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A checkpoint in B-lymphopoiesis related to Notch resistance

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ABSTRACT

While murine B- and T-lymphopoiesis require overlapping molecules, they occur in separate organs: the bone marrow (BM) and the thymus, respectively. The BM microenvironment is incapable of supporting T-lymphopoiesis because of insufficient interactions of Notch1 with delta-like ligand (Dll). Notch1/Dll interactions also play a role in the suppression of B-lymphopoiesis in the thymus. However, it is still unclear whether the Notch1/Dll interaction alone explains why the thymus does not support B-lymphopoiesis. In this study, we compared the precursor population colonizing the thymus with that in the BM by culturing them on stromal cells expressing abundant Dll1. We demonstrated that Flt3$^+$ Il7r$^+$ B220$^+$ Cd19$^+$ BM cells gave rise to B cells under this condition. We defined them as resistant to Dll1. In the thymus, Dll1-resistant cells were undetectable. This suggested that the absence of Dll1-resistant cells might explain the absence of B-lymphopoiesis in the thymus.

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1. Introduction

Studies with gene knockout or mutant mice revealed that the developments of B cells and T cells are regulated by overlapping molecules: Cxcl12, interleukin 7 (Il7), Flt3 ligand (Flt3l) and Kit ligand (Kitl) [1]. Nevertheless, B- and T-lymphopoiesis take place in separate organs: the bone marrow (BM) and the thymus, respectively.

Microenvironments structured in the BM are incapable of supporting T-lymphopoiesis. The reason for this is that interactions of Notch1 with delta-like ligands (Dlls), Dll1 or Dll4, are indispensable for the development of T cells [2–8]. Notch1/Dll interactions are infrequent in the BM. Mice reconstituted with BM cells carrying a constitutively active form of *Notch1* display development of Cd4$^+$ Cd8a$^+$ cells in the BM [2]. On the other hand, Notch1/Dll interactions also explain why the thymus does not support B-lymphopoiesis. Conditional knockout of *Notch1* or *Dll4* in the thymus leads to intrathymic B-lymphopoiesis, suggesting that Notch1/Dll interactions suppress the development of B cells [3–7]. Multipotent hematopoietic precursor generates T cells instead of B cells in the presence of abundant Dlls [8]. In Cd19$^+$ BM cells, active form of *Notch1* or Dll1 induces degradation of Tcf3/E2A and Stat5, which are involved in B-lymphopoiesis [9–12].

It is well documented that the Notch1/Dll interactions distinguish the thymic microenvironment from that of the BM. However, features of the hematopoietic precursor population colonizing the thymus are not fully understood. In this study, we assessed whether the differences of the precursors in the BM and the thymus explain why B-lymphopoiesis does not occur in the thymus.

We cultured freshly isolated BM cells and thymocytes from C57BL/6 mice on OP9 stromal cells forced to express Dll1 (OP9-DL1) [8]. It was revealed that Flt3$^+$ Il7r$^+$ B220$^+$ Cd19$^+$ BM cells gave rise to B cells even in the presence of abundant Dll1. We defined them as B cell precursors resistant to Dll1. While precursors possessing the potential for B-lymphopoiesis and B220$^+$ Cd19$^+$ IgM$^+$ cells were confirmed, Dll1-resistant cells were undetectable in the thymus. The absence of Dll1-resistant cells and the Notch1/Dll interactions might in combination explain why the thymus hardly supports B-lymphopoiesis.

2. Materials and methods

2.1. Mice

C57BL/6 (Japan CLEA, Yokohama, Japan) and a transgenic mouse strain, C57BL/6-Tg(IghelMD4)4Ccg/J (hereafter referred to as MD4) [13], at 3–10 weeks of age were used for the experiments in accordance with the guidelines of the Animal Care and Use Committee of Tottori University. Littermates negative for *IghelMD4* (hereafter referred to as non-tg) were used as control for MD4 mice. MD4 mice were kindly provided by Drs. T. Kurosaki (iFReC, Osaka Univ.) and M. Hikida (Kyoto Univ.) with the permission of Dr. C.C. Goodnow (Australia National Univ.).

2.2. Cell lines

Stromal cell lines, OP9-DL1 and control OP9 (OP9-ctrl), were maintained as monolayers in MEM-alpha medium (Gibco-BRL,

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Grand Island, NY) supplemented with 20% FBS (Gibco-BRL) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin [Meiji Chemical, Tokyo, Japan]) in accordance with previous reports [8,14]. OP9-DL1 and OP9-ctrl were kindly provided by Dr. Y. Takahama (Tokushima Univ.) with the permission of Dr. J.C. Zúñiga-Pflücker (Toronto Univ.).

