International Immunology, Vol. 14, No. 6, pp. 637–645

Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision

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Keywords: gene targeting, Notch, RBP-J, T lymphocyte

Abstract

The transcription factor recombination signal binding protein-J (RBP-J) functions immediately downstream of the cell surface receptor Notch and mediates transcriptional activation by the intracellular domain of all four kinds of Notch receptors. To investigate the function of RBP-J, we introduced loxP sites on both sides of the RBP-J exons encoding its DNA binding domain. Mice bearing the loxP-flanked RBP-J alleles, RBP-J$^{f/f}$, were mated with Mx-Cre transgenic mice and deletional mutation of the RBP-J gene in adult mice was induced by injection of the IFN-α inducer poly(I)–poly(C). Here we show that inactivation of RBP-J in bone marrow resulted in a block of T cell development at the earliest stage and increase of B cell development in the thymus. Lymphoid progenitors deficient in RBP-J differentiate into B but not T cells when cultured in 2′-deoxyguanosine-treated fetal thymic lobes by hanging-drop fetal thymus organ culture. Competitive repopulation assay also revealed cell autonomous deficiency of T cell development from bone marrow of RBP-J knockout mouse. Myeloid and B lineage differentiation appears normal in the bone marrow of RBP-J-inactivated mice. These results suggest that RBP-J, probably by mediating Notch signaling, controls T versus B cell fate decision in lymphoid progenitors.

Introduction

In adulthood all blood cells including T lymphocytes are derived from hematopoietic stem cells (HSC) accommodated in the bone marrow (1). According to the current model of T lymphopoiesis, HSC first differentiate into common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) in the bone marrow (2,3). While CLP in bone marrow mainly commit to the B lineage and produce B lymphocytes through pro-B, pre-B and immature B cell stages (4,5), those CLP migrating to thymus will mainly generate T lymphocytes there (6,7). The differentiation of CLP in the thymus is a genetically controlled process involving several important cell fate specification steps (8,9). At the early stage of T cell differentiation in thymus, the microenvironment of the thymus provides signals to drive CLP to commit preferentially to the T cell lineage, but not B or other lineages. The newly T lineage-committed thymocytes are negative for T cell markers CD3, CD4 and CD8 (triple negative), and can be further divided into subpopulations according to their CD44 and/or CD25 expression. Thus triple-negative and CD44$^+$CD25$^-$ (also c-kit$^+$) thymocytes represent the earliest T cell precursors in thymus. These cells differentiate into CD44$^+$CD25$^+$ cells and start to rearrange their TCR genes at the β, γ and δ loci. Thymocytes having successful TCR rearrangements at the γ and δ loci differentiate into γδ T cells, while those with successful TCR rearrangements at the β locus adopt an αβ T cell fate and further rearrange their TCR gene at the α locus. The newly formed αβ TCR-bearing thymocytes express CD4 and CD8 co-receptors, and are called double-positive (DP) thymocytes. These cells will undergo vigorous negative and positive selections, and differentiate into mature CD4$^+$ T_h cells upon interaction with peptide–MHC class II complex or CD8$^+$ cytotoxic T cells upon interaction with peptide–MHC class I complex.

Several signaling pathways have been implicated in the regulation of proliferation and differentiation of thymocytes (10,11). Among them, Notch, an evolutionarily conserved

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Transmitting editor: K. Sugamura

Received 25 January 2002, *accepted* 5 March 2002

pathway frequently involved in cell fate specification, has recently drawn wide attention for its emerging role in T cell development (12,13). Early studies have shown that abnormal activation of Notch might be an etiological reason of some human T cell leukemia (14). This has got support from the results of Pear *et al*. (15) that transplantation of bone marrow cells expressing an activated form of Notch1 exclusively induced T cell neoplasms in the recipient mice. Detailed analysis has shown that expression of activated Notch1 in early lymphopoiesis may have influenced B versus T lineage determination in bone marrow, i.e. a portion of CLP in the bone marrow adopted a T cell fate at the expense of reduction in B lineage commitment (16,17). This is consistent with a recent loss of function experiment performed by Radtke *et al*. (18), who created Notch1 conditional knockout mice using the Cre–loxP system. By induction of Notch1 deletion in bone marrow, they found that inactivation of Notch1 in bone marrow induced a blockade of T cell development at a very early stage, while B cell development is promoted in the thymus (18). These data, supported by other evidence published recently (19–22), collectively prove that Notch1 is essential for T lineage commitment and inhibitory for B lineage commitment from CLP. In addition to an essential function in T versus B cell fate determination, Notch signaling is also proposed to regulate the differentiation of T lineage-committed precursors into $CD4^+$ or $CD8^+$ cells (23). For example, tissue-specific over-expression of Notch intracellular domain in thymocytes or thymoma cells has induced significant developmental changes including $\alpha\beta$ versus $\gamma\delta$ thymocytes (24) and CD4 versus CD8 lineage determination (25–27), as well as the maturation and apoptosis of CD4 and CD8 DP thymocytes (28–30). However, these findings have not been supported by loss of function experiments (31).

