Notch 1–deficient Common Lymphoid Precursors Adopt a B Cell Fate in the Thymus

Anne Wilson, H. Robson MacDonald, and Freddy Radtke

Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland

Abstract

We have recently reported that Notch 1, a member of the Notch multigene family, is essential for the development of murine T cells. Using a mouse model in which Notch 1 is inactivated in bone marrow (BM) precursors we have shown that B cells instead of T cells are found in the thymus of BM chimeras. However, it is not clear whether these B cells develop by default from a common lymphoid precursor due to the absence of Notch 1 signaling, or whether they arise as a result of perturbed migration of BM-derived B cells and/or altered homeostasis of normal resident thymic B cells.

In this report we show that Notch 1–deficient thymic B cells resemble BM B cells in phenotype and turnover kinetics and are located predominantly in the medulla and corticomedullary junction. Peripheral blood lymphocyte analysis shows no evidence of recirculating Notch1$^{-/-}$ BM B cells. Furthermore, lack of T cell development is not due to a failure of Notch1$^{-/-}$ precursors to home to the thymus, as even after intrathymic reconstitution with BM cells, B cells instead of T cells develop from Notch 1–deficient precursors. Taken together, these results provide evidence for de novo ectopic B cell development in the thymus, and support the hypothesis that in the absence of Notch 1 common lymphoid precursors adopt the default cell fate and develop into B cells instead.

Key words: Notch 1 • T cell development • B cell development • lineage commitment • cell fate

Introduction

Notch proteins have been shown to play crucial roles in binary cell fate decisions in many developmental systems (1). In mammals, four Notch receptors and five transmembrane bound ligands have been identified to date (1–3). Several reports have suggested a role for Notch family members in T cell development (for reviews, see references 4–7). Notch 1, 2, and 3 as well as the ligands Jagged 1 and 2 have been shown to be expressed in thymocytes and thymic stromal epithelium (5, 8). We have recently described mice in which the loxP flanked Notch 1 gene was inactivated in an inducible manner by means of a Cre-recombinase transgene under the control of an IFN-α responsive promoter (9). By reconstituting irradiated mice with a mixture of control and Notch $1^{-/-}$ bone marrow (BM)* we showed that the Notch1 receptor is essential for the development of all conventional T cells in the thymus as well as for the thymus independent intestinal intraepithelial lymphocyte subset (9–11). Instead of T cells, immature B cells of Notch $1^{-/-}$ origin accumulated in the thymus of such BM chimeras. By analogy with the role of Notch genes in invertebrate systems one interpretation of these data is that Notch 1 provides a critical inductive signal that directs a bipotential T/B precursor toward a T cell fate. In the absence of Notch 1 signaling, bipotential precursors would develop into immature B cells in the thymus as a default pathway. Alternatively, the T cell deficiency observed in Notch $1^{-/-}$ BM chimeras might reflect a failure of lymphoid precursors to home from the BM to the thymus. According to this scenario the accumulation of immature B cells of Notch $1^{-/-}$ origin in the thymus would be unrelated to lineage commitment and might reflect either pertubated migration of immature B cells from the BM to the thymus or increased proliferation of resident intrathymic B cells.

The presence of a small number of B cells (0.1–0.2% of total thymic cells), in both the human and murine thymus has been known for some time (12, 13). Resident thymic B cells have a predominantly mature or activated phenotype

Address correspondence to Freddy Radtke, Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges, Switzerland. Phone: 41-21-692-5964; Fax: 41-21-653-4474; E-mail: Freddy.Radtke@isrec.unil.ch

**Abbreviations used in this paper:* BM, bone marrow; BrdU, bromodeoxyuridine; CLP, common lymphoid precursor; DN, double-negative; DP, double-positive; pI-pC, polyI–polyC; SP, single-positive.

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(summarized in reference 14), and are principally located in clusters at the corticomedullary junction or in the medulla (13, 15). Several recent reports have demonstrated the presence of putative B cell progenitors (and hence limited B cell development) in the thymus (16, 17), and have further suggested that thymic B cells may even migrate out to peripheral lymphoid organs (17). In addition, an apparently increased frequency of thymic B cells has been reported in several T cell–deficient mouse models including the TCR$\beta^{-/-}$ mutant (17, 18), and a transgenic mouse overexpressing a human CD3ϵ transgene (CD3ϵ26tg) at high copy number (19, 20). By analogy with these models it is possible that increased accumulation of B cells in the thymus of Notch $1^{-/-}$ mice reflects increased B cell lymphopoiesis as a nonspecific consequence of altered T cell development.

In an attempt to distinguish between these various possibilities we have undertaken a detailed analysis of thymic B cells derived from Notch 1–deficient BM precursors. Our data indicate that these Notch $1^{-/-}$ thymic B cells resemble BM B cells rather than mature peripheral B cells or normal resident thymic B cells in terms of phenotype and turnover kinetics. Moreover, immature Notch $1^{-/-}$ B cells are not detectable in the circulation and can be generated (in the absence of T cells) upon intrathymic injection of Notch $1^{-/-}$ BM precursors. Collectively our data demonstrate that Notch $1^{-/-}$ immature thymic B cells arise in situ and strongly suggest that they develop from bipotential T/B precursors in the absence of inductive Notch 1 signaling.

Materials and Methods

Mice and Cell Suspensions. Notch $1^{lox/lox}$ (control) and Notch $1^{lox/lox+Mx\text{-}Cre}$ (Notch 1 deleted) mice were generated as described previously (9) and used at 8–12 wk of age. These mice are positive for the common leukocyte antigen CD45.2. Activation of the Cre-recombinase was performed as described previously (9, 10). In brief, both Notch $1^{lox/lox}$ and Notch $1^{lox/lox+Mx\text{-}Cre}$ adult mice received four intraperitoneal injections of 250 μg polyI–polyC (pIpC; Sigma-Aldrich) at 2-d intervals. For BM chimeras the mice were killed and the BM prepared for transplantation by T cell depletion 2 d after the last injection. To quantitate deletion of the Notch 1 gene, genomic DNA was prepared from a portion of the T cell–depleted BM cells and deletion efficiency was verified by Southern blot analysis followed by PhosphorImager quantitation. To follow the kinetics of deletion of Notch 1 in adult mice, both Notch $1^{lox/lox}$ and Notch $1^{lox/lox+Mx\text{-}Cre}$ mice were injected with pIpC as described above, and killed for analysis at 7, 14, 21, or 28 d after the last injection.