2.3. Reagents

Hen-egg-lysozyme (Sigma–Aldrich, St. Louis, MO) was conjugated with biotin and used for the detection of IgM and IgD encoded by *IghelMD4*. Recombinant mouse Il7 was purified from culture supernatant of CHO cells engineered to produce it [15]. Monoclonal antibodies used in this study are listed in Supplemental Table 4.

2.4. Cell separations (removal and enrichment) and cell sorting

Separations of surface molecule-expressing cells were performed with the MACS® system in accordance with the instructions of Miltenyi Biotec Inc. (Auburn, CA). Cells that passed through the column were pooled as a removed fraction, and bound cells were collected as an enriched fraction. $Cd19^+$ cells and $Cd19^-$cells in the $Flt3^+$ cell-enriched fraction were purified by cell sorting with EPICS-ALTRA or MoFlo™ XPD (Beckman Coulter, Inc., Hialeah, FL).

2.5. Cultures on OP9-ctrl or OP9-DL1

OP9-ctrl or OP9-DL1 was supplied on 6-well plates (Corning Inc., Corning, NY), 2 days prior to culturing. Freshly isolated BM cells or BM cells separated using MACS® column were seeded on OP9-ctrl or OP9-DL1 at a density of 10^4 cells/well. Thymocytes were cultured at a density of 10^6 cells/well. $Flt3^+$ $Cd19^+$ cells or $Flt3^+$ $Cd19^-$ cells were seeded on OP9-ctrl or OP9-DL1 at a density of 5×10^3 cells/well. Co-cultures were continued for 14 days in MEM-alpha medium supplemented with 20% FBS, antibiotics, Il7 (10 U/ml) and 2-ME (50 μM) (Wako, Osaka, Japan).

2.6. Flow cytometry

Flow cytometry was performed on EPICS-XL (Beckman Coulter, Inc.). Dead cells were excluded by staining with propidium iodide (Sigma–Aldrich). We distinguished OP9 cells from cells derived from cultured BM cells or thymocytes by their forward and side scatter profiles.

3. Results

3.1. Definitions of B cells and T cells in culturing on OP9-ctrl or OP9-DL1

First, we defined B cells and T cells in our cultures (Fig. 1A left and B right). DNA chip assay revealed that OP9-ctrl and OP9-DL1 similarly expressed mRNA encoding Kitl, Flt3l and Notch ligands other than Dll1 (Supplemental Table 1) [16,17]. Il7 was supplemented at 10 U/ml throughout the culturing.

Freshly isolated BM cells (10^4 cells/well) were cultured on OP9-ctrl for 14 days. On average, 86.5% (101.0×10^4 cells) of cells recovered from this culture were $B220^+$ $Cd19^+$ $Cd24a^+$ Kit^- cells (Fig. 1A left, C and D, Supplemental Fig. 1A). Some of them expressed IgM on their surface (surface IgM [sIgM]) (Supplemental Fig. 1A). T cells were induced from thymocytes (10^6 cells/well) on OP9-DL1. $Thy1.2^+$ $Cd44^+$ cells, $Thy1.2^+$ $Cd25^+$ cells and $Thy1.2^+$ $Cd4^+$ $Cd8a^+$ cells were detected in this culture (Fig. 1B right, Supplemental Fig. 1B). We defined $B220^+$ $Cd19^+$ cells as B-lineage cells and $Thy1.2^+$ cells as T-lineage cells in this study.