Notch is a family of transmembrane receptors characterized by an extracellular domain composed of ~36 EGF-like repeats and three LN (for Notch LIN-12) repeats, a transmembrane domain, and an intracellular part consisting of the RAM domain, CDC10/ankyrin repeats and a PEST motif (32,33). Multiple members of Notch family (Notch1–4 in human and mouse) and their ligands (Delta1 and Jagged1 and 2 in mouse) have been identified in mammals (32). At least Notch1–3, Jagged1 and 2 are expressed in the thymus (34). Current understanding of signal transduction events of Notch receptor suggests that when Notch is triggered by association with its ligand, a proteolysis reaction occurs within the transmembrane domain of the receptor, to release the intracellular domain of Notch. The Notch intracellular domain enters the nucleus and serves as a transcription activator. However, because the intracellular domain of Notch does not possess DNA binding activity, it associates with transcription factor Su(H)/RBP-J (suppressor of Hairless/recombination signal binding protein-J), and transactivates promoters containing RBP-J recognition site(s) to regulate cell fate specification and other physiological functions (33). It is noteworthy that the intracellular domains of all four kinds of Notch proteins interact with RBP-J and therefore RBP-J may serve as an integrator of Notch signaling pathway (35).

To further investigate the function of Notch in lymphocyte development, we created RBP-J conditional knockout mice using the Cre-loxP-mediated site-specific recombination strategy (36). Here we show that inactivation of RBP-J in bone marrow induced a blockade of T cell development at the early stage and increased B cell development in the thymus, similar to Notch1 conditional knockout mice. Competitive repopulation assay revealed cell autonomous deficiency of T cell development of RBP-J knockout bone marrow cells. The differentiation of myeloid lineage as well as B lineage is not changed in the bone marrow of RBP-J knockout mouse. These results suggest that RBP-J, probably by mediating Notch signals, controls T versus B cell fate decision in lymphoid progenitors.

Methods

Generation of mice with floxed RBP-J allele

Homologous recombination in embryonic stem (ES) cells was employed to generate mice bearing the floxed RBP-J allele. To construct a targeting vector, the 15-kb *Eco*RI fragment of mouse RBP-J genomic gene previously isolated from 129/Sv (37) mouse was used. The 6-kb *Stu*I–*Bam*HI fragment and the 6.5-kb *Eco*RV–*Eco*RI fragment were used as 5′ and 3′ homologous regions respectively. A loxP site was inserted into the *Bam*HI site upstream of the sixth exon, which encodes the DNA binding domain of RBP-J (38). A second loxP site was inserted into the *Eco*RV site downstream of the seventh exon and a neomycin phosphotransferase-expressing cassette was inserted upstream of the loxP site. The vector was linearized by digestion with *Not*I and electroporated to ES cell line E14 (generously provided by Dr S. Itohara). Transfected cells were cultured in the presence of G418 (300 μg/ml) for 1 week, and G418-resistant clones were picked up, expanded and subjected to DNA preparation. Clones with homologous recombination were identified by Southern blot using a probe (probe A) outside of the 5′ homologous region (Fig. 1A).

Three independent homologous recombinant ES clones were injected into blastocysts of C57BL/6 mice and chimeras were born from each of them. Germline transmission of the floxed RBP-J allele was obtained by crossing chimeras with C57BL/6 mice and confirmed by Southern blot analysis of tail DNA using probe A.

Deletion of RBP-J gene by Cre-mediated recombination

Mice homozygous for floxed RBP-J allele ($RBP\text{-}J^{f/f}$) were crossed with Mx-Cre transgenic mice (Cre gene under the control of Mx promoter, generously provided by Dr K. Rajewsky) and crossed further to get $RBP\text{-}J^{f/f} \times$ Cre mice. The Mx-Cre transgene was detected by PCR using following primers: Cre1: 5′-GGCCCCATGGCATCCAATTTACTGACC-GTACAC-3′; Cre2: 5′-TCGCTCGAGGTGATCGCCATCTTC-CAGCAG-3′. To induce Cre-mediated deletion of the RBP-J gene, 6- to 8-week-old $RBP\text{-}J^{f/f} \times$ Cre mice were injected i.p. with 300 μg poly(I)–poly(C) (Sigma) for 4 times at 2-day interval. Mice were then injected with the same amount of poly(I)–poly(C) for 4 more times at 1-week intervals (eight injections in total). Two days after the last injection the mice were sacrificed for further analysis. Deletion of the floxed RBP-J allele and wild-type RBP-J exons was detected by PCR using the following primers: Floxed1: 5′-GAAGGTCGGTTGA-CACCAGATAGC-3′; Floxed2: 5′-GCAATCCATCTTGTTC-