CD45.1$^+$ C57BL/6 congenic mice were purchased from The Jackson Laboratory, and maintained on antibiotic containing water after lethal (1,000 rads) or sublethal (550 rads) irradiation as described previously (9). TCR$\beta^{-/-}$ mice were purchased from The Jackson Laboratory and the CD3ϵ26 tg (19) mice were the gift of Dr. Georg Holländer, Kantonspital, Basel, Switzerland. Thymocyte, BM, spleen, and LN cell suspensions were prepared and stained for FACS® by standard procedures. PBLs were prepared by collecting several drops of blood from the tail vein into a tube containing two drops Heparin to prevent clotting. After diluting to 1 ml in PBS, the suspension was underlayed with 1 ml Lymphoprep M (Cedarlane Laboratories) and centrifuged for 15 min at room temperature at 700 *g*. Lymphocytes were collected from the interface, washed in PBS, and stained for FACS® following standard proceedures.

FACS® Analysis and Monoclonal Antibody Conjugates. Four color flow cytometric analysis was performed as described previously (9, 11). The following monoclonal antibodies were purchased from BD PharMingen: CD45.1-FITC and –biotin (clone A20); CD45.2-FITC, and –biotin (clone 104); CD4-CyChrome (clone RM4–5), CD19-FITC (clone 1D3), BP-1-PE (clone 6C3), class II (anti-IA^b)-PE (clone M5/114.15.2), Sca-1-FITC (clone D7), and CD43-FITC (clone S7). AA4.1-biotin (clone 493) was the gift of Dr. Antonius Rolink, Basel Institute for Immunology, Basel, Switzerland and IgM (a+b)-biotin (50:50 mixture of RS1.3 and MB 86) was the gift of Dr. Hans Acha-Orbea, Ludwig Institute for Cancer Research, Lausanne, Switzerland. CD8α-Cy5, CD25-Cy5, CD45.1-Cy5, and CD45.2-Cy5 were purified from hybridoma supernatants (clones 53.6.7, PC61, A20.1, and 104, respectively) and conjugated in this laboratory using Cy-5 (Amersham Pharmacia Biotech). CD45.2-PE (clone A20) was conjugated in this laboratory using purified protein purchased from BD PharMingen and the PE conjugation kit (Prozyme). Biotinylated antibodies were revealed with Streptavidin-PE (Caltag), Streptavidin-CyChrome (BD PharMingen), or Streptavidin-allophycocyanin (APC) (Molecular Probes) conjugates. FACS® analysis was performed on a FACSCalibur™ flow cytometer (Becton Dickinson), and data analysis using CELLQuest™ software (Becton Dickinson).

Intrathymic Injection and BM Chimeras. Intrathymic injections were performed as described (21–23). Briefly, CD45.1$^+$ C57BL/6 congenic female mice were sublethally irradiated (550 rads) then anaethestized with a mixture of Ketamine hydrochloride (1.2 mg/100 g body weight) (Ketaset; Bristol Myers Co.) and Dormicom (0.12 mg/100 g body weight), a muscle relaxant, injected intraperitoneally. After verifying that they were adequately anethestized, the skin over the lower cervical and upper thoracic region was cut and pulled aside to reveal the chest wall. The upper third of the sternum was bisected longitudinally to reveal the thymus and the left thymic lobe injected with 10 μl of PBS containing 10^6 total BM cells using a 100 μl Hamilton syringe equipped with a 30G needle and mounted on a Chaney adaptor (Polylabo SA.) to control the volume delivered. The chest wall and skin were closed with surgical wound clips to maintain thoracic pressure. The mouse was gently warmed under an infrared lamp until the effects of the anesthetic wore off. Mice were maintained on antibiotic containing water until killed for analysis between 21 and 24 d later.

Individual thymic lobes were analyzed for the contribution of donor or host cells to reconstitution by staining with antibodies to CD45.2 and CD45.1, respectively. The phenotype of CD45.2$^+$ donor derived cells was further analyzed by FACS® with antibodies directed against T cell (CD4, CD8, TCRβ), and B cell (B220, IgM and AA4.1) specific markers. Mixed BM chimeras were prepared as described previously (9, 10) and analyzed 2–3 mo after reconstitution.

Bromodeoxyuridine Uptake to Assess Cell Turnover Rate. Mice were injected intraperitoneally with a single dose (180 μg) of bromodeoxyuridine (BrdU; Sigma-Aldrich) at the time points indicated, then fed continuously with water containing 800μg/ml BrdU and 5% glucose. Surface staining for FACS® was performed as described above using PE-, CyChrome-, Cy5-, APC-, or biotin-conjugated antibodies. Fixation, permeabilization, DNase treatment, and intracellular staining with anti–Brdu-FITC anti-

body was performed using the BrdU labeling kit (BD PharMingen). Cells stained in the same manner but isolated from an uninjected mouse were used in all cases as negative controls for BrdU staining. Percent BrdU uptake for each population was measured from at least five individual experiments and results are expressed as mean ± SD.