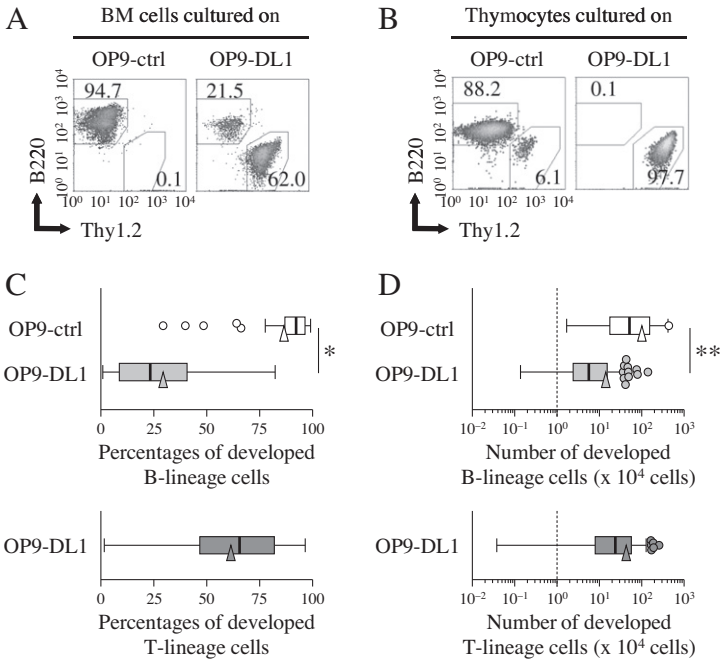

Fig. 1. Development of B-lineage cells from BM cells in the presence of abundant Dll1. Freshly isolated (A, C, D) BM cells and (B) thymocytes were cultured on OP9-ctrl or OP9-DL1. After 14 days, expressions of B220 and Thy1.2 on recovered cells were analyzed by flow cytometry. (A and B) Expressions of B220 and Thy1.2 on recovered cells from cultures of (A) BM cells or (B) thymocytes were evaluated by flow cytometry. Numbers in the plots show percentages of cells within each region ($B220^+$ or $Thy1.2^+$ region). Representative data are shown. (C and D) (C) Percentages and (D) numbers of B- (upper) and T-lineage cells (lower) recovered from cultures of BM cells on OP9-ctrl ($n = 37$) or OP9-DL1 ($n = 81$) are shown in the box-and-whisker plots. Outliers are presented by circles. Triangles in the plots are placed at the mean values. *p-value = 4.1×10^{-24}, **p-value = 4.8×10^{-10}.

3.2. BM cells give rise to B cells in the presence of abundant Dll1

To evaluate the populations of B and T cell precursors colonizing the BM and the thymus, cultures of thymocytes on OP9-ctrl (10^6 cells/well) and BM cells on OP9-DL1 (10^4 cells/well) were performed. Thymocytes gave rise to B-lineage cells at low frequency on OP9-ctrl (Fig. 1B left). This indicated the potential for B-lymphopoiesis in the thymus [18]. On OP9-DL1, BM cells predominantly developed to T-lineage cells (61.4%, 43.0×10^4 cells) (Fig. 1A right, C and D, Supplemental Fig. 1C). Interestingly, B-lineage cells were also recovered from this culture (29.4%, 14.1×10^4 cells) (Fig. 1A right, C and D Supplemental Fig. 1C).

Notch1 signaling is known to repress the transcriptions of genes related to B-lymphopoiesis: *Pax5*, *Ebf1* and so on [19]. Dll1 induces the degradation of Tcf3/E2A and Stat5 in Cd19$^+$ BM cells [9,10]. This suggested that populations in the BM contained B cell precursors resistant to the inhibitory effect of Dll1: Dll1-resistant B cell precursors (Dll1^R-Bps).

3.3. Dll1^R-Bps are Il7r$^+$ B220$^+$ Cd19$^+$ BM cells

To characterize Dll1^R-Bp, we evaluated expressions of five markers on its cell surface [20]: B220 [21], Cd19 [22], Il7r [23], Flt3 [24] and glutamyl aminopeptidase (hereafter referred to as Bp-1) [25]. We assessed whether Dll1^R-Bps were depleted by removing each molecule associated with BM cells. Numbers of developed B- and T-lineage cells from each removed fraction were calculated (Supplemental Tables 2 and 3).

While the fraction from which B220$^+$ cells were removed (B220$^+$ cell-removed fraction) developed to B-lineage cells on OP9-ctrl, this fraction failed to generate B-lineage cells on OP9-DL1 (Fig. 2). This indicated that Dll1^R-Bps were depleted by removing B220$^+$ cells. Enriched B220$^+$ cells (B220$^+$ cell-enriched fraction) predominantly gave rise to B-lineage cells on OP9-DL1 (Fig. 2, Supplemental Tables 2 and 3). Similar results were obtained when we separated BM cells on the basis of the expression of Cd19 or Il7r (Supplemental Tables 2 and 3). These results indicated that Dll1^R-Bps were Il7r$^+$ B220$^+$ Cd19$^+$ BM cells.

3.4. Most Dll1^R-Bps are depleted by removing Flt3$^+$ BM cells

In 3 of 4 independent experiments, Dll1^R-Bps were completely depleted by removing Flt3$^+$ cells (Supplemental Table 2). In one experiment, 0.9×10^4 B-lineage cells developed from the Flt3$^+$ cell-removed fraction (Supplemental Table 2). The Flt3$^+$ cell-enriched fraction efficiently gave rise to B-lineage cells on OP9-DL1 (Supplemental Table 2). These data suggested that Dll1^R-Bps were mostly depleted by removing Flt3$^+$ cells, and pooled in the enriched fraction.