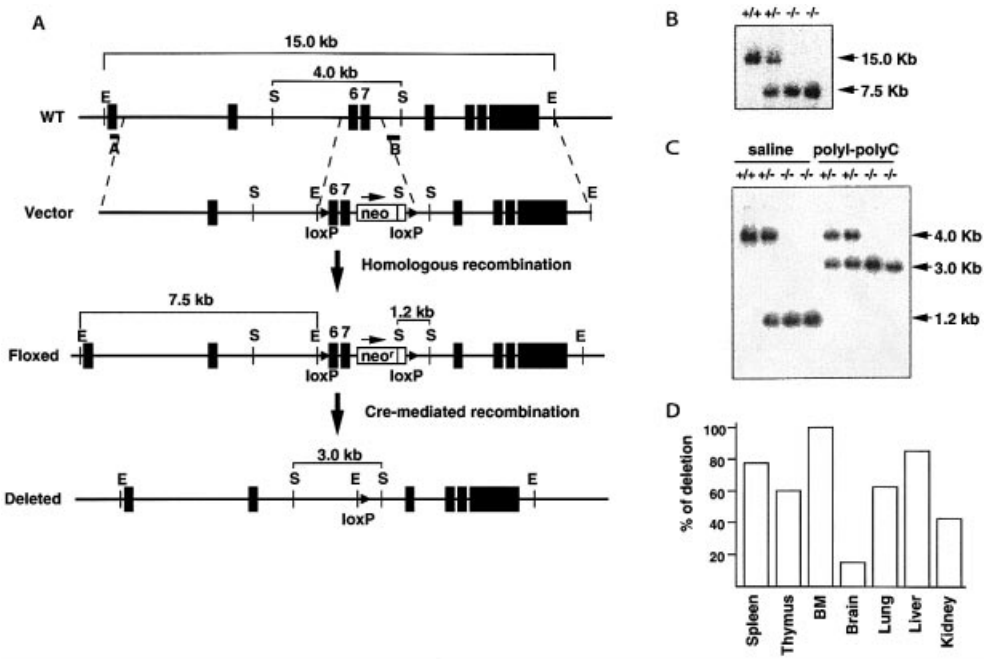

Fig. 1. Conditional gene knockout of RBP-J. (A) Schematic representatives of the wild-type RBP-J gene in mice (WT), targeting vector (Vector), floxed RBP-J allele (Floxed) and deleted RBP-J allele induced by Cre recombinase (Deleted). After homologous recombination, the 15.0-kb *Eco*RI fragment of the RBP-J gene will become 7.5 kb when probed with probe A and the 4.0-kb *Sph*I fragment will become 1.2 kb when probed by probe B. Cre-mediated recombination at the loxP sites will further convert the 1.2-kb *Sph*I fragment to 3.0 kb. (B) Southern blot analysis of *Eco*RI-digested mouse tail DNA using probe A, showing germline transmission of the floxed RBP-J allele. The wild-type allele is 15.0 kb while the floxed allele is 7.5 kb. (C) Southern blot analysis of DNA from bone marrow of mice with wild-type RBP-J (+/+), heterozygous floxed RBP-J (+/–) and homozygous floxed RBP-J (–/–) genotypes injected with saline or with poly(I)–poly(C). Adult mice (8–10 weeks old) were injected 4 times i.p. at 2-day intervals with 300 μg of poly(I)–poly(C), followed by four more injections at 1-week intervals. DNA was prepared from bone marrow cells, digested with *Sph*I, separated with gel electrophoresis, blotted and probed with probe B. (D) Efficiency of Cre-mediated RBP-J deletion in different organs induced by poly(I)–poly(C). Mice were injected as in (C). DNA from indicated organs was digested with *Sph*I and analyzed with probe B. Hybridization signals were quantified and the deletion efficiency was calculated as the percentage of signals of deleted alleles in the total signals.

AATGGCC-3′; WT1: 5′-GTTCTTAACCTGTTGGTCGGAACC-3′; WT2: 5′-GCTTGAGGCTTGATGTTCTGTATTGC-3′. The loxP-mediated deletional mutation of RBP-J gene was further confirmed by Southern blot analysis using probe B (Fig. 1A). Animal experiments were performed in accordance with the guidelines of the Graduate School of Medicine, Kyoto University.

Flow cytometry

Single-cell suspension was prepared from thymus or bone marrow and resuspended in PBS(–) supplemented with 2% FCS and 0.02% NaN_3. Cells were stained for 30 min with the antibodies listed below: anti-CD3–allophycocyanin (APC) (145-2C11), anti-CD4–APC (G.K1.5), anti-CD4–phycoerythrin (PE) (G.K1.5), anti-CD8–APC (53-6.7), anti-CD8–FITC (53-6.7), anti-B220–APC (BA3-6B2), anti-B220–FITC (BA3-6B2), anti-B220–PE (BA3-6B2), anti-Mac1–APC (M1-70), anti-CD25–FITC (7D4), anti-CD44–PE (Pgp-1), anti-CD24–PE (M1/69), anti-CD19–PE (1D3), anti-CD43–FITC (S7), anti-mouse immature lymphocytes–FITC (AA4.1), anti-TCRαβ–FITC (H57–597), anti-TCRγδ–PE (GL3), anti-CD45.1–PE (A10) and anti-CD45.2–FITC (104). Goat anti-mouse IgM–FITC was from Southern Biotechnology Associates (Birmingham, AL). Stained cells were washed and analyzed using a FACSCalibur with CellQuest software version 3.1 (Becton Dickinson, San Jose, CA). Debris, erythrocytes and dead cells were excluded from the analysis by forward and side scatter and propidium iodide gating. The data collected from 5×10^4 cells were analyzed.

In vitro colony forming assay

Single-cell suspension of bone marrow was prepared and 2×10^5 cells were cultured in α-MEM medium supplemented with 30% FCS, 2 mM l-glutamine, 0.1 mM 2-mercaptoethanol, 10 mg/ml BSA and 1.2% methylcellulose. The following cytokines were also included in the culture medium: for myeloid colonies, IL-3 (200 U/ml), granulocyte colony stimulating factor (200 U/ml), erythropoietin (2 U/ml), stem cell factor (50 ng/ml), thrombopoietin (10 ng/ml) and IL-6 (20 ng/ml); for B colonies, stem cell factor (50 ng/ml) and IL-7 (10 U/ml). Cells were cultured for 8 (myeloid colonies) or 13 (B colonies) days and colonies were counted under a microscope.