Results

Accumulation of Immature B Cells in the Thymus of Notch 1–deficient Mice. In a previous study (9) we have shown that inducible inactivation of Notch 1 in the BM (with an IFN-α–responsive Mx-Cre transgene) results in a complete block in T cell development. Instead of T cells, immature B cells of Notch 1–deficient origin are found in the thymus. These findings clearly showed that expression of the Notch 1 protein is essential in early T cell lineage commitment and suggested that lymphocyte precursors adopt a B cell fate in the thymus in the absence of Notch 1 signals. All of the above data were generated in a mixed BM chimera system which requires large numbers of mice and a long period to establish stable chimerism. To facilitate further studies we have used an adult deletion model in which 4–8-wk-old adult mice carrying the floxed Notch 1 gene together with (Notch $1^{-/-}$), or without (control) the Mx-Cre transgene are treated with pIpC. In Mx-Cre–positive mice the floxed Notch 1 gene is deleted while in control mice the gene is left intact due to the absence of the Cre-recombinase. Although deletion in different organs is of variable efficiency, it is close to 100% in the BM (9). As the vast majority of immature thymocytes are generated from incoming BM precursors over a short period of time (10–14 d; reference 24), we are now able to analyze the effects of Notch 1 deletion on intrathymic T cell development. In addition, this system also allows a more detailed analysis of the rate of appearance and phenotype of the immature B cell population in the thymus.

As shown in Fig. 1 A, the total number of thymocytes rapidly decreases over 4 wk ($138 \pm 23 \times 10^6$ down to $10 \pm 5 \times 10^6$), with a concomitant increase in the percentage of double-negative (DN) thymocytes from 2% of total thymus in control pIpC-treated mice (21 d after deletion) to 31% in the thymus of Notch 1–deleted mice after 28 d. In agreement with the mixed BM chimera studies performed previously, this DN population is largely composed of $CD19^+B220^+$ B cells. This Notch 1–deficient B cell population increases in total number from $0.24 \pm 0.13 \times 10^6$ per thymus to between 2 and 6×10^6 (3.6 ± 1.6), so that by 28 d after deletion it comprises ~85% of the DN thymus subset. Furthermore, a significant percentage of these Notch 1–deficient B cells express lower levels of B220 and CD19 consistent with their immature phenotype.

Fig. 1 B shows a comparison of BM B cells and B cells found in the thymus of Notch 1–deficient mice 28 d after deletion. The staining patterns for B220 versus IgM and B220 versus CD43 on BM and Notch 1–deficient thymus look very similar suggesting that B lineage cells of equivalent differentiation status are present in both the BM and the thymus in the absence of Notch 1.

Four-Color FACS® Analysis Was Used to Investigate in Detail the Phenotype of Thymic Notch 1–deficient B Cells Compared with BM B Cells and Peripheral LN B Cells. Fig. 1 C shows a comparison of thymic B cells found in both the mixed BM chimeras and in the adult deletion model (21 d after deletion) together with the pattern of expression of the same markers on LN or BM B cells. Notch 1–deficient thymic B cells obtained from either mixed BM chimeras or adult deleted mice show heterogeneous surface expression of the mature B cell markers IgM, IgD, and Sca-1 similar to BM B cells and in contrast to LN B cells. Furthermore, Notch 1–deficient thymic B cells are also heterogeneous for expression of the immature B cell markers, AA4.1, BP-1, and CD25 which are normally not expressed by mature LN B cells but have heterogeneous expression patterns on BM B cells.

Notch 1–deficient B Cells Increase in Number in the Thymus Concomitant with a Loss of T Cell Precursors. During adult life the thymus is continually seeded by $CD44^+117^+$ Sca-1^+ lin-negative lymphocyte precursors entering from the BM. Once in the thymus these precursors undergo a series of phenotypic changes characterized by transient expression of CD25 and loss of CD44 and CD117. Thus, early DN precursors progress through sequential $CD44^{hi}$ $CD25^-CD117^{hi}$ (DN 1), $CD44^{hi}CD25^+CD117^{hi}$ (DN 2), $CD44^{lo}CD25^+CD117^-$ (DN 3), and $CD44^{lo}CD25^-$ $CD117^-$ (DN 4) stages (25). As shown in Fig. 2, the number of DN 1, DN 2, and DN 3 thymocytes decreases rapidly following deletion of Notch 1, consistent with a complete block in T cell development from a BM precursor cell. As expected, the kinetics of disappearance of DN 1 and DN 2 precursors is faster than that of the DN 3 subset in Notch 1–deficient mice (Fig. 2), consistent with their earlier appearance and disappearance during normal thymus development. By day 28 after deletion there are virtually no identifiable thymic precursor cells. Interestingly, this progressive loss of Notch $1^{-/-}$ T cell precursors is mirrored by a concomitant increase in B cells in the thymus. In contrast, there is no change in the number of resident thymic B cells nor in any of the DN precursor subsets in control mice treated with pIpC (data not shown).

Immature B Cells Are Not Recirculating from the BM to the Thymus in Notch 1–deficient Mice. One simple explanation for the apparent accumulation of immature B cells in the thymus of Notch 1–deficient mice could be that treatment with pIpC to induce the IFN-α required to activate Cre-recombinase–mediated deletion of Notch 1 induces a wave of migration of immature B cells from the BM to the thymus. If this were the case, a large number of immature B cells would be detectable in the peripheral blood of pIpC-treated mice. To rule out this possibility, PBLs from Notch 1–deficient and control mice were analyzed for the presence of immature B cells by FACS® at various times between 2 and 28 d after deletion.

