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Earliest hematopoietic progenitors at embryonic day 9 preferentially generate B-1 B cells rather than follicular B or marginal zone B cells

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ABSTRACT

The lymphoid potential of the hematopoietic system is observed as early as embryonic day 9 (E9) before transplantable hematopoietic stem cells (HSCs) appear at E11 in mice. However, it is largely unknown as to which cell fraction is responsible for the initial wave of lymphopoiesis and whether these earliest lymphocytes make any contributions to the adult lymphoid system. We previously isolated the earliest hematolymphoid progenitors at E9 that had $CD45^+$c-$Kit^+AA4.1^+$ phenotypes. In this study, the differentiation potency into B cell subsets of the E9 hematolymphoid progenitors was examined in detail. In culture, E9 hematolymphoid progenitors produced $B220^{-/low}$ B cell progenitors in striking contrast to adult BM c-Kit^+Sca-1^+Lin^- cells. Upon *in vivo* transplantation, B cell progenitors derived from E9 hematolymphoid progenitors preferentially differentiated into the B-1 B lymphocyte subset, whereas their differentiation into B-2 B lymphocyte subsets [follicular B (FoB), marginal zone B (MZB) cells] was inefficient. Of note, these donor B lymphocytes permanently repopulated in host mice, even if adult mice were used as recipients. These results suggest that B cell progenitors produced from an initial wave of definitive hematopoiesis before authentic HSCs appear could be a permanent source for, at least, the B-1 B lymphocyte subset.

1. Introduction

Hematopoietic stem cells (HSCs) are a subset of cells that are capable of self-renewal and differentiation into all mature blood cell lineages. In mice, HSCs are experimentally defined as cells that can repopulate within the body over a long term after their transfer into adults. During mouse ontogeny, the first HSCs appear at embryonic day 11(E11) in the fetal liver, placenta, aorta–gonad–mesonephros region, and yolk sac (YS) [1]. However, hematopoietic progenitors that can give rise to both lymphoid and myeloid lineage cells but lack adult-repopulating capability are present even before the appearance of HSCs.

Several studies, including our own, have demonstrated the presence of lymphomyeloid hematopoietic progenitors in E9.5 embryos and embryos prior to E9.5 [2–6]. Although it is not known whether these cells act as pre-HSCs or constitute only a transient cell population, it has been shown that E9 cells could repopulate mice when these cells were transferred into embryonic or fetal mice [2,4], which suggested that at least some of the cells at E9 functioned as pre-HSCs. In addition, recent studies using cell tracking techniques revealed that mature myeloid cells derived from hematopoietic cells produced before the appearance of HSC might be sustained in the adult [7,8].

With increasing age, HSCs change their characteristics, including their cell division kinetics, cell surface molecule expressions, cytokine dependency, and cell differentiation properties [9]. Regarding cell differentiation properties, fetal liver (FL) HSCs are more prone to generate myeloid cell lineages (especially erythroid lineage cells) as compared with the adult bone marrow (BM) HSC compartment [10]. Even within adult BM, HSCs shift to myeloid-biased differentiation with age, although this phenomenon can be partly explained by the selective expansion of myeloid-biased HSCs over non-biased HSCs [11].

Studies also showed the alternation in differentiation tendency within the lymphocyte subsets along with the developmental stage [12–15]. It is known that fetal and adult T progenitors have different propensities in their Vγ usage [12]. Both cell intrinsic and non-intrinsic mechanisms are likely to contribute to the generation of the difference [13]. Also, among B lineage cells, it is well known that fetal cells are a more potent source of B-1 cells that reside in the peritoneal and pleural cavities as compared with adult hematopoietic tissues [16–19], although BM and spleen contain

Abbreviations: E, embryonic day; FL, fetal liver; FoB, follicular B; MZB, marginal zone B; YS, yolk sac.

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B-1-specified progenitors [20,21] and stem/progenitor cells that have the capacity to generate B-1 B lymphocytes [22,23].

At the pre-FL stage, it is known that the AGM region, placenta, and YS contain B progenitors [3,4,6,24]. Although these foci contained the B cell source (especially B-1 B cells), it is not well known as to which cell compartments generate these B lymphocytes. Detailed analyses have not been performed regarding their differentiation tendency toward either B-1 B or conventional B cells (B-2 B cells). In this study, we carefully examined the lymphoid potentials of E9 hematolymphoid progenitors that we previously isolated and describe their unique B cell differentiation potential.