Fetal thymus organ culture (FTOC)

Thymic lobes were harvested from embryos of ICR mice at day 14.5 of gestation and cultured in FTOC medium (RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 1 × MEM non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin), at the interface between a floating

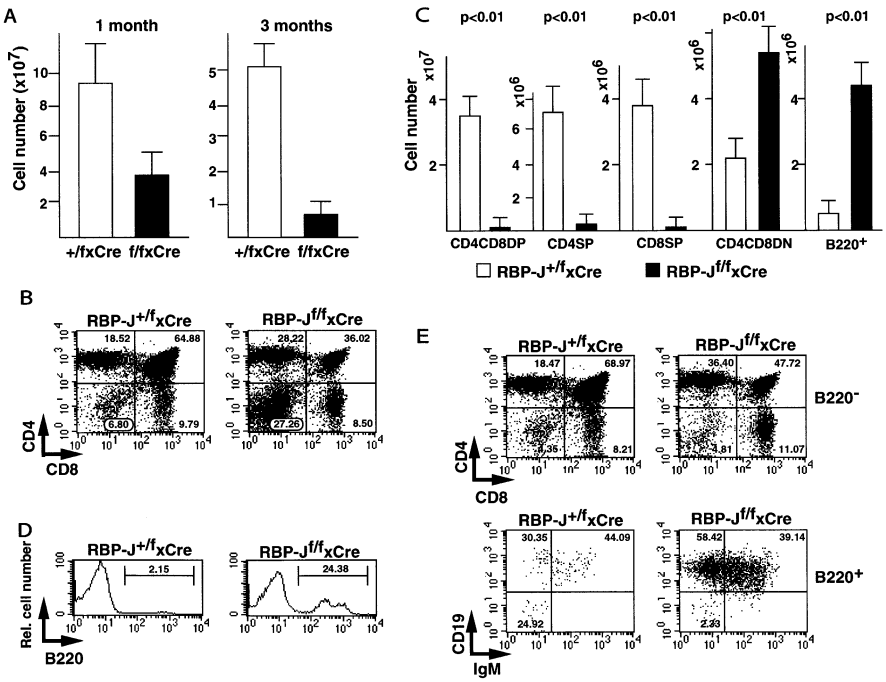

Fig. 2. T cell development is blocked and B cell development is promoted in the thymus of poly(I)–poly(C)-treated RBP-J$^{f/f}$ × Cre mice. Groups of five adult mice with the RBP-J$^{+/f}$ × Cre (w/f) or RBP-J$^{f/f}$ × Cre (f/f) genotype were injected with poly(I)–poly(C) as in Fig. 1(C). After 1 or 3 months after the first injection, single-cell suspensions were prepared from the thymi and analyzed. (A) Total thymocyte numbers (average ± SD) of RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre mice 1 or 3 months after poly(I)–poly(C) injection. (B) Thymocytes were stained with anti-CD4–PE and anti-CD8–FITC antibodies, and analyzed by FACS 1 month after the poly(I)–poly(C) injection. (C) Absolute numbers (average ± SD) of thymocyte subsets of RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre mice treated with poly(I)–poly(C) for 3 months. The statistical significance was calculated using Student's *t*-test. (D) Thymocytes from RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre mice were stained with FITC-conjugated anti-B220 antibody and analyzed by FACS 1 month after the poly(I)–poly(C) injection. (E) Analysis of B220^{-} and B220^{+} cells in the thymi of RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre mice after poly(I)–poly(C) treatment for 1 month. Gated B220^{-} or B220^{+} thymocytes were stained either with antibodies against CD4 and CD8 (upper panels) or with antibodies against CD19 and IgM (lower panels).

Nucleopore polycarbonate filter (Corning, Acton, MA) and 5% CO_2-humidified air as described (39). Bone marrow cells (5 × 10^5 for one lobe) were cultured with 2′-deoxyguanosine-treated thymic lobes in a hanging-drop for 24 h and were further organ-cultured for 2 weeks. Organ-cultured thymocytes were analyzed by flow cytometry as above.

Bone marrow transplantation

Bone marrow cells were prepared from normal C57BL/6-SJL mice (CD45.1) and either RBP-J$^{f/f}$ × Cre or RBP-J$^{+/f}$ × Cre mice (CD45.2) injected with poly(I)–poly(C). Recipients were irradiated for 9 Gy and then injected i.v. with 5–10 × 10^6 bone marrow cells that were a mixture at a 1:1 ratio of cells from CD45.1 (normal C57BL/6-SJL) and CD45.2 (RBP-J$^{f/f}$ × Cre or RBP$^{+/f}$ × Cre) donors. Mice were maintained on aqueous antibiotics (1.1 g/l of neomycin sulfate) and analyzed after 2 months.

Results

Conditional knockout of RBP-J

The DNA binding domain of RBP-J is encoded by the sixth exon (38). The domain is essential for its function, as demonstrated by site-directed mutagenesis (40) and gene knockout experiments (37). To establish mice with conditional gene-targeted RBP-J, a loxP site was inserted upstream of the sixth exon and the other downstream of the seventh exon together with a neomycin phosphotransferase gene-expressing cassette (PGK-neo) (Fig. 1A). ES cells were electroporated with the targeting construct and selected with G418. G418-resistant clones were picked up and screened by Southern blot analysis using probe A, which detects a 7.5-kb *Eco*RI fragment for the homologous recombinant allele but a 15-kb fragment for the wild-type allele. Three homologous recombinant ES clones were injected into blastocysts of C57BL/6 mice to generate chimeric mice. All of them gave germline transmission of the targeted allele, as determined by Southern blot analysis of mouse tail DNA (Fig. 1B). Mice with either homozygous (RBP-J$^{f/f}$) or heterozygous (RBP-J$^{+/f}$) floxed RBP-J alleles showed no visible developmental change without Cre recombinase expression.

To induce the deletional inactivation of RBP-J by Cre expression, the mice with the floxed RBP-J allele were bred with the Mx-Cre transgenic mice in which the Cre recombinase gene is under the control of the Mx promoter (36). Deletion of the floxed RBP-J exons was induced upon injection of IFN-α inducer poly(I)–poly(C) and examined by Southern blot analysis using probe B (Fig. 1A), which detects 4.0-, 1.2- and 3.0-kb *Sph*I fragments for the wild-type, floxed and deleted RBP-J alleles respectively. As shown in Fig. 1(C and D), injection of poly(I)–poly(C) induced almost complete

deletion of the floxed RBP-J fragment in the bone marrow, while deletion efficiency in other examined tissues varied and ranged from 15 to 80%.