Flt3 is reported to be repressed by Pax5, which promotes the transcription of *Cd19* [26,27]. This suggested that Dll1^R-Bps might express Flt3 at low intensity. In pre-cultured Flt3$^+$ cell-enriched fractions, B220$^+$ cells and Cd19$^+$ cells were concentrated (Supplemental Fig. 2A and B). CFU-Il7s consisting of Il7r$^+$ B220$^+$ Cd19$^+$ cells were more frequent in the Flt3$^+$ cell-enriched fraction than the B220$^+$ cell- or Cd19$^+$ cell-enriched fraction (Supplemental Fig. 2C) [28,29]. About 3% of B220$^+$ cells apparently expressed Flt3, and the remainder expressed Flt3 at low intensity (Supplemental Fig. 2D). Dll1^R-Bps should consist of extensive Flt3$^+$ cells and few or only a minority of Flt3$^-$ cells.

3.5. Dll1^R-Bps consist of Bp-1$^-$cells and Bp-1$^+$ cells

The removal of Bp-1$^+$ cells incompletely depleted Dll1^R-Bps. On OP9-DL1, removing Bp-1$^+$ cells prior to culturing slightly reduced the number of developed B-lineage cells, and Bp-1$^+$ cell-enriched fraction generated B-lineage cells efficiently (Supplemental Tables 2 and 3). These data indicated that Dll1^R-Bps are a heterogeneous population of Bp-1$^-$ cells and Bp-1$^+$ cells.

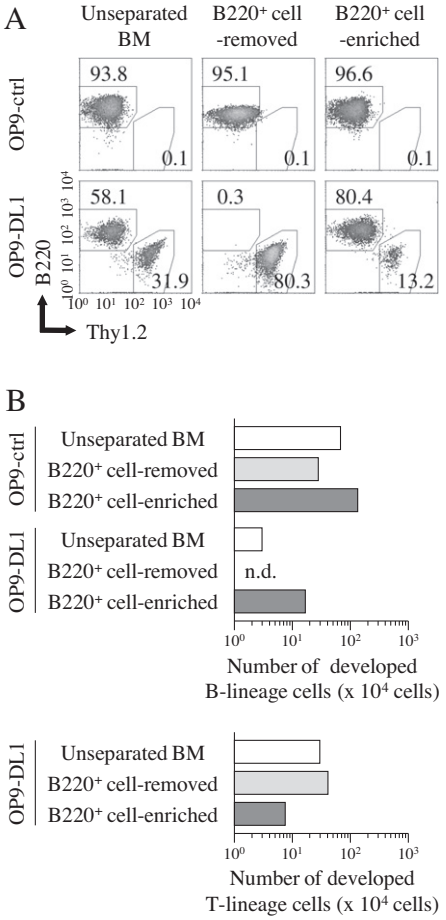

Fig. 2. Removal of B220$^+$ BM cells resulted in depletion of Dll1^R-Bps. B220$^+$ BM cells were removed or enriched using MACS® system. After 14 days, cells in cultures were harvested and analyzed by flow cytometry. Representative data from three independent experiments are shown. (A) Expressions of B220 and Thy1.2 on recovered cells from indicated cultures were evaluated by flow cytometry. Numbers in corners of plots show percentages of cells within each region. (B) Numbers of recovered B- (upper) and T-lineage cells (lower) from indicated cultures are shown. n.d.: not detected.

Bp-1 is expressed on B220$^+$ Cd19$^+$ BM cells transiently in the process of B-lymphopoiesis [20,25]. Bp-1$^+$ cells arise from Il7r$^+$ B220$^+$ Cd19$^+$ Bp-1$^-$ cells, and give rise to Il7r$^-$ B220$^+$ Cd19$^+$ Bp-1$^-$ cells [20]. Bp-1$^-$ Dll1^R-Bps were suggested to be staged prior to Bp-1$^+$ Dll1^R-Bps.

3.6. Ig transgenic mice have severely reduced number of Dll1^R-Bps in the BM

Dll1^R-Bps were suggested to be staged at the time when rearrangements of *Ig* loci occur [20]. In *Ig*-tg MD4 mice, the transgene *IghelMD4* allows cells to express functional sIgM specific for HEL (Igh-6.5) without the rearrangement of endogenous *Ig* (Igh-6.4) (Supplemental Fig. 3A and B) [13]. The number of B220$^-$ BM cells was normal, and MD4 BM cells developed to a number of T-lineage cells comparable to that of non-tg BM cells (Supplemental Fig. 4A and C). B220$^+$ BM cells were significantly reduced in MD4 mice, which was explained by a decreased number of Cd19$^+$ sIgM$^-$ cells and cells expressing sIgM at intermediate intensity (sIgM^{int})