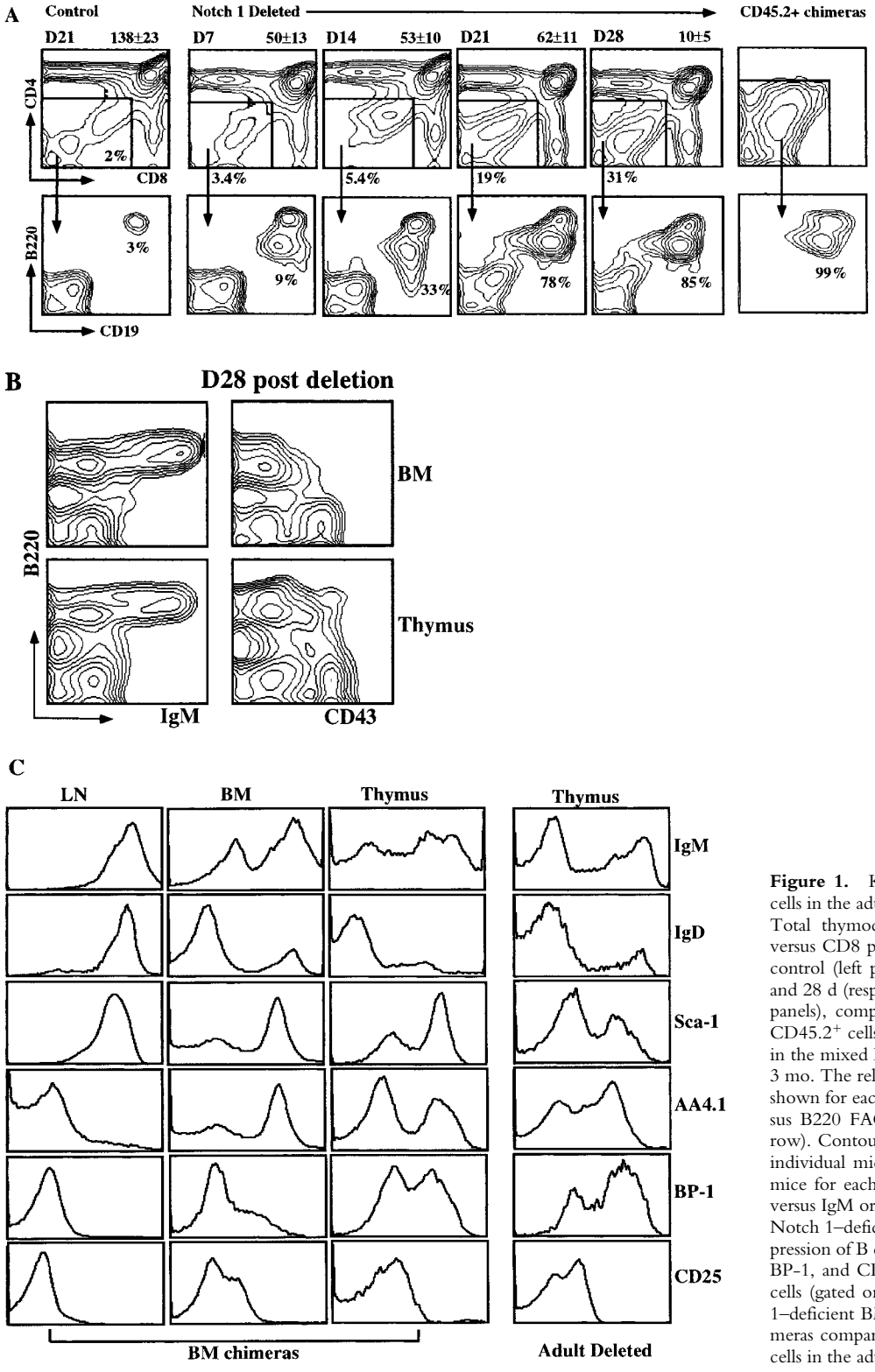


Figure 1. Kinetics of emergence of immature B cells in the adult thymus after Notch 1 deletion. (A) Total thymocyte numbers ($\times 10^6$) and the CD4 versus CD8 profiles (top row) of adult thymi from control (left panel), Notch 1 deleted at 7, 14, 21, and 28 d (respectively) after pIpC injection (middle panels), compared with the profile obtained from CD45.2$^+$ cells derived from Notch 1–deficient BM in the mixed BM chimera system (right panel) after 3 mo. The relative percentage of DN thymocytes is shown for each time point as well as the CD19 versus B220 FACS® analysis on gated DNs (bottom row). Contour plots are representative examples of individual mice. Data are mean ± SD from 8–12 mice for each time point. (B) Expression of B220 versus IgM or CD43 on total BM and thymus from Notch 1–deficient mice 28 d after deletion. (C) Expression of B cell markers, IgM, IgD, Sca-1, AA4.1, BP-1, and CD25 on gated LN, BM, or thymic B cells (gated on B220$^+$CD19$^+$) in CD45.2$^+$ Notch 1–deficient BM-derived cells from mixed BM chimeras compared with Notch 1–deficient thymic B cells in the adult deletion model.

Only mature phenotype B cells were detected in the blood of both control and Notch 1–deficient mice during this time period (Fig. 3, A and B, and data not shown). This result shows that no aberrant recirculation of immature B cells is detectable after pIpC treatment.

Notch 1–deficient Thymic B Cells Are Turning Over at a Similar Rate to BM B Cells and Not Peripheral B Cells. Immature BM B cells are turning over rapidly while resting mature B cells in the peripheral lymphoid organs are not dividing. To determine if the immature B cells accumulat-

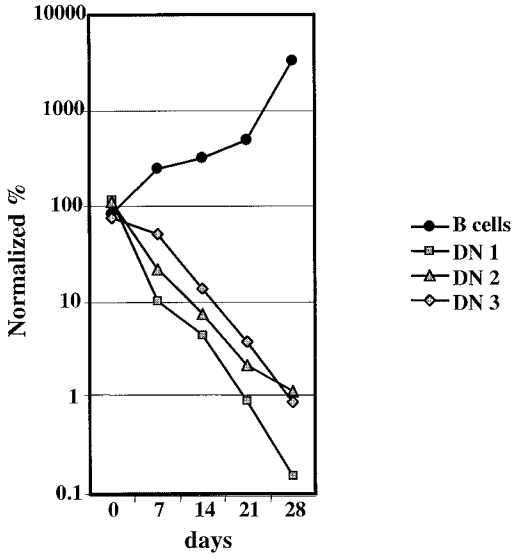
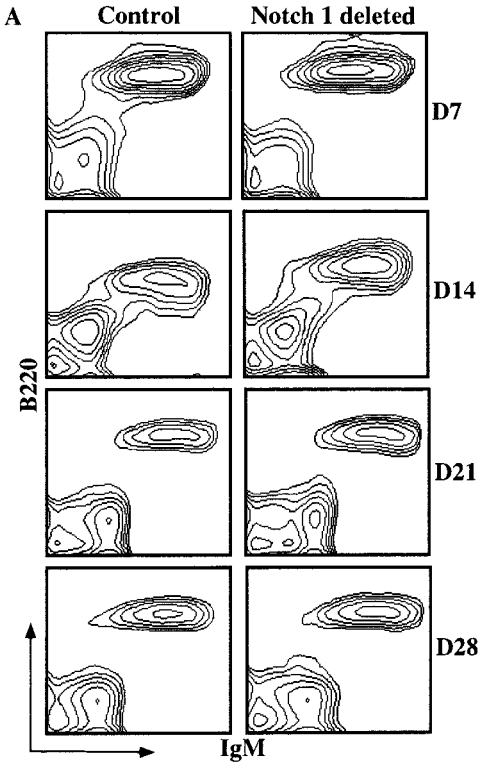