Block of T cell development and promotion of B cell development in the thymus of RBP-J$^{f/f}$ × Cre mice after Cre induction

Adult RBP-J$^{f/f}$ × Cre mice were injected with poly(I)–poly(C) to induce the expression of Cre recombinase, with RBP-J$^{+/f}$ × Cre mice as a control. No visible gross change was noticed in either RBP-J$^{f/f}$ × Cre or RBP-J$^{+/f}$ × Cre mice after injection. Given that RBP-J is the main nuclear target molecule of the Notch signaling pathway and conditional knockout of Notch1 in bone marrow induced an early T cell developmental block in thymus (18), we examined T cell differentiation in the thymus of the RBP-J conditional knockout mice. One month after the first injection of IFN-α inducer, the size of the thymus of RBP-J$^{f/f}$ × Cre mice became smaller than that of the control mice (not shown) and total numbers of thymocytes decreased significantly in RBP-J$^{f/f}$ × Cre mice as compared with the control (Fig. 2A). Three months after IFN induction, total thymocyte numbers were further decreased in RBP-J$^{f/f}$ × Cre mice, to one-eighth of control mice. These results suggest abnormality of T cell development in RBP-J$^{f/f}$ × Cre mice after induction of Cre recombinase.

We examined thymocyte subpopulations of RBP-J$^{f/f}$ × Cre and control mice by FACS. One month after injection, we observed a significant reduction in the CD4 and CD8 DP fraction, a significant increase in the CD4 or CD8 single-positive (SP) fraction, and a large increase in the CD4 and CD8 double-negative (DN) fraction of thymocytes (Fig. 2B). Three months after the first injection, absolute numbers of each population were compared. We detected a drastic decrease in the CD4 and CD8 DP population and CD4 or CD8 SP populations, with a large increase of the CD4 and CD8 DN population (Fig. 2C). These data indicate that inactivation of RBP-J blocks differentiation of T lymphocytes in thymus.

Conditional knockout of Notch1 in bone marrow results in T versus B cell fate decision abnormality in the thymus (18): T cell development is blocked at early stage and B cell differentiation is permitted or promoted in the thymus. We therefore analyzed B cell differentiation in the thymus of RBP-J conditional knockout mice. Staining of thymocytes with anti-B220 revealed a significant increase in B220+ thymocytes in RBP-J$^{f/f}$ × Cre but not in control RBP-J$^{+/f}$ × Cre mice 1 month after induction of Cre expression (Fig. 2C and D), and 3 months later most of the thymocytes were B220$^+$ cells (data not shown). Most of these cells also express a B cell marker CD19 and a significant part of them express IgM as well (Fig. 2E, lower panels), indicating they are of the B cell lineage. These cells account for the increase of the DN thymocytes (Fig. 2E, upper panels). Taken together, these results suggest that knockout of RBP-J in lymphocyte progenitors induced B cell development at the expense of T cell development in the thymus.

Analysis of T and B cell precursors in the thymus of RBP-J conditional knockout mouse

We then asked which stage of T cell development is blocked when RBP-J is inactivated. T cell differentiation in the thymus

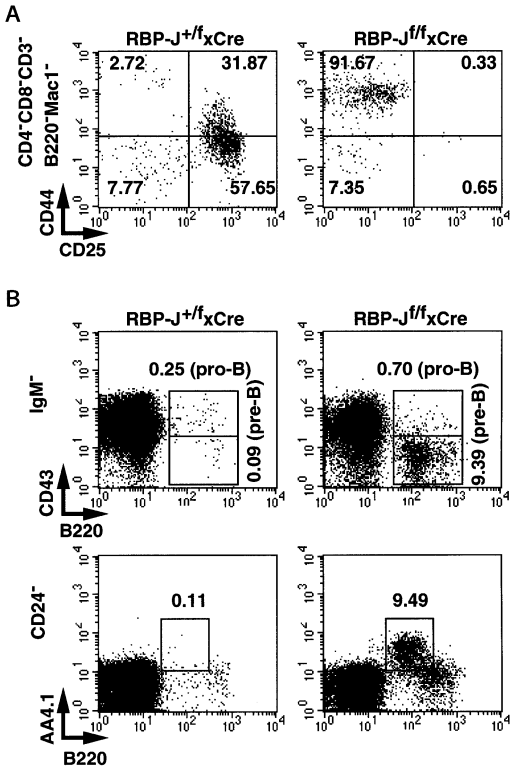

Fig. 3. Analysis of T and B cell precursors in the thymi of RBP-J$^{f/f}$ × Cre and control mice induced by poly(I)–poly(C). Adult RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre mice were injected with poly(I)–poly(C) for 1 month. (A) Thymocytes were gated to eliminate CD4$^+$, CD8$^+$, CD3$^+$, B220$^+$ and Mac1$^+$ cells, and stained for CD44 and CD25. (B) B cell development in the thymus of RBP-J conditional knockout mouse. (Upper panels) Thymocytes were gated for IgM$^-$ cells, and stained with CD43 and B220. (Lower panels) CD24$^-$ cells were stained for AA4.1 and B220. Percentages of pre-B, pro-B and CD24$^-$ AA4.1$^+$B220$^+$ cell fractions are shown.