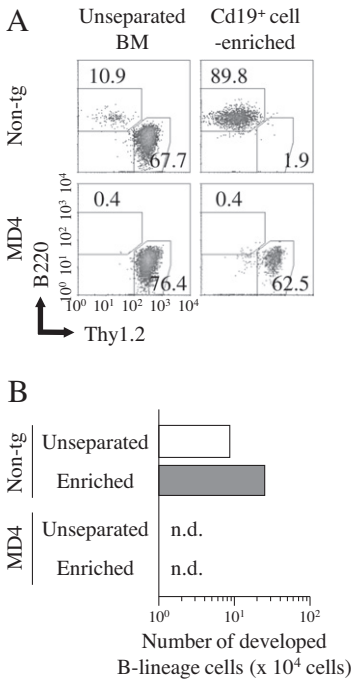

Fig. 3. MD4 mice have severely reduced number of Dll1^R-Bps. BM cells from MD4 mice or non-tg mice were cultured on OP9-DL1. (A) Expressions of B220 and Thy1.2 on recovered cells from each culture were assessed by flow cytometry. Numbers in corners of plots show percentages of cells within each region. (B) Numbers of B-lineage cells recovered from indicated cultures are shown. Data are the representative data from three independent experiments. n.d.: not detected.

(Supplemental Fig. 3C and D, 4A and B). Cells at the rearrangement events should be severely reduced in MD4 mice.

From MD4 BM cells, few B-lineage cells were generated on OP9-DL1 (Fig. 3). Since MD4 mice have fewer CFU-Il7s (Supplemental Fig. 4D), we cultured enriched Cd19$^+$ MD4 BM cells on OP9-DL1. Even the Cd19$^+$ cell-enriched fraction from MD4 mice failed to give rise to B-lineage cells on OP9-DL1 (Fig. 3). This suggested that MD4 mice had drastically reduced Dll1^R-Bps in their BM. Decreased B220$^+$ Cd19$^+$ sIgM$^{-/int}$ cells would explain the decrease of Dll1^R-Bps in MD4 mice.

3.7. Flt3$^+$ Cd19$^+$ BM cells are functional Dll1^R-Bps

To evaluate the developmental potential of Dll1^R-Bps directly, we purified Cd19$^+$ cells in the Flt3$^+$ cell-enriched fraction (Flt3$^+$ Cd19$^+$ cells) with a cell sorter. On OP9-ctrl, both Flt3$^+$ Cd19$^-$ cells and Flt3$^+$ Cd19$^+$ cells developed to B-lineage cells (Supplemental Fig. 5). On OP9-DL1, Flt3$^+$ Cd19$^-$ cells developed to T-lineage cells, and Flt3$^+$ Cd19$^+$ cells mostly gave rise to B-lineage cells (Supplemental Fig. 5A). These data indicated that Flt3$^+$ Cd19$^+$ cells were functionally Dll1^R-Bps, and that Dll1^R-Bps efficiently generated B-lineage cells even in the presence of abundant Dll1.

3.8. Little evidence of Dll1^R-Bps in the thymus

Finally, we assessed the presence of Dll1^R-Bps in the thymus. In the thymus, we detected B220$^+$ Cd19$^+$ sIgM$^+$ cells at 0.24% (data not shown). Precursors possessing the potential for B-lymphopoiesis were also present in the thymus (Fig. 1B left).

Since unseparated 10^6 thymocytes generated few B-lineage cells on OP9-DL1 (Fig. 1B right), more detailed analyses were carried out. Flt3$^+$ thymocytes were enriched, and only 0.19%, 0.049% and 0.073% of total flowed cells bound to the column (Table 1). We detected Cd19$^+$ cells at 1.0, 0.64 and 0.88% in the Flt3$^+$ thymocyte-enriched fractions (Table 1). Flt3$^+$ Cd19$^+$ cells were only 0.00095% on average in the thymus (Table 1). Thymic Flt3$^+$ Cd19$^+$ cells were purified and cultured on OP9-DL1, and they did not expand and generated no B-lineage cells either on OP9-DL1 or on OP9-ctrl (data not shown). These results suggested the absence or the extremely low frequency of Dll1^R-Bps in the thymus. The absence of Dll1^R-Bps would explain why the thymus does not support B-lymphopoiesis.

4. Discussion

In this study, we confirmed B cell precursors generating B-lineage cells even in the presence of over-expressed Dll1. Dll1^R-Bps were detected in the BM, and there was little evidence of Dll1^R-Bps in the thymus. Separations of BM cells prior to culturing revealed that Flt3$^+$ Il7r$^+$ B220$^+$ Cd19$^+$ BM cells were Dll1^R-Bps. Dll1^R-Bps might be staged at the rearrangement events of *Ig* loci.