2. Materials and methods

2.1. Mice

C57BL/6JJcl mice were purchased from CREA Japan Inc. EGFP transgenic mice [C57BL/6-Tg (CAG-EGFP)] and BALB/cA-*Rag2*$^{-/-}$/*IL2Rγc*$^{-/-}$ mice, originally purchased from the Central Institute for Experimental Animals, were maintained in the Institute of Laboratory Animals of Mie University. All animal experiments were performed according to Mie University guidelines for laboratory animals.

2.2. Cell preparation from E9 embryos and adult bone marrow

The caudal halves of embryos proper and yolk sacs obtained from C57BL/6J females time mated with EGFP transgenic males were incubated with 1 mg/mL of collagenase (Wako) in Hanks' solution supplemented with 2% FBS for 30 min at 37 °C. After incubation, single cell suspensions prepared by pipetting were used for the experiments. Adult bone marrow cells were obtained by flushing femurs of 8- to 20-week-old EGFP transgenic female mice.

2.3. Cell sorting and analysis

All cell sorting experiments were performed on FACSAria (BD). Cells were analyzed with a FACSAria or FACSCanto II (BD). Data analysis was performed using the FlowJo software (Tree Star). The following antibodies were used for cell sorting and analysis: AA4.1 (eBioscience), B220 (RA3-6B2, BioLegend), CD4 (GK1.5, BioLegend), CD5 (53-7.3, eBioscience), CD8 (53-6.7, BioLegend), CD11b (M1/70, BioLegend), CD19 (6D5, BioLegend), CD21 (7E9, BioLegend), CD23 (B3B4, BioLegend), CD45 (30-F11, eBioscience), c-Kit (2B8, eBioscience), Gr-1 (RB6-8C5), IgD (11-26c.2a, BioLegend), IgM (II/41, eBioscience), Sca-1 (E13-161.7, BioLegend), and Ter119 (BioLegend).

2.4. Cell culture

For B-cell induction, cells were cultured on OP9 stromal cells in RPMI-1640 containing 5% FBS and 0.05 mM 2ME in the presence of 10 ng/mL rmIL-7 (Miltenyi) [25]. Cells were cultured for 2 weeks and then used for transplantation.

2.5. Transplantation

GFP-labeled B cell progenitors were retroorbitally injected into unconditioned 4 to 8-week-old *Rag2*$^{-/-}$/*IL2Rγc*$^{-/-}$ mice [26].

2.6. Elisa

For a sandwich mouse IgM ELISA, Nunc-Immuno plates (MaxiSorp) were coated with capture antibodies (R6-60.2, BD). After incubation with serially diluted serum samples, the plates were incubated with biotin-conjugated II/41 monoclonal antibodies (eBioscience), and then streptavidin-conjugated horseradish peroxidase (BD). A SureBlue TMB Microwell Peroxidase substrate (KPL) was used for detection. After adding a stop solution, serum IgM levels were determined by end point assays at a wavelength of 450 nm.

2.7. Rosette formation assay

To prepare bromelain-treated mouse red blood cells (RBCs), RBCs from the peripheral blood of C57BL/6 mice at a concentration of 5×10^8/mL were incubated with 10 mg/mL of bromelain (Sigma) for one hour at 37 °C. After washing three times in Hanks' solution supplemented with 2% FBS, bromelain-treated or untreated RBCs (5×10^6) were incubated with 5×10^3 FACS-sorted donor-derived B-1 B cells in 0.25 mL of Hanks' solution supplemented with 2% FBS for 15 min at 4 °C. Images were captured using an inverted microscope (Olympus IX71) equipped with an Olympus DP71 camera.

3. Results

In E9.5 embryos, cells with the CD45$^+$c-Kit$^+$AA4.1$^+$ cell surface phenotype possess the capacity for lymphoid and myeloid differentiation [6]. It was previously shown that E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cells gave rise to αβ and γδ T lymphocytes *in vitro* in an unbiased manner comparable to the adult bone marrow (BM) c-Kit$^+$Sca-1$^+$Lin$^-$ stem cell compartment [6]. However, the differentiation capability of E9.5 hematolymphoid progenitors into distinct B lymphocyte subsets was not examined in detail. Thus, we sorted E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cells, cultured them on stromal cell lines under lymphocyte culture conditions, and evaluated their descendants for their cell surface phenotypes by flow cytometry. As shown in Supplemental Fig. 1, E9.5 hematolymphoid progenitors efficiently produced CD19$^+$ B lymphocytes and proceeded to the IgM$^+$ stage comparable to adult BM c-Kit$^+$Sca-1$^+$Lin$^-$ cells. Interestingly, E9.5 hematolymphoid progenitors preferentially produced B lineage cells in which the expression of B220, a pan-B lymphocyte marker, was negative to low, although adult BM progenitors produced B220^{high} B lymphocytes. Culture on distinct stromal cells (OP9 and ST2 cells) gave the same results.