Figure 2. Concomitant increase in thymic B cells and decrease in DN 1, 2, or 3 immature thymocytes in adult Notch 1–deleted mice. Total numbers of thymic B cells or DN 1 (lineage negative, $CD117^+44^+25^-$), DN 2 ($CD117^+44^+25^+$), and DN 3 ($CD117^-44^-25^+$) thymocytes from Notch 1 adult deleted mice (at various times after deletion) were normalized to the equivalent subset from pIpC-treated control mice which was taken as 100%. Data are mean values of 8–12 mice per time point.



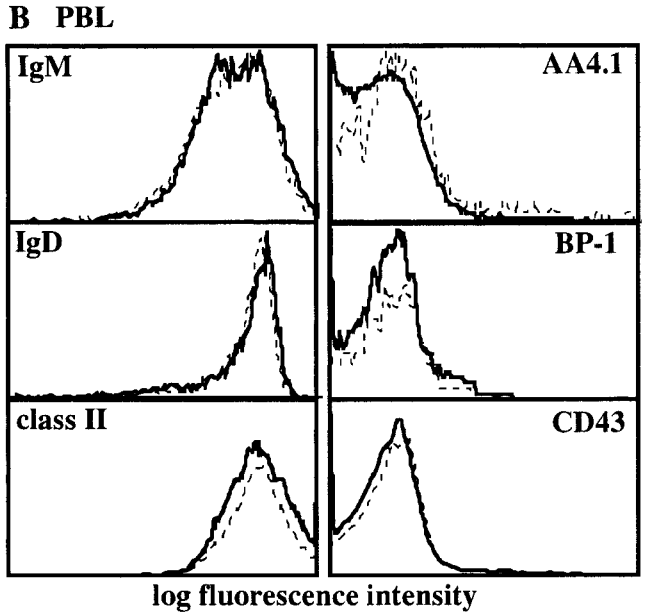


Figure 3. Mature phenotype of circulating B cells in $Notch1^{-/-}$ mice. (A) Expression of B220 versus IgM on PBLs isolated from pIpC-treated control or Notch 1–deficient mice on days 7, 14, 21, and 28 after deletion. (B) Expression of IgM, IgD, class II, AA4.1, BP-1, and CD43 on gated $B220^+CD19^+$ B cells isolated from PBLs from control (dotted line) compared with Notch 1 deleted (solid line) 21 d after deletion. Data for each part of Fig. 3 are representative FACS® profiles from experiments in which six mice of each genotype were analyzed at each time point.

ing in the thymus of Notch 1–deficient mice are in fact undergoing de novo development in situ. Notch 1–deleted mice were injected with a single dose of BrdU and subsequently fed BrdU in their drinking water for various times. Cells that have divided during the period of BrdU administration are specifically detected by intranuclear FACS® staining with FITC-conjugated anti-BrdU antibodies together with surface staining to identify specific subpopulations. As shown in Fig. 4, peripheral B cells (PBL and spleen) from both control and Notch 1–deleted mice show very little BrdU uptake over a 48-h period consistent with their resting status. In contrast, a 20-fold increase in BrdU labeling kinetics is observed in BM B cells from both control or Notch 1–deleted mice. While normal resident thymic B cells in control mice have slightly faster BrdU labeling kinetics than resting mature B cells, Notch 1–deficient thymic B cells label with BrdU 30-fold more rapidly. Indeed, the BrdU labeling curve of Notch 1–deficient thymic B cells is almost overlapping with that of BM B cells from either control or Notch 1–deficient mice.

Notch 1–deficient BM Precursors Produce Immature B Cells Instead of T Cells after Intrathymic Injection. To formally rule out the possibility that the lack of T cell development in the thymus is due to a failure of Notch 1–deficient BM derived precursor cells to home to the thymus, Notch $1^{-/-}$ or control BM cells were injected directly into one lobe of the thymus of sublethally irradiated $CD45.1^+$ congenic mice. Approximately 3 wk after injection the thymi were analyzed for the presence of donor-derived cells. As shown in Fig. 5 A, a population of $CD45.2^+$ cells derived from control or Notch 1–deficient BM can be detected in the

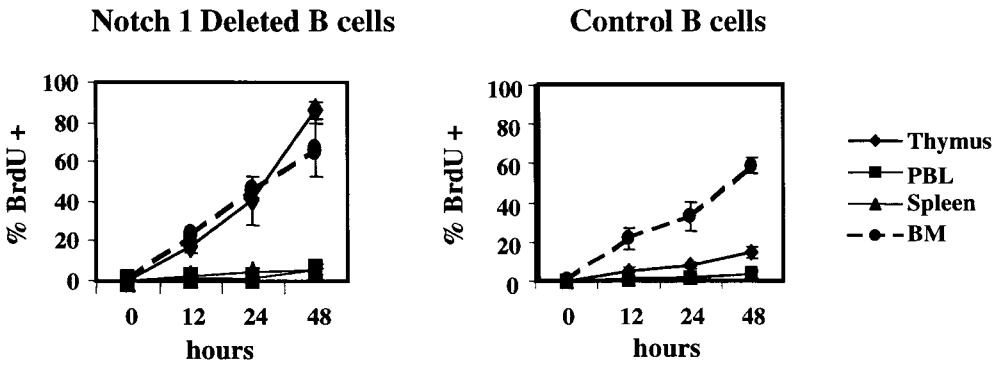


Figure 4. Rapid turnover of Notch1$^{-/-}$ thymic B cells. Percentage of BrdU$^+$ B cells (gated as B220$^+$CD19$^+$) from thymus, PBLs, spleen, and BM isolated from control (right panel) or Notch 1–deleted (14 d after deletion, left panel) mice after 12, 24, or 48 h continuous labeling. Data are mean ± SD of 4–8 mice per time point.