Only a portion of BM cells were resistant to Dll1. In three independent experiments, the frequencies of Dll1^R-Bps were 4.4×10^{-4}, 13.8×10^{-4} and 6.9×10^{-4} (K. Okuyama, unpublished observation). This fact was well demonstrated in the femurs of MD4 mice. As shown in Fig. 3, although T cell precursors, Cd19$^-$ sIgM$^-$ cells and Cd19$^+$ sIgM$^+$ cells, were intact in the femurs of MD4 mice, Dll1^R-Bps were almost absent in MD4 mice. The reduced number of Cd19$^+$ sIgM$^{-/int}$ cells was suggested to cause the lack of Dll1^R-Bps in MD4 mice. The resistance to Dll1 might be exerted by Cd19$^+$ sIgM$^{-/int}$ cells but not Cd19$^-$ sIgM$^-$ cells or Cd19$^+$ sIgM$^+$ cells.

It is important to determine what contributes to the Dll1 resistance in Dll1^R-Bps. There are at least two ways to create resistance to Dll1. One is to weaken the signaling via Notch1/Dll. Notch1 signaling represses the transcription of *Pax5*, and Pax5 suppresses the expression of *Notch1* [19,30]. In mice reconstituted with BM cells carrying a constitutively active form of *Notch1*, B-lymphopoiesis was reported to be impaired [2]. It is possible that Dlls require a certain density of Notch1 on the cell surface to function as an inhibitor of B-lymphopoiesis. Since the transcription of *Cd19* is regulated by Pax5, Dll1^R-Bps should express Pax5 [27]. Pax5 might enable Dll1^R-Bps to give rise to B cells on OP9-DL1. Zinc finger and BTB domain containing 7a (Zbtb7a: also known as *Lrf*) was reported to repress the signaling via Notch1 [31]. Zbtb7a is also a candidate for a repressor of the expression of Notch1 in Dll1^R-Bps.

Table 1
Number of Flt3$^+$ Cd19$^+$ cells in the BM and the thymus.

Organ	Exp. #	Percentage of indicated cell			Number of Flt3$^+$ Cd19$^+$ cell/mouse
		Flt3$^+$ cell/whole cell (%)	Cd19$^+$ cell/total Flt3$^+$ cell (%)	Flt3$^+$ Cd19$^+$ cell/whole cell (%)	
BM	–	3.9	86.0	3.4	4.0×10^6
Thymus	1	0.19	1.0	0.002	3.2×10^3
	2	0.049	0.64	0.00031	2.5×10^2
	3	0.073	0.88	0.00064	8.7×10^2
	Average	0.10	0.84	0.00095	1.4×10^3

Flt3$^+$ cells were purified from the BM or the thymus, and percentages of Cd19$^+$ cells in the enriched fractions were assessed by flow cytometry. Numbers of Flt3$^+$ Cd19$^+$ thymocytes per mouse were calculated. Total mass of the thymus (two lobes) was 1.7×10^8, 0.78×10^8, and 1.4×10^8 cells in the experiment.

On the other hand, factors that promote the development or the expansion of B cells might also be able to provide Dll1 resistance. In the thymus, B cells displaying a unique phenotype ($Cd5^+$ $Cd8a^+$) are detected at a low frequency [32–34]. It was reported that Il7 and Flt3l promoted expansions of thymic B cells [35]. Stat5 is a downstream target of Il7r, and constitutively activated *Stat5* was reported to induce intrathymic B-lymphopoiesis [36]. Although we could not confirm effects of Flt3 or Flt3l, a supplement of Il7 did increase the number of B-lineage cells that developed on OP9-DL1 by our culturing method (data not shown). Kit is also known to facilitate the expansion of B cell precursors [37]. While B-lineage cells developed in both Kit-dependent and -independent manners on OP9-ctrl the B-lymphopoiesis on OP9-DL1 was highly dependent on Kit signaling. In the presence of antagonistic antibody against Kit, B cells did not develop from BM cells on OP9-DL1 (data not shown). The expansion of $Dll1^R$-Bps or B cells derived from $Dll1^R$-Bps might be ensured by the signaling via Il7r and Kit.