It has been known that hematopoietic cells in E9.5 embryos and in embryos prior to E9.5 do not repopulate mice if standard intravascular (i.v.) cell transfer is used. However, it was reported that direct injection of E9.5 cells into busulfan-conditioned neonatal liver allowed donor cells to repopulate [4]. Another report demonstrated that engraftment of the paraaortic splanchnopleura under the kidney capsules of adult mice, before overt hematopoietic differentiation occurred, allowed the development of B cells in host mice [3]. Even isolated hematolymphoid progenitors do not engraft adult and neonatal mice if standard i.v. injection is used [6]. To overcome this engraftment capability problem, we investigated whether transplanting B progenitors that had been produced in culture would allow us to determine their *in vivo* contributions to B lymphocyte subsets.

E9.5 hematolymphoid progenitors isolated from the YS or caudal halves of embryo propers were cultured for 2 weeks. Whole cells in culture were then injected into adult *Rag2*/*IL2Rγc* deficient mice. This method resulted in the reconstitution of B lymphocyte subsets and helped to overcome the engraftment problem of E9-derived committed hematopoietic cells into adult host mice. Besides the constant B lineage reconstitution, donor-derived T lymphocytes were sporadically observed in approximately 20% mice analyzed, suggesting that some T lymphocyte progenitors were maintained in stromal cell cultures. GFP was used to detect

donor cells throughout the experiments. In some donor cells the GFP transgene was silenced, but there were no differences in the readout patterns between GFP$^+$ cells and GFP-silenced cells in all of these experiments. E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cell-derived B cells repopulated the spleen and peritoneal cavity (Fig. 1).

In the spleens of 8 of the 12 mice that were transplanted with E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cell-derived B progenitors, no CD23$^+$CD21^{mid}-IgM^{low} follicular (Fo) B cells or CD23$^-$CD21^{high}IgM^{high} marginal zone (MZ) B cells were produced (Fig. 1A, Table 1, Supplemental Table 1). In these mice, the cells that repopulated the spleen had relatively higher surface IgM expression and were positive for CD5 (Fig. 1A). Therefore, these were B-1 lineage cells. In 4 of the 12 mice that were transplanted with E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cell-derived B progenitors, these cells contributed to B-2 B lineage cells (Fo and MZ B lymphocyte subsets) in the spleen (Table 1, Fig. 2, Supplemental Table 1). Three of these 4 mice had both Fo and MZ B cells, and only one mouse had Fo B cells alone (Table 1).

In the peritoneal cavity, E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cell-derived B progenitors repopulated the CD23$^-$IgM^{high} B-1 B subset in all mice analyzed (12 mice total), but rarely contributed to the CD23$^+$IgM^{low} B-2 B subset (4 of 12 mice) in the peritoneal cavity (Fig. 1B). CD23$^-$IgM^{high} B-1 B lymphocytes possessed the IgD^{low}B220^{low}-Mac1$^+$ cell surface phenotype, which showed that these cells were typical B-1 B lymphocytes (Fig. 1C). The majority of cells within the

