7A) (23, 24). B1 cells are additionally distinguished from B2 cells by their higher IgM expression (Fig. 7A) (23, 24). To further examine functional differences between CLPs and kit−CLPs, we assessed their potential to generate B1 B cells by measuring relative B1 potential as the ratio between B1 and B2 cells in the PC (see Fig. 7A for representative analysis gates). Both in sublethally irradiated C57BL/6 and Rag1−/− hosts, the B1/B2 ratio of transferred kit CLPs was significantly stronger than that of CLPs and MPPs. The B1/B2 ratio of LSKF− cells appeared somewhat higher than that of CLPs and MPPs in both types of recipients, but this difference failed to reach statistical significance both using parametric and nonparametric tests (Fig. 7B, 7C). Similar to splenic B2 cells, Sca1 expression was higher on B1 cells derived from the kit−CLPs than from LSKF− cells (shown in Fig. 7D for Rag1−/− competitively repopulated with LSKF− cells and kit−CLPs). We also note that the B1/B2 ratio was similar in CLPs, MPPs, or LSKF− cells into sublethally irradiated C57BL/6 mice. Sca1 expression calculated as the ratio between the geometric mean of Sca1 fluorescence of kit−CLPs, CLPs, MPPs, or LSKF−-derived cells and that of host cells of the same phenotype in each recipient (n = 6; *significantly different from LSKF− cells; **significantly different from MPPs and LSKF− cells). E, Expression of Sca1 on donor-derived B lineage cells 3 wk after transfer 1500 CD45.1+ CLPs, kit−CLPs, or MPPs together with 1500 CD45.1+CD45.2+LSKF− cells into sublethally irradiated Rag1−/− mice. Sca1 expression calculated as the ratio between the geometric mean of Sca1 fluorescence of kit−CLPs, CLPs−, and MPPs-derived cells and that of LSKF−-derived cells of the same phenotype in each recipient (n = 3; *significantly different from LSKF− cells).

Discussion

We have shown here that progenitors in three distinct populations, CLPs, kit−CLPs, and LSKF− cells, display quantitatively different potentials to give rise to various types of mature B cells. The LSKF− fraction contains a B cell precursor that is functionally distinct from CLPs and kit−CLPs and gives rise in the short term to a FO-biased B cell development program. Alternatively, it is possible that the HSCs within this fraction give rise to such a B cell precursor in the short term. CLPs and kit−CLPs are more MZ-biased than LSKF− cells, in particular in conditions of lymphopenia, whereas kit−CLPs have the strongest B1 potential. Distinct progenitor populations give rise to a maximal output of mature B cells, such as B1 cells, at different time points, dependent on the overall maturation state of the respective progenitor populations (51). We observed, however, that all populations investigated in this study are capable of generating mature B cells within 3 wk of transfer and that the ratios between the types of mature B cell generated at this early time point differ remarkably. These qualitative differences in B cell potential are surprising. Harman et al. (22), for example, reported that each donor...
population (CLPs and Kit-CLPs) generated B cell subsets at comparable frequencies 3 mo after transfer into nonlymphopenic B6 hosts. However, a problem in the interpretation of later time points with respect to qualitative differences in B cell potential is that local homeostatic forces have had an opportunity to alter the fate of mature cells. It has been shown previously that small resting B cells can assume a MZ phenotype in conditions of lymphopenia. Furthermore, B1 cells can undergo homeostatic proliferation (23), and very likely, MZ B cells can do the same, as in conditions where B cell production has ceased because of conditional deletion of Rag1, MZ and B1 compartments are maintained (36). Therefore, any differences in the intrinsic potential of distinct progenitor populations may become more difficult to detect at later time points because of the effect of local homeostatic forces. Thus assessment of B cell potential at an early time point more likely reveals intrinsic differences in B cell potential. Another caveat in the interpretation of our data, and of any experiments probing the potential of progenitor populations in vivo, is that adoptive transfer into lymphopenic or conditioned hosts may not reflect the function and potential of these progenitors in steady state. However, we also observed differences of the MZ/FO ratio after adoptive transfer of Sca1+ and Sca1− iB cells into lymphocyte-replete, unconditioned hosts, which is as close to steady state as is currently possible.