One remaining issue is whether $Dll1^R$-Bps also give rise to B cells *in vivo*. Though intrathymic transplantations of BM cells from control or *Notch1*-deficient mice have been performed [4], intrathymic B-lymphopoiesis from control BM cells was not referred to. It was reported that Dll4 was a physiological ligand for Notch1 on T cell precursors in the thymus [5,6]. Although Dll4 was superior to Dll1 in terms of inducing T-lymphopoiesis, either Dll could repress the development of B cells from multipotent hematopoietic precursors [38]. We cultured $Cd19^+$ BM cells on OP9 expressing abundant Dll4, and $Cd19^+$ BM cells could give rise to B-lineage cells on them (preliminary data). This suggested that $Dll1^R$-Bps should be resistant to Dll4.

In the thymus, we could detect $B220^+$ $Cd19^+$ $sIgM^+$ cells (0.24%) and precursors possessing the potential for B-lymphopoiesis (in an experiment, the frequency was $0.7–1.8 \times 10^{-6}$) (Fig. 1B left). However, the percentages of $Flt3^+$ $Cd19^+$ cells were extremely low and $Dll1^R$-Bps could not be confirmed in the thymus (Table 1). The frequency of $Dll1^R$-Bps was suggested to be less than 8×10^{-8} in the thymus. Multipotent hematopoietic precursors or T cell precursors migrate from the BM to the thymus through the peripheral blood stream [39]. Peripheral blood mononuclear cells gave rise to T cells on OP9-DL1. However, $Dll1^R$-Bps could not be detected in the peripheral blood (K. Okuyama, unpublished observation). It suggests that $Dll1^R$-Bps might be rarely released into the peripheral blood from the BM, and therefore do not seed the thymus. Although some molecules playing a role in retaining hematopoietic stem and precursor cells have been reported, which molecule involve in anchoring Dll1R-Bps in the BM is still uncovered [1,20]. We concluded that Notch1/Dll interactions and the absence of $Dll1^R$-Bps should collaboratively explain why the thymus does not support B-lymphopoiesis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.072.