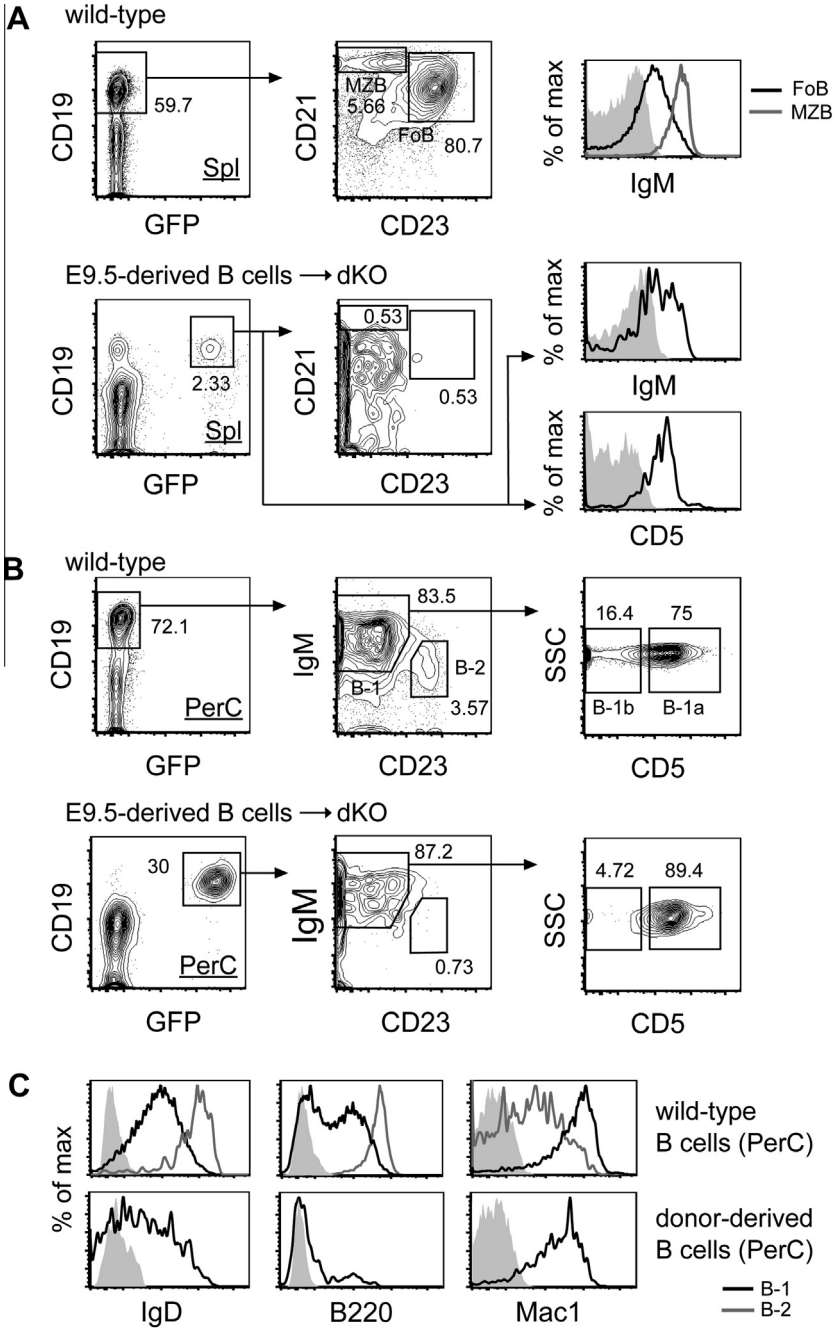

Fig. 1. Preferential generation of B-1 B lymphocytes from E9 hematolymphoid progenitors. B cell progenitors produced *in vitro* from E9 hematolymphoid progenitors were transplanted into *Rag2*$^{-/-}$/*IL2Rγc*$^{-/-}$ (dKO) mice. B cell fractions in spleen (Spl) (A) and peritoneal cavity (PerC) (B and C) were analyzed. B cell fractions in wild-type mice are shown for comparisons (top panels in A–C). Numbers indicate the percentages within the parent population. Grey shadows in histograms are staining controls. Representative results for 8 mice (of the 12 total mice analyzed) that were repopulated only with B-1 B lineage cells are shown. Plots are the results obtained for mice that were transplanted with B progenitors from YS hematolymphoid progenitors at 5 months post-transplantation.

Table 1
Repopulation of B cell subsets after transplantation of E9.5-derived B progenitors.

Cell source	No. cells transplanted	Weeks post-transplant	Repopulation of B cell subsets (repopulated mice/total mice analyzed)		
			B-1	FoB	MZB
E9.5	5×10^5	21	2/2	1/2	0/2
CH[a] KA45[b]	1×10^6	3–28	4/4	0/4	0/4
E9.5	1×10^6	12	1/1	1/1	1/1
YS KA45	2×10^6	23	2/2	1/2	1/2
	7×10^6	23	1/1	0/1	0/1
	1×10^7	6–16	2/2	1/2	1/2
Unfractionated	5×10^5	10	2/2	0/2	ND
E9.5 YS	7×10^5	13	1/1	0/1	0/1
	1×10^6	11–12	2/2	1/2	1/2

[a] CH: caudal halves of the embryos proper.
[b] KA45: $CD45^+$c-$Kit^+AA4.1^+$ cells.