starts from the CD4 and CD8 DN stage. This population is further subdivided according to their expression of CD44 and CD25 antigens. Thus CD44$^+$CD25$^-$ cells represent the most immature thymocytes, which differentiate into CD44$^+$CD25$^+$ and then CD44$^-$CD25$^+$ cells (8,9). We examined the subpopulations of early thymocytes that do not express CD3, CD4, CD8, B220 or Mac-1 with anti-CD44 and anti-CD25 antibodies. As shown in Fig. 3(A), while in a control mouse a significant portion of CD3$^-$CD4$^-$CD8$^-$B220$^-$Mac-1$^-$ cells were CD25$^+$ pre-T cells, a RBP-J$^{f/f}$ × Cre mouse injected with poly(I)–poly(C) had few CD25$^+$ cells and the majority of the CD3$^-$CD4$^-$CD8$^-$B220$^-$Mac-1$^-$ cell population were of the CD44$^+$CD25$^-$ phenotype. This suggests that inactivation of RBP-J arrested T cell development at the CD44$^+$CD25$^-$ pro-T or earlier stage.

We also analyzed different stages of B cell development in the thymus by FACS. The most immature B cell precursors express AA4.1 antigen, but are CD24$^-$ (4,5). This population is significantly increased in the thymus of RBP-J$^{f/f}$ × Cre mice after poly(I)–poly(C) injection, as compared with the control mice (Fig.3B, lower panels). Pro-B (IgM$^-$B220$^+$CD43$^+$) and pre-B (IgM$^-$B220$^+$CD43$^-$) cells are also increased in the

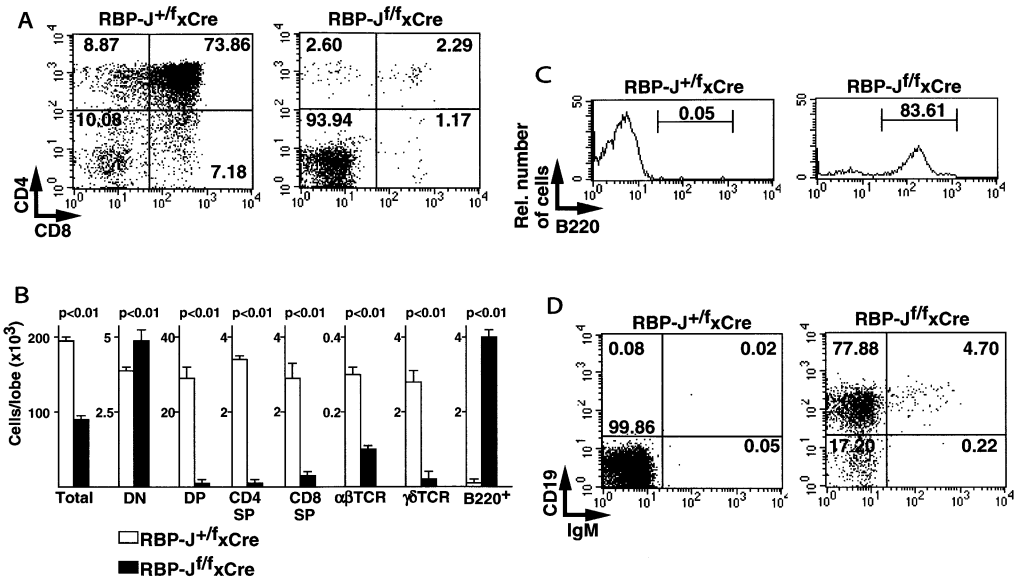

Fig. 4. Bone marrow cells from mice with inactivation of RBP-J differentiate into B but not T cells in FTOC. Adult mice with the indicated genotypes were injected with poly(I)–poly(C) as in Fig. 1(C). Bone marrow cells were cultured with fetal thymic lobes by hanging-drop FTOC for 14 days. (A) Organ-cultured lymphocytes from RBP-J$^{f/f}$ × Cre or control mice were stained for CD4 and CD8. (B) Absolute cell numbers per lobe (average ± SD) were calculated for total cells, CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$ (CD4SP), CD8$^+$ (CD8SP), T cells with αβ TCR (αβTCR), T cells with γδ TCR (γδTCR) and B220$^+$. The statistical significance was calculated using Student's *t*-test. (C and D) The majority of cells differentiated from RBP-J-deficient bone marrow in FTOC are of the B lineage. Cells were stained with anti-B220–FITC (C) and anti-CD19 plus anti-IgM (D) antibodies.

thymus of RBP-J$^{f/f}$ × Cre mice, as shown in Fig. 3(B). These results provided further evidence that lymphoid progenitors preferentially selected the B cell fate at the expense of the T cell fate in the thymus of RBP-J$^{f/f}$ × Cre mice.

Abnormality in T versus B cell fate decision in RBP-J knockout mouse is cell autonomous

Because RBP-J is a universally expressed transcription factor, we next addressed the question whether the abnormality in T versus B cell fate decision in the thymus of RBP-J knockout mouse is intrinsic to the progenitor cells or due to environmental cues. We cultured bone marrow cells from RBP-J$^{f/f}$ × Cre or control mice after injection of poly(I)–poly(C) in 2′-deoxyguanosine-treated fetal thymic lobes by hanging-drop FTOC (39). Organ-cultured thymocytes were analyzed by flow cytometry after 2 weeks. Bone marrow cells from the control mice eventually differentiated into immature and mature thymocytes in the organ culture system, as shown by CD4 and CD8 staining, and few cells were B220+ (Fig 4). In the thymic lobes cultured with bone marrow cells from induced RBP-J$^{f/f}$ × Cre mice, however, most cells differentiated into B cell lineage—expressing CD19 with some cells also expressing IgM. T cell differentiation is largely blocked—cells expressing CD4 and/or CD8, as well as cells bearing αβ or γδ TCR, are sharply decreased compared with the cells derived from the control mouse (Fig. 4B). These results indicate that the lymphoid progenitors in the RBP-J-deficient bone marrow preferentially select the B cell fate rather than the T cell fate in normal thymic microenvironment.