thymus after intrathymic reconstitution. In agreement with previous reports, immature CD4$^+$CD8$^+$ double-positive (DP) and mature CD4$^+$ single-positive (SP) and CD8$^+$ SP cells of donor origin are observed in the thymus injected with control BM cells (23). The distribution of these subsets is skewed toward DP and mature SP thymocyte subsets (Fig. 5 B) with few, if any immature DN, and no CD25$^+$ thymic precursors (data not shown), consistent with the fact that intrathymic injection of total BM gives rise to a single wave of reconstitution rather than permanent reconstitution as is the case with normal BM seeding. In contrast, no CD4- or CD8-expressing cells are produced in the thymus after intrathymic injection of Notch 1–deficient BM (Fig. 5 B). Instead, these CD45.2$^+$ Notch 1–deficient BM precursors give rise to immature B cells expressing CD19, B220, and AA4.1 (Fig. 5 C), as observed in adult deleted thymus, whereas CD45.2$^+$ control donor-derived BM did not. Although the absolute number of donor-derived control or Notch 1–deficient cells was quite variable after intrathymic reconstitution the percentage of B cells in the thymus was very reproducible (0.4 ± 0.5 versus 91 ± 8 for control and Notch 1$^{-/-}$ BM, respectively). These data demonstrate that Notch 1$^{-/-}$ BM precursors adopt a B (but not T) cell fate upon intrathymic transfer and thus imply that the immature B cells seen in the thymus of adult deleted (or chimeric) mice are the progeny of Notch 1–deficient common lymphoid precursors (CLPs) that have migrated to the thymus.

Notch 1–deficient Thymic B Cells Do Not Resemble Thymic B Cells Detected in Other T Cell–deficient Mouse Models. A small number of B cells have previously been detected in the normal thymus (16). However, the phenotype of these B cells is predominantly that of mature or activated cells and the number is extremely small (around 0.24 ± 0.13 × 10^6 per thymus). In addition, in at least two T cell–deficient mouse models (TCRβ$^{-/-}$ and CD3ε26 tg), increased numbers of B cells have been described in the thymus (17, 20). To eliminate the possibility that Notch 1–deficient thymic B cells only accumulate due to a relative lack of normal T cell development, we have compared the number and phenotype of thymic B cells in adult Notch 1–deleted mice with those found in the thymus of either TCRβ$^{-/-}$ or CD3ε26tg mice. As shown in Fig. 6, the thymus of Notch 1–deficient mice contains >10-fold more B cells than age-matched TCRβ$^{-/-}$ or CD3ε26tg mice. Moreover the majority of thymic B cells in both TCRβ$^{-/-}$ or CD3ε26tg mice are mature IgM$^+$ BP-1$^-$, AA4.1$^-$ CD19$^+$ B220$^+$ B cells, while those found in the Notch 1–deleted thymus express heterogeneous levels of IgM, BP-1, and AA4.1 (Fig. 6).

Localization of Notch 1–deficient Thymic B Cells to the Thymus Medulla. Immunohistochemical analysis of frozen sections of thymi of mixed BM chimeras (Fig. 7) show that CD45.2$^+$ cells derived from control BM are spread throughout the thymus cortex and medulla consistent with their comprising all immature and mature thymus

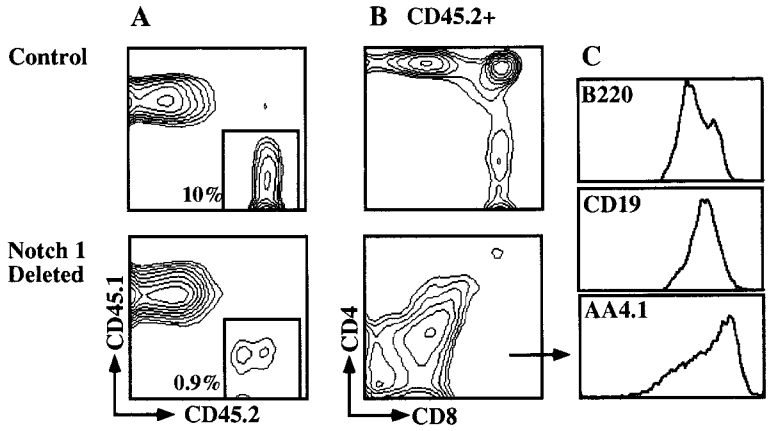


Figure 5. Notch1$^{-/-}$ BM cells give rise to B cells upon intrathymic injection. (A) Expression of CD45.2 (donor derived) versus CD45.1 (host derived) in the thymus 21 d after intrathymic injection of control (top panel) or Notch 1–deficient (bottom panel) BM. Contour plots contain around 500,000 events. (B) Expression of CD4 versus CD8 in CD45.2$^+$ (donor derived) thymocytes 21 d after intrathymic injection of control (top) or Notch 1–deleted (bottom) BM. Contour plots contain around 10,000 and 5,000 events, respectively. (C) Expression of B220, CD19, and AA4.1 on CD45.2$^+$CD4$^-$CD8$^-$ (donor derived) thymocytes 21 d after intrathymic injection of Notch 1–deleted BM. Data for all parts of Fig. 5 are representative stainings from three independent experiments.