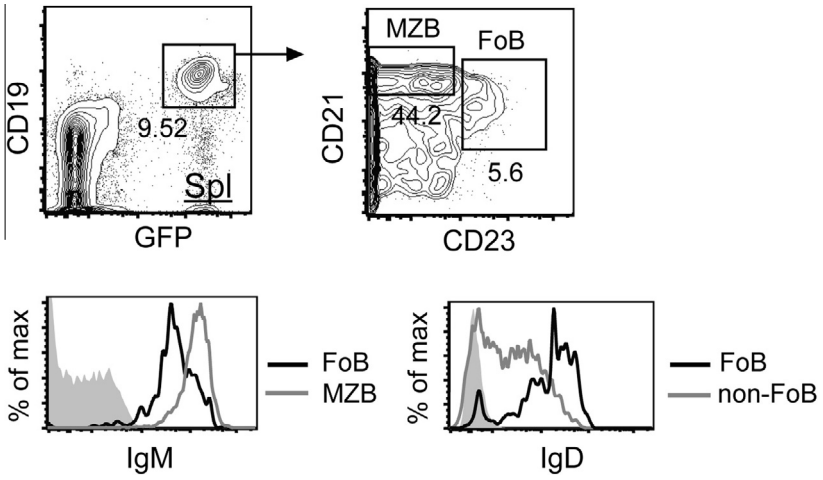

Fig. 2. Generation of B-2 B lymphocytes from E9 hematolymphoid progenitors. B cell progenitors produced *in vitro* from E9 hematolymphoid progenitors were transplanted into *Rag2*$^{-/-}$/*IL2Rγc*$^{-/-}$ mice. Representative results for 4 mice (of 12 total mice analyzed) that were repopulated with B-2 B lineage cells are shown. Plots are the results obtained for the spleen (spl) from a mouse at 12 weeks post-transplantation of B progenitors from YS hematolymphoid progenitors. Numbers indicate the percentages within the parent population. Grey shadows in histograms are staining controls.

B-1 B subset were $CD5^+$ B-1a cells and some B-1 cells were $CD5^-$ B-1b cells (Fig. 1B). Eight mice that lacked B-2 subsets in the peritoneal cavity also lacked spleen B-2 B lymphocytes (Fo B and MZ B subsets).

In contrast to these results, B lymphocytes produced from the E13.5 FL and adult BM stem cell compartments (c-Kit^+Sca-1^+Lin^- cells) repopulated Fo B and MZ B lymphocytes in the spleen and B-1a, B-1b, and B-2 B lymphocytes in the peritoneal cavity in all mice analyzed (FL, $n = 3$; BM, $n = 4$) using the same protocol (Fig. 3, Table 2, Supplemental Fig. 2, Supplemental Table 1), Therefore, E9.5 hematolymphoid progenitors had a strongly biased differentiation tendency toward the B-1 B lymphocyte subset compared to the FL and BM stem cell compartments. These results also indicated that it was unlikely that B-2 B lymphocyte progenitors were selectively lost during cell culture on OP9 stromal cells. Because B-2 B lymphocytes persist in the lymphoid tissues over a year in the absence of B cell influx from the bone marrow [27,28], it was also unlikely that these cell subsets were lost over time in mice. Indeed, only one of 3 mice that were transplanted with E9.5 $CD45^+$c-$Kit^+AA4.1^+$ cell-derived B progenitors had B-2 B lineage cells when mice were analyzed relatively earlier at 3–6 weeks post-transplantation.

When isolated from either the caudal half of the embryo proper or from the YS, E9.5 $CD45^+$c-$Kit^+AA4.1^+$ cells preferentially produced B-1 B lymphocytes (Table 1). Therefore, this tendency was common to E9.5 hematolymphoid progenitors regardless of their tissue origin. To examine whether this was a property restricted to $CD45^+$c-$Kit^+AA4.1^+$ cells and was not shared with possible alternative sources of lymphocytes at E9.5 (more immature compartments or minor subsets of cells), B lymphocytes produced from whole unfractionated yolk sac cells were tested for their capability to differentiate into B lymphocyte subsets. As shown in Table 1, unfractionated cells showed the same propensity. Therefore, this was a property of the entire B cell source of E9.5. Repopulated B cells were maintained in mice for a long-term period of >20 weeks, which indicated that B-1 B cells derived from E9.5 hematolymphoid progenitors could be sustained throughout a lifetime (Table 1).