References

[1] T. Nagasawa, Microenvironmental niches in the bone marrow required for B-cell development, Nature Reviews Immunology 6 (2006) 107–116.
[2] J.C. Pui, D. Allman, L. Xu, et al., Notch1 expression in early lymphopoiesis influences B versus T lineage determination, Immunity 11 (1999) 299–308.
[3] F. Radtke, A. Wilson, G. Stark, et al., Deficient T cell fate specification in mice with an induced inactivation of Notch1, Immunity 10 (1999) 547–558.
[4] A. Wilson, H.R. MacDonald, F. Radtke, Notch1-deficient common lymphoid precursors adopt a B cell fate in the thymus, Journal of Experimental Medicine 194 (2001) 1003–1012.
[5] K. Hozumi, C. Mailhos, N. Negishi, et al., Delta-like 4 is indispensable in thymic environment specific for T cell development, Journal of Experimental Medicine 205 (2008) 2507–2513.
[6] U. Koch, E. Fiorini, R. Benedito, et al., Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment, Journal of Experimental Medicine 205 (2008) 2515–2523.
[7] T.B. Feyerabend, G. Terszowski, A. Tietz, et al., Deletion of Notch1 converts pro-T cells to dendritic cells and promotes thymic B cells by cell-extrinsic and cell-intrinsic mechanisms, Immunity 30 (2009) 67–79.
[8] T.M. Schmitt, J.C. Zúñiga-Pflücker, Induction of T cell development from hematopoietic progenitor cells by Delta-like-1 in vitro, Immunity 17 (2002) 749–756.
[9] Z. Huang, L. Nie, M. Xu, et al., Notch-induced E2A degradation requires CHIP and Hsc70 as novel facilitators of ubiquitination, Molecular and Cellular Biology 24 (2004) 8951–8962.
[10] L. Nie, S.S. Perry, Y. Zhao, et al., Regulation of lymphocyte development by cell-type-specific interpretation of Notch signals, Molecular and Cellular Biology 28 (2008) 2078–2090.
[11] K. Beck, M.M. Peak, T. Ota, et al., Distinct roles for E12 and E47 in B cell specification and the sequential rearrangement of immunoglobulin light chain loci, Journal of Experimental Medicine 206 (2009) 2271–2284.
[12] S. Malin, S. McManus, C. Cobaleda, et al., Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development, Nature Immunology 11 (2010) 171–179.
[13] C.C. Goodnow, J. Crosbie, S. Adelstein, et al., Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice, Nature 334 (1988) 676–682.
[14] T. Nakano, H. Kodama, T. Honjo, Generation of lymphohematopoietic cells from embryonic stem cells in culture, Science 265 (1994) 1098–1101.
[15] T. Sudo, M. Ito, Y. Ogawa, et al., Interleukin 7 production and function in stromal cell-dependent B cell development, Journal of Experimental Medicine 170 (1989) 333–338.
[16] K. Nagino, O. Nomura, Y. Takii, et al., Ultrasensitive DNA chip: gene expression profile analysis without RNA amplification, Journal of Biochemistry 139 (2006) 697–703.
[17] A. Murata, K. Okuyama, S. Sakano, et al., A Notch ligand, Delta-like 1 functions as an adhesion molecule for mast cells, Journal of Immunology 185 (2010) 3905–3912.
[18] A. Sambandam, I. Maillard, V.P. Zediak, et al., Notch signaling controls the generation and differentiation of early T lineage progenitors, Nature Immunology 6 (2005) 663–670.
[19] S. Höflinger, K. Kesavan, M. Fuxa, et al., Analysis of Notch1 function by in vitro T cell differentiation of Pax5 mutant lymphoid progenitors, Journal of Immunology 173 (2004) 3935–3944.
[20] R.R. Hardy, P.W. Kincade, K. Dorshkind, The protean nature of cells in the B lymphocyte lineage, Immunity 26 (2007) 703–714.
[21] R.L. Coffman, I.L. Weissman, B220: a B cell-specific member of the T200 glycoprotein family, Nature 289 (1981) 681–683.
[22] A. Rolink, E. ten Boekel, F. Melchers, et al., A subpopulation of $B220^+$ cells in murine bone marrow does not express CD19 and contains natural killer cell progenitors, Journal of Experimental Medicine 183 (1996) 187–194.
[23] T. Sudo, S. Nishikawa, N. Ohno, et al., Expression and function of the interleukin 7 receptor in murine lymphocytes, Proceedings of the National Academy of Sciences of the United States of America 90 (1993) 9125–9129.
[24] J. Adolfsson, R. Månsson, N. Buza-Vidas, et al., Identification of $Flt3^+$ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: a revised road map for adult blood lineage commitment, Cell 121 (2005) 295–306.
[25] M.D. Cooper, D. Mulvaney, A. Coutinho, et al., A novel cell surface molecule on early B-lineage cells, Nature 321 (1986) 616–618.
[26] M.L. Holmes, S. Carotta, L.M. Corcoran, et al., Repression of Flt3 by Pax5 is crucial for B-cell lineage commitment, Genes and Development 20 (2006) 933–938.
[27] Z. Kozmik, S. Wang, P. Dörfler, et al., The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP, Molecular and Cellular Biology 12 (1992) 2662–2672.
[28] S.I. Hayashi, T. Kunisada, M. Ogawa, et al., Stepwise progression of B lineage differentiation supported by interleukin 7 and other stromal cell molecules, Journal of Experimental Medicine 171 (1990) 1683–1695.
[29] K.S.R.S. Tudor, K.J. Payne, Y. Yamashita, et al., Functional assessment of precursors from murine bone marrow suggests a sequence of early B lineage differentiation events, Immunity 12 (2000) 335–345.
[30] A. Souabni, C. Cobaleda, M. Schebesta, et al., Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1, Immunity 17 (2002) 781–793.

[31] T. Maeda, T. Merghoub, R.M. Hobbs, et al., Regulation of B versus T lymphoid lineage fate decision by the proto-oncogene LRF, Science 316 (2007) 860–866.
[32] S.I. Mori, M. Inaba, A. Sugihara, et al., Presence of B cell progenitors in the thymus, Journal of Immunology 158 (1997) 4193–4199.
[33] K. Akashi, L.I. Richie, T. Miyamoto, B lymphopoiesis in the thymus, Journal of Immunology 164 (2000) 5221–5226.
[34] I. Ferrero, F. Anjuère, P. Martín, et al., Functional and phenotypic analysis of thymic B cells: role in the induction of T cell negative selection, European Journal of Immunology 29 (1999) 1598–1609.
[35] H.J. McKenna, P.J. Morrissey, Flt3 ligand plus IL-7 supports the expansion of murine thymic B cell progenitors that can mature intrathymically, Journal of Immunology 160 (1998) 4801–4809.
[36] C.A. Goetz, I.R. Harmon, J.J. O'Neil, et al., Restricted STAT5 activation dictates appropriate thymic B versus T cell lineage commitment, Journal of Immunology 174 (2005) 7753–7763.
[37] M. Ogawa, Y. Matsuzaki, S. Nishikawa, et al., Expression and function of c-kit in hemopoietic progenitor cells, Journal of Experimental Medicine 174 (1991) 63–71.
[38] M. Mohtashami, D.K. Shah, H. Nakase, et al., Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes, Journal of Immunology 185 (2010) 867–876.
[39] A. Krueger, H. von Boehmer, Identification of a T lineage-committed progenitor in adult blood, Immunity 26 (2007) 105–116.