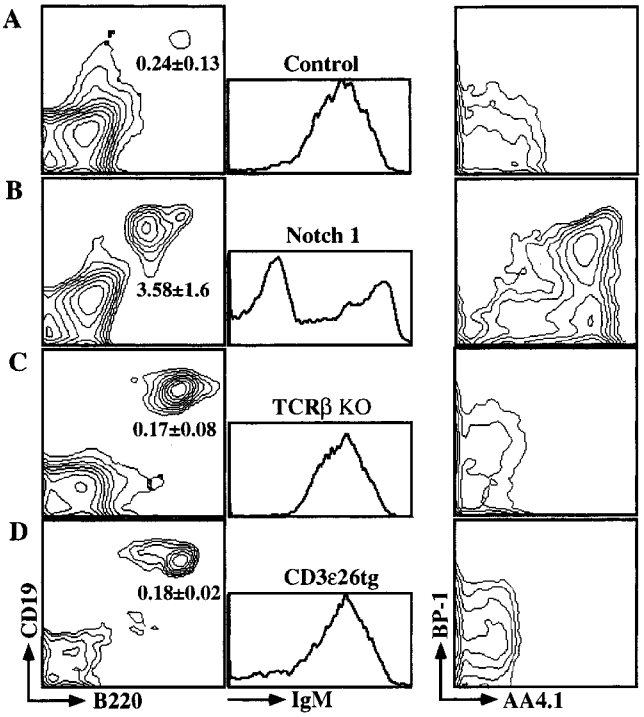


Figure 6. Comparison of thymic B cells in Notch1$^{-/-}$ versus other T cell–deficient mouse models. Number and phenotype of thymic B cells in (A) control, (B) day 28 adult Notch 1 deleted, (C) CD3ϵ26tg, and (D) TCRβ-deficient mice. CD19 versus B220 profiles on total thymus (left panels), expression of IgM (middle panels), and BP-1 versus AA4.1 (right panels) on gated B220^{+} B cells. The numbers are mean ± SD of total B cells ($\times 10^6$) per thymus from 7–10 mice of each genotype.

subsets. In contrast, all CD45.2^{+} cells derived from Notch 1–deficient BM are found localized in clusters throughout the medulla, and none are detectable in the cortex or outer cortical regions. As expected, staining of serial sections with a B cell–specific antibody (B220) shows that virtually all the CD45.2^{+} cells from Notch 1–deficient BM are indeed B cells. Thus similar to the normal resident thymic B cell population (14, 15), immature Notch 1$^{-/-}$ thymic B cells are localized to the thymic medulla and corticomedullary region.

Discussion

We have previously shown that in the absence of Notch 1 signaling in BM precursors, no T cells are produced either in the thymus or extrathymically in the gut (9, 11). At the same time, a heterogeneous population of B cells is observed accumulating in the thymus (9). One interpretation of these data is that a Notch 1–deficient CLP adopts a B cell fate instead of a T cell fate in the thymus. This hypothesis is in agreement with the previously well established function of Notch family genes in invertebrate bipotential cell fate decisions (1, 26, 27). However, an alternative possibility that cannot be excluded is that Notch 1–deficient precursors are unable to home from the BM to the thymus and consequently can no longer give rise to mature T cells.

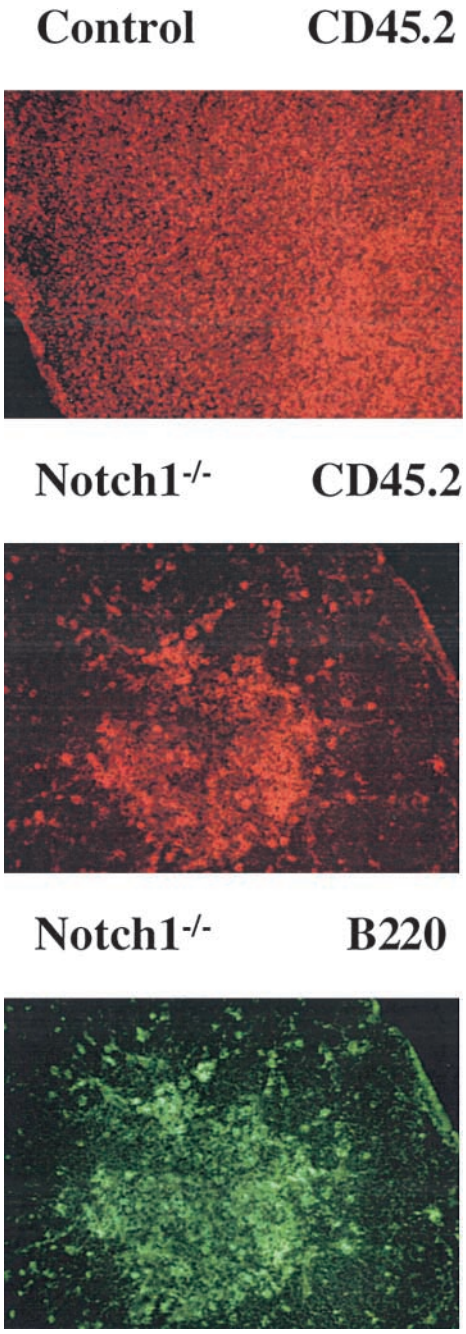


Figure 7. Tissue localization of intrathymic Notch1$^{-/-}$ B cells in mixed BM chimeras. Expression of CD45.2 (top two panels) and B220 (bottom panel) on serial sections of thymi reconstituted with control (top panel) or Notch 1 deleted (bottom panels) BM precursors.

If this were the case, the existence of a heterogeneous B cell population in the thymus could be the result of different scenarios which may or may not be dependent on Notch 1 deficiency in BM precursors, such as recirculation of BM B cells via the blood to the thymus (17) or enhanced development of resident thymic B cell precursors as a consequence of increased availability of thymic niches in the absence of normal T cell development and expansion (17, 20).