The progeny of CLPs and Kit-CLPs does not only differ from that of LSKF− cells in terms of MZ/FO ratio, but also in Sca1 expression, as B cells derived from CLPs and Kit-CLPs express more Sca1 than those derived from the LSKF− fraction. MPPs share B cell differentiation characteristics with both LSKF− cells and CLPs and are therefore likely a heterogeneous transitional population with respect to early B cell development. Before the iB stage, however, Sca1 expression is similar in cells derived from CLPs, Kit− CLPs, MPPs, or LSKF− cells. Because Sca1 is induced by inflammatory cytokines (52), it is possible that Sca1 expression is a reflection of differential response of the cells to stress, either caused by irradiation or by lymphopenia. However, the adoptive transfer experiments of iB cells were performed in unconditioned, lymphocyte-replete host, arguing against this notion. As Sca1 is an activation marker (48), it is also possible that CLPs and Kit− CLPs generate intrinsically more “activated” B cells and are therefore more readily recruited into the MZ population, which have a more activated phenotype than FO B cells (25, 29). However, the absence of progenitor origin-dependent differences in LPS-induced proliferation, CD86 and MHC II expression, as well as in anti-IgM-induced Ca2+ flux suggest that the activation status of Sca1hi and Sca1lo B cells may be a reflection of lineal origin. The difference in Sca1 expression is too

**FIGURE 4.** Representative example of Sca1 expression. Histograms of Sca1 expression on donor-derived iB, T1, MZ, and FO B cells in Rag1<sup>−/−</sup> mice 3 wk after competitive co-transplantation of 1500 LSKF<sup>−</sup> cells with 1500 CLPs, Kit<sup>−</sup>CLPs, or MPPs (blue, LSKF<sup>−</sup>; red, Kit<sup>−</sup>CLP, CLP, or MPP; representative of three independent experiments).
transferred Sca1+ and Sca1− iB cells among donor-derived cells 4 days (in one of the four experiments, 6 days) after adoptive transfer of Sca1+ and Sca1− iB cells. The same phenotype (MZ or FO) in each recipient (Fig. 5). Transfer into CD45.1+ into unconditioned CD45.2+ C57BL/6 mice (Fig. 6). The observed variation in differentiation ratios among conditioned hosts, as demonstrated by the transplants of iB cells (see Fig. 6). The observed variation in differentiation ratios among CLPs, kit− CLPs and LSKF− cells, whereas the MZ/FO ratio of LSKF− derived cells was equally low despite varying degrees of B cell reconstitution by competing MPPs, CLPs, or kit− CLPs. Finally, the difference in MZ/FO ratio between both programs is also observed in unconditioned hosts, as demonstrated by the transplants of iB cells (see Fig. 6). The observed variation in differentiation ratios among CLPs, kit− CLPs and LSKF− cells must therefore be caused by cell intrinsic mechanisms.

If all B cells were derived from CLPs, then no matter which progenitor population upstream from CLPs (MPPs or LSKF− cells) is transferred in vivo, the various types of peripheral B cells should be recovered in similar ratios. The only expected difference would be a developmental delay of the earliest compared with the most differentiated population. Our observations do not fit this model, as we find that CLPs, kit− CLPs and LSKF− cells have intrinsically distinct potentials to generate mature B cells. We suggest a model where LSKF− B cell progenitors generate a FO-biased B cell population with a Sca1 expression at the lower end of the Sca1 distribution on mature B cells, whereas CLPs and kit− CLPs belong to a program that generates B cell populations with a stronger tendency to generate MZ B cells and with a Sca1 expression at the upper end of the Sca1 distribution on mature B cells. Importantly, CLP and kit− CLP-derived B cell development is more responsive to lymphopenia than LSKF− derived B cell development, further underscoring the functional distinction between both programs. As such, our findings have two major implications. First, this model differs from models of early hematopoietic differentiation where at each successive stage of differentiation, dichotomous cell fate choices lead to progressive specification of progenitors to a given lineage (1). This model was already challenged by the observations that erythroid and mega-karyocytic potential is lost before HSCs reach the MPP stage (2) and that T cell development independent of CLPs is possible (14–18). These controversies are not yet resolved, however (3, 10, 11). A second major implication of our observations is that progenitor cell origin adds another layer of control to B cell fate

**FIGURE 5.** Functional analysis of FO and MZ B cells according to progenitor origin. A, LPS-induced proliferation, measured by [3H]thymidine incorporation, of MZ and FO B cells generated from 1500 CLPs, kit− CLPs, and LSKF− cells 3 wk after transfer into Rag1−/− mice (n = 3 independent experiments, all differences between MZ and FO B cells; p < 0.05). B, Ca2+ flux, as measured by ratiometric analysis of Indo-1 fluorescence gated on FO and MZ B cells generated 3 wk after adoptive transfer of 1500 CD45.1+ CLPs (upper panel) and LSKF− cells (lower panel) into Rag1−/− mice. Wt CD45.2+ C57BL/6 spleen cells were added to the samples as an internal control and analyzed according to the same MZ/FO gates.