Finally, we examined whether the B-1 B cells produced from E9.5 hematolymphoid progenitors were functional. Serum samples were obtained from the peripheral blood of transplanted mice and assayed for the presence of natural IgM antibodies. Mice repopulated by E9.5 $CD45^+$c-$Kit^+AA4.1^+$ cell-derived B progenitors contained natural antibodies in the peripheral blood comparable to wild-type mice or mice repopulated by BM-derived B progenitors (Fig. 4A). Because B-1 B lymphocytes are known to be the predominant producers of natural antibodies against phosphatidylcholine and as many as 5–15% of B-1 B lymphocytes react to phosphatidylcholine [29,30], we also examined whether B-1 B cells produced from E9.5 $CD45^+$c-$Kit^+AA4.1^+$ cells had this antigen specificity using a rosette formation assay.

In this assay, B-1 B cells with specificity for phosphatidylcholine aggregate around bromelain-treated mouse red blood cells with this antigen exposed on their surfaces and form rosettes. As shown

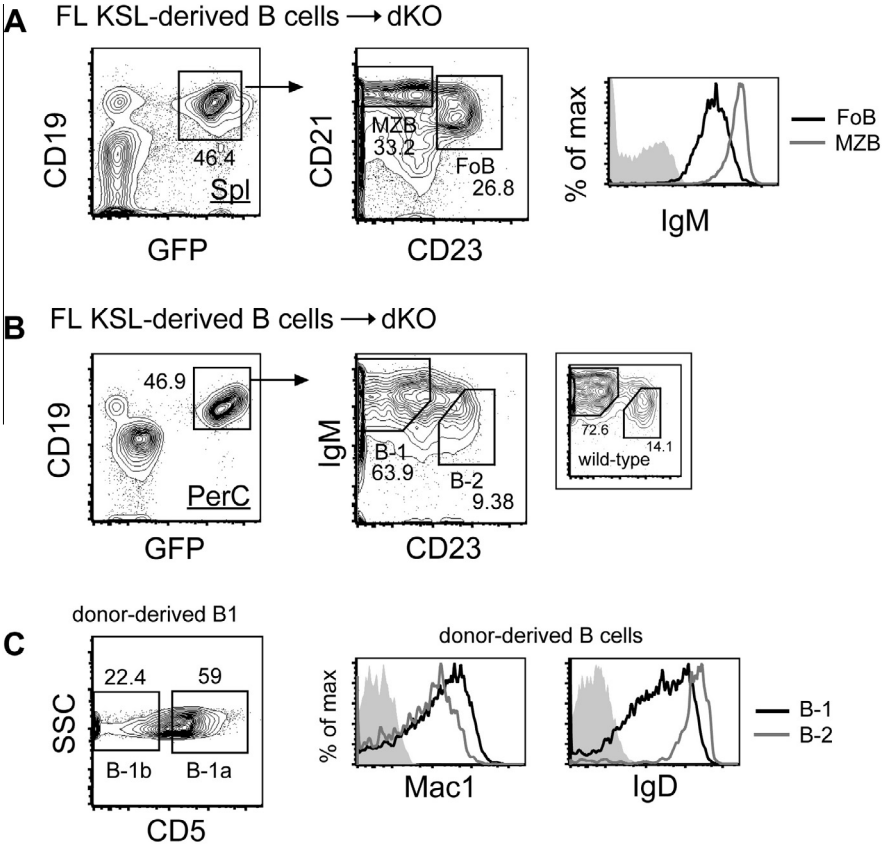

Fig. 3. FL stem cell compartment generates B-1 and B-2 B lymphocytes. B progenitors produced *in vitro* from E13.5 FL c-Kit$^+$Sca-1$^+$Lin$^-$ cells were transplanted into *Rag2*$^{-/-}$/*IL2Rγc*$^{-/-}$ (dKO) mice. B cell fractions in spleen (Spl) (A) and peritoneal cavity (PerC) (B and C) were analyzed. B cell fractions in wild-type mice are shown for comparisons at right for CD23/IgM (B). Numbers indicate the percentages within the parent population. Grey shadows in histograms are staining controls. Representative results obtained for 3 mice are shown. Plots are the results obtained for a mouse that had been transplanted with B progenitors from E13.5 FL c-Kit$^+$Sca-1$^+$Lin$^-$ cells at 11 weeks post-transplantation.