Our results to date demonstrating a strict requirement for Notch 1 in T cell development have been obtained using a competitive mixed BM chimera system. As inducible deletion of Notch 1 is close to 100% in the BM, this system provides an ideal model to evaluate the capacity of BM precursor cells to regenerate all hematopoietic lineages (9). However, BM chimeras are not optimal to address such questions as kinetics and population turnover, as these populations may be influenced by the altered environment of the irradiated host. Therefore, we have developed an alternative model in which Notch 1 is inducibly deleted in the BM of adult mice and the thymus analyzed at various time points after deletion. As the thymus is continually seeded from BM precursors during adult life the complete turnover of all populations of thymocytes occurs roughly once every 3–4 wk (23, 24, 28–31). In this way it is possible to follow the loss (or appearance) of defined intrathymic populations in a sequential manner if there is a complete block in the influx of BM CLPs to the thymus, or if those that do enter are unable to differentiate further along the T cell developmental pathway. Moreover, if a CLP adopts a default cell fate in the thymus, it should be possible to follow the kinetics of appearance of cells belonging to the default pathway. The thymic phenotype obtained in the Notch 1 adult deletion model resembles that already described in the mixed BM chimeras with respect to inhibition of T cell development, as well as the phenotype and accumulation of thymic B cells (9). However, the block in T cell development is less complete in adult Notch 1–deleted mice than in BM chimeras. The residual T cells present in adult deleted mice presumably remain either because their intrathymic precursors have not undergone Notch 1 deletion (which is much less efficient in the thymus than the BM) or due to the fact that their further development and/or maturation is no longer Notch 1 dependent. In the latter context we have recently shown that deletion of Notch 1 at the $CD25^+$ DN stage (using a Cre-recombinase transgene driven by a CD4 minigene promoter) does not detectably influence subsequent thymus development (32).

Notch 1–deficient thymic B cells can be first detected as a distinct population expressing lower levels of both CD19 and B220 (compared with resident B cells) around 7 d after deletion. Subsequently, their number increases to around $2–6 \times 10^6$ at 28 d. This accumulating population of B cells expresses several markers specific for immature B cells, such as BP-1, AA4.1, and CD25, is predominantly negative for mature B cell markers such as Sca-1, IgD, and class II, and is heterogeneous for IgM. This phenotypic analysis of Notch $1^{-/-}$ thymic B cells clearly identifies cells resembling all stages of BM B cell development up until surface expression of IgD (33–36), and is consistent with de novo ectopic B cell development in the thymus. This is in stark contrast to the small population of resident thymic B cells that can be isolated from normal thymus and which is predominantly composed of $CD19^{hi}$, $B220^{hi}$, IgM^+, IgD^+, $Sca\text{-}1^+$, $AA4.1^-$, $BP\text{-}1^-$, $CD25^-$ cells (14). Although putative B cell precursors have been described in normal thymus (16, 17), their phenotype is different to that observed in the adult deleted Notch 1–deficient thymus (16), and their absolute number is 10-fold less.

One simple explanation for the appearance of immature Notch 1–deficient B cells in the thymus is that there is an influx of recirculating BM B cells after pIpC induction of IFN-α (which is required to mediate Cre-recombinase deletion). If this were the case, one might expect to see immature B cells in the blood of both Notch 1–deficient and control mice, and perhaps immature B cells in the thymus of control mice as well. However, between 2 and 28 d after pIpC treatment no immature phenotype B cells have been observed in the blood of either Notch 1–deficient or control mice.

Another possibility is that whenever T cell development is severely impaired in the thymus, an increase in thymic B cells can be observed, perhaps due to an increase in the number of niches available for precursor seeding (17, 20). In the wild-type thymus the presence of normal numbers of T cells would exert an inhibitory effect on thymic B cell development. If this is the case, one could expect to find an increase in thymic B cells in all mouse models in which T cell (but not B cell) development is impaired at an early stage. Moreover, the developmental status and phenotype of those B cells should be comparable to those developing from Notch 1–deficient precursors. However, in the only two models in which such an increase in thymic B cells has been reported, the phenotype observed is essentially that of mature B cells, and the relative increase in B cell numbers is much less than in the Notch 1–deficient thymus (17, 20). Furthermore, the similar heterogeneous immature phenotype and dramatic increase in number of thymic B cells observed in Notch $1^{-/-}$ mixed BM chimeras, where the overall size of the thymus is normal (9), rules out an important role for availability of "niches" in ectopic B cell development in the thymus.

A unique advantage of the adult Notch 1 deletion model is that it permits analysis of the kinetics of loss of intrathymic T cell precursor subsets expressing CD117 and/or CD25 (DN 1, 2, and 3). The timing of this progressive decrease of T cell precursors confirms and extends previous studies using [^{3}H]thymidine uptake (28–30, 37, 38) and BrdU uptake (31), which have defined the time taken for a BM precursor to differentiate into a DP and subsequently SP mature thymocyte. Although no precursor/product studies have been performed, the kinetics of loss of T cell precursors and simultaneous accumulation of immature B cells are consistent with a model in which immature Notch 1–deficient B cells develop from CLP in the thymus as the default cell fate in the absence of Notch 1 signaling.

More direct evidence for the hypothesis that Notch 1–deficient thymic B cells arise in situ comes from a kinetic analysis of BrdU uptake. In comparison to mature, peripheral B cells, (or indeed, normal resident thymic B cells) Notch 1–deficient thymic B cells have a 10-fold faster rate of BrdU labeling. Indeed, the labeling kinetics of Notch 1–deficient thymic B cells is almost identical to that of BM B cells, strongly suggesting that they mature ectopically in the thymus.

Perhaps the most compelling argument that Notch 1 signaling controls the fate of bipotent T/B precursors in the thymus is provided by intrathymic reconstitution studies. Whereas (as expected) control BM precursors give rise exclusively to T lineage cells after intrathymic transfer, Notch $1^{-/-}$ BM precursors only give rise to immature B cells, with a phenotype indistinguishable from those seen in adult Notch 1–deleted mice. The complete absence of T cell reconstitution after intrathymic transfer of Notch $1^{-/-}$ BM precursors formally demonstrates that defective thymic homing of CLP per se cannot account for the block in T cell development in Notch 1–deficient mice. Furthermore, the efficient generation of immature B cells from intrathymic transferred Notch $1^{-/-}$ BM shows that a Notch 1–deficient precursor (most likely a CLP) can progress along an ectopic B cell developmental pathway in the thymus.

Taken together, our data strongly support the hypothesis that Notch 1–deficient CLPs which are unable to continue along the T cell developmental program due to the absence of Notch 1 signals, take the default cell fate decision, and undergo de novo B cell development in the thymus.

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