**FIGURE 6.** Potential of Sca1+ and Sca1− iB cells. A, Representative example of Sca1, IgM, and AA4.1 expression of sorted Sca1+ and Sca1− iB cells from the BM of C57BL/6 mice. More than 95% of the cells fell in the sort windows in each experiment. B, Representative example of the detection of donor cells 4 days after transfer of 5.105 CD45.1+ iB cells into an unconditioned CD45.2+ C57BL/6 host. C, Expression of CD21 and CD23 on donor-derived cells 4 days after adoptive transfer of and Sca1+ and Sca1− iB cells from congenic CD45.1+ mice into unconditioned CD45.2+ C57BL/6 mice. D, Fraction of MZ B cells among donor-derived cells 4 days (in one of the four experiments, 6 days) after adoptive transfer of Sca1+ and Sca1− iB cells from congenic CD45.1+ into unconditioned CD45.2+ C57BL/6 mice (n = 4; p = 0.006). E, Average Sca1 expression on FO and MZ B cells generated from adoptively transferred Sca1+ and Sca1− iB cells, calculated as the ratio between geometric mean of Sca1 fluorescence of donor-derived cells and that of host cells of the same phenotype (MZ or FO) in each recipient (n = 4).
FIGURE 7. B1 potential of progenitor populations. A, Representative analysis windows for the detection of B1 and B2 cells in the PC of transplant-ant cell mice (the example shown is a sublethally irradiated C57BL/6 host transplanted with kit+ CLPs). B, B1/B2 ratio in donor-derived cells in the PC 3 wk after transfer of 3000 CLPs, kit+ CLPs, MPPs, or LSKF− cells into sublethally irradiated C57BL/6 mice (n = 6; significantly different from CLPs and MPPs cells). C, B1/B2 ratio in donor-derived cells in the PC 3 weeks after transfer of 1500 CD45.1+ CLPs, kit+ CLPs, or MPPs together with 1500 CD45.1+CD45.2− LSKF− cells into sublethally irradiated Rag1−/− mice (n = 4; significantly different from CLPs and MPPs). D, Representative example of Sca1 expression on B1 cells derived from LSKF− cells and from kit+CLPs in a Rag1−/− recipient competitively transplanted with both populations. E, Nested DNA PCR for the rearrangement of Vµ11 and VµQ52 in B1 cells isolated by cell sorting from Rag1−/− mice after adoptive transfer of CLPs, kit+ CLPs, or LSKF− cells.

determination. Although Ig gene rearrangements are not yet completed at the CLP, kit+ CLP, MPP, or LSKF− stage, these observations do not rule out a role for BCR signaling (25, 26, 29–35), however, as the various differentiation programs may differ in the expression of proteins that regulate BCR signaling.

The lineage relation between kit+ CLPs and CLPs is unclear. Both populations generate B cells with elevated MZ/FO ratio and Sca1 expression and therefore likely belong to the same B cell differentiation program. However, in many respects, kit+ CLPs appear more “committed” to this program, because their MZ/FO ratio was higher than that of CLPs, at least in Rag1−/− mice, and as in C57BL/6 hosts, kit+ CLPs but not CLPs also generated Sca1+ iB cells. These findings, in addition to their lower proliferative capacity (21) would place kit+ CLPs downstream of CLPs. However, kit+ CLPs have a broader differentiation potential, because they can generate B1 cells more efficiently than do CLPs. Furthermore, kit+ CLPs are also characterized by lower expression of Rag genes (21, 22) compared with CLPs. As Rag genes are progressively upregulated during lymphoid commitment (7, 19, 20), these expression data would place kit+ CLPs upstream of CLPs. It is possible that CLPs and kit+ CLPs both originate from a progenitor within the MPP population and represent distinct, parallel progenitors within the same differentiation program.

In conclusion, the findings presented here indicate that progenitor origin contributes to cell fate choices of mature B cells and suggest that CLP-independent B cell development programs may exist.

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Disclosures

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References