Table 2
Repopulation of B cell subsets after transplantation of fetal liver and adult bone marrow c-Kit$^+$Sca-1$^+$Lin$^-$ cell-derived B progenitors.

cell source	No. cells transplanted	Weeks post-transplant	Repopulation of B cell subsets (repopulated mice/total mice analyzed)		
			B-1	FoB	MZB
E13.5	5×10^5	8–11	2/2	2/2	2/2
FL KSL[a]	2×10^6	11	1/1	1/1	1/1
Adult	6×10^5	14	2/2	2/2	2/2
BM KSL	6×10^6	13	2/2	2/2	2/2

[a] KSL: c-Kit$^+$Sca-1$^+$Lin$^-$ cells.

in Fig. 4B and C, B–1 B cells isolated from mice repopulated with E9.5-derived B progenitors exhibited reactivity with bromelain-treated mouse red blood cells, but not with untreated red blood cells. This demonstrated that B-1 B cells derived from E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cells had normal B-1 B cell function.

4. Discussion

Although it has been shown that E9 cells could be the source of B-1 B cells, it is unknown as to which cell fraction are responsible for the B-1 B cell population. Godin et al. showed that transplanting the splanchnopleura of the lateral plate mesoderm from E8.5-E9 embryos under the kidney capsule provided for the development of B-1 B lymphocytes [3]. However, it is likely that the source of B-1 B lymphocytes was uncommitted mesodermal cells and their descendants were unknown because the budding of hematopoietic cells occurs later in the tissues that descend from this region.

Yoshimoto et al. showed that injecting cells from E9 into the peritoneal cavities of neonate mice allowed these donor cells to contribute to the B-1 and MZ B cell subsets [31]. This report also showed that VE-cadherin$^+$ endothelial cells, but not CD41$^+$ hematopoietic cells, could have been the source of B cells. This conflicted with our data in which we demonstrated that the committed definitive hematopoietic cells (CD45$^+$c-Kit$^+$AA4.1$^+$) that were also positive for CD41 were the source of B lymphocytes [6]. Because CD41 is widely expressed, including the progenitors of primitive erythrocytes, and CD45$^+$c-Kit$^+$AA4.1$^+$ cells represent a subset of the CD41$^+$ fraction, their report might have underestimated the differentiation potential of CD41$^+$ cell populations. Before arriving at a conclusion, it is necessary to carefully determine whether VE-cadherin$^+$ cells and CD45$^+$c-Kit$^+$AA4.1$^+$ cells are developmentally related or are exclusive cell populations.

Although the authors showed that E9 cells lacked the differentiation potential for Fo B cells, but possessed that for MZ B cells, we observed some minor contributions to Fo B lymphocytes along