To evaluate quantitatively the ability of RBP-J-deficient bone marrow cells to repopulate thymus and generate T lymphocytes *in vivo*, we performed mixed bone marrow transplantation assay. Bone marrow cells were prepared from poly(I)–poly(C)-induced RBP-J$^{f/f}$ × Cre mice or control mice (CD45.2 haplotype) and were mixed with equal numbers of bone marrow cells from C57BL/6-SJL mice (CD45.1 haplotype). The mixed cells were transplanted into lethally irradiated mice. Thymocytes were analyzed 2 months after bone marrow transplantation. In mice receiving the cells from control mouse and wild-type mouse, two kinds of bone marrow cells repopulated the thymus approximately equally, as shown by staining with anti-CD45.1 and anti-CD45.2 antibodies (Fig. 5A). However, in mice receiving the cells from poly(I)–poly(C)-induced RBP-J$^{f/f}$ × Cre mouse and wild-type mouse, most thymocytes are derived from the wild-type bone marrow (CD45.1). The small population of thymocytes derived from RBP-J-deficient bone marrow (CD45.2) is not of T but of B lineage cells, because most of them are negative for CD4 and/or CD8, but express B220 surface marker (Fig. 5B). Therefore, lymphoid progenitors lacking RBP-J could not differentiate into T lineage cells, but rather selected the B cell fate in the thymus.

Differentiation of myeloid and B lineage in bone marrow of RBP-J conditional knockout mice appears normal

Notch has been suggested to play a role in myeloid and B lineage differentiation (reviewed in 41). We therefore examined myeloid and B cell development in the bone marrow of poly(I)–poly(C)-induced RBP-J$^{f/f}$ × Cre mice using an *in vitro* colony-forming assay. The results showed colony-forming progenitors of myeloid and B cell lineages are not remarkably changed in the bone marrow of RBP-J$^{f/f}$ × Cre mice (Fig. 6).

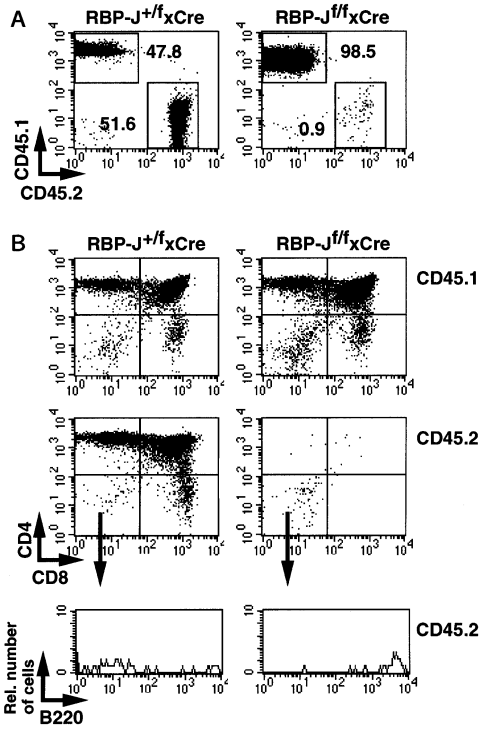

Fig. 5. Competitive repopulation assay. Lethally irradiated mice were repopulated with bone marrow cells from wild-type C57BL/6-SJL (CD45.1) mice mixed with those from poly(I)–poly(C)-induced RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre (CD45.2) mice at 1:1 ratio. Thymocytes were analyzed 2 months later. (A) FACS analysis of thymocytes stained with CD45.1 and CD45.2 antibodies. Relative contributions of competing CD45.1 versus CD45.2 donor bone marrow cells are shown. (B) CD45.1 (wild-type derived) and CD45.2 (RBP-J$^{+/f}$ × Cre- or RBP-J$^{f/f}$ × Cre-derived) thymocytes were stained for CD4 and CD8 expression (upper panels). Lower panels, CD4$^-$ and CD8$^-$ cells in the CD45.2 population were stained with APC-conjugated B220.

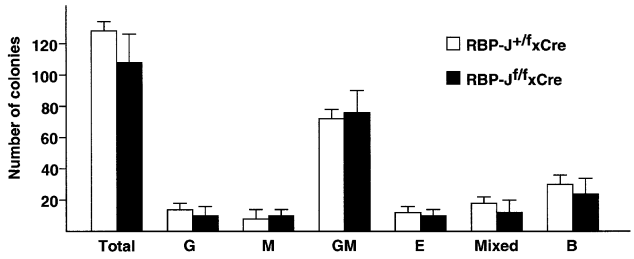

Fig. 6. *In vitro* colony-forming assay of myeloid and B lineage progenitors of RBP-J conditional knockout mice. A single-cell suspension was prepared from the bone marrow of RBP-J$^{+/f}$ × Cre and RBP-J$^{f/f}$ × Cre mice injected with poly(I)–poly(C). Cells were cultured with cytokines in methylcellulose for 8 (for myeloid colonies) or 13 (for B colonies) days, and colonies were counted under a microscope and numbers (average ± SD) of colonies are shown (G, granulocyte colonies; M, monocyte colonies; GM, colonies with granulocytes and monocytes; E, erythrocyte colonies; Mixed, colonies with granulocytes, monocytes and erythrocytes; B, B cell colonies).