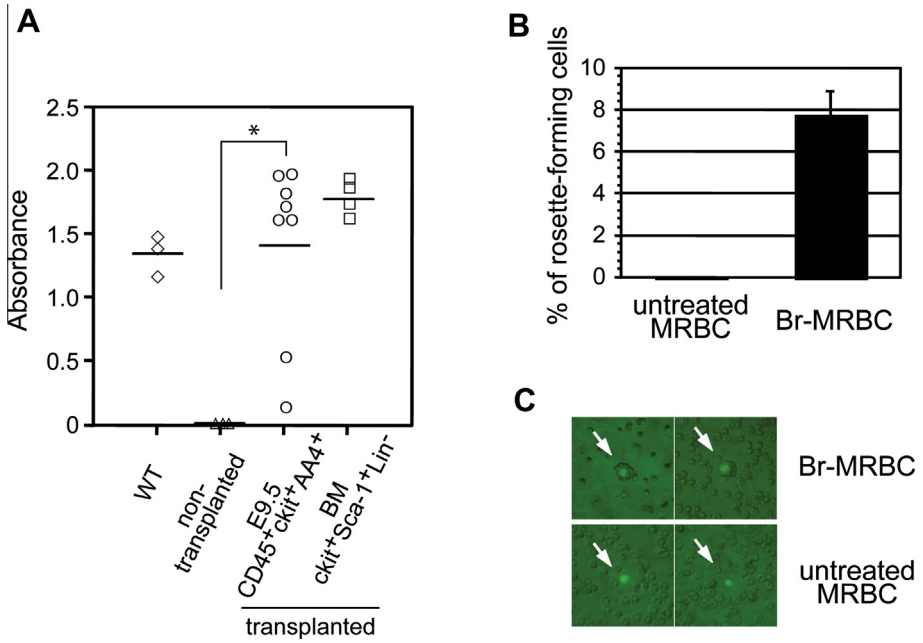

Fig. 4. E9 hematolymphoid progenitors generate functional B-1 B lymphocytes. (A) Peripheral blood samples obtained from mice repopulated with E9 CD45$^+$c-Kit$^+$AA4.1$^+$ cell-derived (n = 8) and BM c-Kit$^+$Sca-1$^+$Lin$^-$ cell-derived (n = 4) B lymphocytes were assayed for serum IgM levels by ELISA. Results obtained for mice that had >1% donor chimerism are shown. As controls, serum IgM levels in wild-type mice (n = 3) and non-transplanted *Rag2*$^{-/-}$/*IL2R*γ*c*$^{-/-}$ mice (n = 3) were determined. Representative results of two independent experiments using the same samples are shown. $^*p$ = 0.008. (B and C) B-1 B cells isolated from mice repopulated with E9.5 CD45$^+$c-Kit$^+$AA4.1$^+$ cell-derived B progenitors (GFP-labeled) were incubated with bromelain-treated mouse red blood cells (Br-MRBCs) or untreated MRBCs. The fraction of rosette-forming cells among GFP$^+$ cells was determined by counting 100 GFP$^+$ cells in each well (B). Results are means ± S.D.s of triplicate wells. Representative results of two independent experiments are shown. Photomicrographs in (C) show representative images of isolated GFP-labeled B-1 B cells that formed rosettes with Br-MRBCs (arrows in upper panels). GFP-labeled B-1 B cells did not form rosettes with untreated MRBCs (arrows in lower panels).

with MZ B cells. In adult B lymphopoiesis, Fo B and MZ B lymphocytes are considered to share common precursor cells. Consistent with this model, in this study Fo B and MZ B cells appeared together in most cases. Therefore, it was likely that there was a common precursor stage for Fo B and MZ B cells, at least with our experimental set-up. Because it was not shown in the report how frequently the MZ B cell subset was observed compared to B-1 B cells, it is difficult to judge whether E9 cells repopulated MZ B cells comparable to B-1 B cells. Further studies are required to determine whether E9 mice contain heterogeneous cell populations or whether the resulting pattern of repopulation in the report was generated by incomplete engraftment into Fo B cells under distinct experimental settings.

In any event, our data demonstrated that B cell progenitors derived from the earliest committed hematolymphoid progenitors at E9 had a strong bias toward differentiation into B-1 B lymphocytes, and the differentiation into B-2 B lymphocyte subsets (Fo B and MZ B) was not efficient compared with that of the FL and BM stem cell fractions. Because FL cells are more prone to generate the B-1 B cell subset as compared to adult BM cells [17–19], multipotent hematopoietic stem/progenitor cells might gradually change their cell intrinsic developmental potential from being B-1 B-biased to being B-2 B-biased as the developmental stages progress. This suggests that the characteristics of multipotent stem/progenitor cells change in a multi-step manner rather than a simple switch from fetal- to adult-type cells [32].

Although the direct injection of the CD45$^+$c-Kit$^+$AA4.1$^+$ cells into adult mice do not allow the repopulation, the descendant B cell progenitors repopulated adult host mice. This indicated that lineage-restricted progenitors might repopulate adult host mice without the engraftment capability problem and suggested that the engraftment problem into adult mice could be attributed to the homing capability of multipotent progenitors into adult host environments. If the committed progenitors of other hematopoietic cell lineages could repopulate adult mice using this method, it would become possible to determine the complete differentiation capability of E9 hematopoietic progenitors. This is an important question that needs to be examined to clarify the contributions of hematopoietic cells produced before the appearance of HSCs into adult blood cell types.

Because the B lymphocytes derived from CD45$^+$c-Kit$^+$AA4.1$^+$ cells, which are the earliest definitive hematopoietic progenitors at E9, could repopulate adult host mice over a long term, E9 hematopoietic progenitors could be the source for, at least, the adult B-1 B lymphocyte pool. Further studies are needed to determine the contributions of E9 definitive hematopoietic progenitors, FL stem cells, and B-1-restricted BM and splenic progenitors to the adult B-1 B lymphocyte pool.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.073.

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