Flow cytometry did not show any defect either in myeloid or B cell lineage development in the bone marrow of RBP-J$^{f/f}$ × Cre mice injected with poly(I)–poly(C) (data not shown). This suggests that RBP-J may not be essential for the development of these two lineages in the bone marrow. On the other hand, B cell development in spleen of RBP-J$^{f/f}$ × Cre mice injected with poly(I)–poly(C) was defective. Marginal zone B cells were drastically reduced while follicular B cells increased slightly (data not shown), in agreement with studies on RBP-J$^{f/f}$ × CD19-Cre mice (data not shown).

Discussion

Disruption of RBP-J in mice (37) results in a change of embryonic development very similar to Notch1 gene knockout mice (42,43): delayed and disorganized somitogenesis resulting in embryonic lethality around day 10 of gestation. This makes it impossible to analyze the function of RBP-J in later gestational stages and in the adult animals. In the current study, we created conditional gene knockout mice of RBP-J using the Cre–loxP system, in which the exons encoding the DNA binding domain of RBP-J are flanked by loxP sites. Without Cre expression, either heterozygous or homozygous floxed conditional knockout mice did not show any visible developmental abnormality. However, when a Cre transgene driven by Mx promoter is introduced and induced for Cre expression, the flanked exons are deleted. This animal model will be useful for studying the function of RBP-J in late fetal and adult stages. Moreover, because RBP-J mediates the transcriptional activation activity of all four kinds of Notch receptors, this mouse will also be helpful for the studying of the function of Notch receptors *in vivo*.

The deletion efficiency varies among different tissues using the Mx promoter-driven Cre-expressing transgene. The deletion is almost complete in the bone marrow. The phenotype of RBP-J inactivation in bone marrow is reminiscent of Notch1 conditional knockout. While generation of myeloid lineage and B lineage cells appears normal in the bone marrow of RBP-J conditional knockout mice, T cell development is blocked at an early stage and B cell development is induced or permitted in thymus. Analysis of early precursors has shown that T cell development is blocked at or before the CD44$^+$CD25$^-$ stage and B cell precursors increased in the thymus of RBP-J conditional knockout mouse. These changes are intrinsic to bone marrow progenitor cells because even in the normal thymic microenvironment B but not T cells were generated in the thymus, as shown by *in vitro* FTOC and *in vivo* mixed bone marrow transplantation experiments. Taking together, these results suggest that RBP-J, by mediating functions of Notch receptors, is essential for T versus B cell fate decision of lymphoid progenitors.

Our and others' results suggest that activation of the Notch–RBP-J pathway promotes T cell fate and suppress B cell fate determination. Triggering of Notch receptors by association with ligands initiates a proteolytic reaction in the transmembrane domain of the receptor, resulting in release of their intracellular domain. Two downstream signal transduction pathways have been speculated to participate in the regulation of lymphopoiesis by Notch (44). One is RBP-J dependent. The cleaved intracellular domain of Notch enters the nucleus and associates with RBP-J to activate transcription of T cell

differentiation-related genes. One candidate molecule downstream of RBP-J is the basic helix-loop-helix transcription factor HES1, because disruption of HES1 also blocked T cell development, as shown by Tomita *et al.* (45,46). The other pathway, mediated by inhibition of transcription factor E47, is supposed to be RBP-J independent (47). This pathway might be important for suppression of B cell differentiation by Notch, because E47 is essential for B lymphopoiesis (48). Thus, inactivation of Notch1 in CLP will block commitment to the T lineage due to failure to activate of RBP-J and increase commitment of the B lineage through insufficient suppression of E47 by the Notch intracellular domain. Our results, however, suggest that inactivation of RBP-J not only blocks T cell differentiation but also induces B cell differentiation in the thymus. This suggests that Notch may suppress B cell development through other molecules than E47.

Multiple Notch receptors and ligands are expressed in the thymus, and may fulfill distinct or overlapping roles in thymocyte differentiation (33). Overexpression of the intracellular domain of the Notch1 receptor in thymoma cells, bone marrow cells or mouse thymus has shown that Notch signaling might be involved in cell fate decision of T versus B cells, versus γδ T cells and CD4 versus CD8 T cells (24–27). In addition, Notch was also reported to be involved in the regulation of apoptosis and T cell maturation in the thymus (28–30). However, so far only the control of T versus B cell fate decision by Notch1 has been supported from both gain and loss of function experiments. When Notch1 was inactivated after lymphoid progenitors commit to the T cell lineage by introducing a CD4 promoter-driven Cre transgene into Notch1 conditional knockout mice, no remarkable change was observed in either αβ/γδ or CD4/CD8 T cell differentiation in the thymus (31). One possible explanation is that Notch1 function is redundant for the later stage of T cell development after lineage commitment. However, it is also possible that different Notch receptors regulate different aspects of thymocyte differentiation. Our study shows that RBP-J is indispensable for T lineage commitment from lymphoid progenitors and this animal model will be also useful for the analysis of other Notch functions in T cell development, because RBP-J mediates transcriptional activation of all four kinds of Notch receptors.

Acknowledgements

We thank Ms T. Taniuchi for help in the generation of gene knockout mice, and Ms Y. Doi, Ms E. Inoue, Ms M. Nakata and Ms A Nakano for excellent technical support. We are grateful to Dr S. Itohara for providing ES cell line E14 and Dr K. Rajewski for Mx-Cre transgenic mice. This work was supported by grants by Center for Excellence grant from the Ministry of Education, Science, Sports and Culture, and the Agency for Science and Technology of Japan.

Abbreviations

APC	allophycocyanin
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
DN	double negative
DP	double positive
ES	embryonic stem
FTOC	fetal thymus organ culture
HSC	hematopoietic stem cells
PE	phycoerythrin
RBP-J	recombination signal binding protein-J
SP	single positive

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