Notch1 Deficiency Dissociates the Intrathymic Development of Dendritic Cells and T Cells

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Abstract

Thymic dendritic cells (DCs) form a discrete subset of bone marrow (BM)-derived cells, the function of which is to mediate negative selection of autoreactive thymocytes. The developmental origin of thymic DCs remains controversial. Although cell transfer studies support a model in which T cells and thymic DCs develop from the same intrathymic pluripotential precursor, it remains possible that these two types of cells develop from independent intrathymic precursors. Notch proteins are cell surface receptors involved in the regulation of cell fate specification. We have recently reported that T cell development in inducible Notch1-deficient mice is severely impaired at an early stage, before the expression of T cell lineage markers. To investigate whether development of thymic DCs also depends on Notch1, we have constructed mixed BM chimeric mice. We report here that thymic DC development from Notch1

12/2 BM precursors is absolutely normal (in terms of absolute number and phenotype) in this competitive situation, despite the absence of Notch1

12 T cells. Furthermore, we find that peripheral DCs and Langerhans cells are also not affected by Notch1 deficiency. Our results demonstrate that the development of DCs is totally independent of Notch1 function, and strongly suggest a dissociation between intrathymic T cell and DC precursors.

Key words: Notch1 • dendritic cell • cell fate • T cell • development

Introduction

Dendritic cells (DCs) play a key role in T cell immune responses by endocytosing, processing, and presenting foreign antigens to specific T cells (1, 2). All DCs originate from bone marrow (BM) precursors, but it is not clear whether these precursors differentiate and mature to DCs in the BM itself, or whether they migrate to and differentiate within other organs.

Different lines of evidence point to the existence of two types of DCs in the mouse. Although all DCs express CD11c and MHC class II, the two subsets can be distinguished according to their differential expression of cell surface markers such as CD8α, M-ac-1, and DEC-205. The CD8α+M-ac-1+DEC-205 subset of DCs is thought to be of lymphoid origin, whereas the CD8α+M-ac-1+DEC-205−DCs are believed to be myeloid derived. In the spleen, where the function of DCs is to present foreign antigens to specific T cells, both subpopulations of DCs are present. However, thymic DCs whose function is to mediate negative selection of autoreactive thymocytes (3) constitute only the lymphoid-related subset (4–6).

The development of thymic DCs and T cells has been reported to be closely linked via an intrathymic common precursor (6, 7). In support of this hypothesis, intrathymic transfer of either early T cell precursors (CD4loCD44+CD25−CD117+) or progenitor T cells (CD4+CD44+CD25−CD117+) into sublethally irradiated mice results in the production of both thymic DCs and mature T cells. However, the next downstream population (defined as CD4−, CD44−CD25−, CD117+) has lost DC potential and generates only T cells (8). These data are consistent with a lymphoid-related origin for thymic DCs, and support the existence of a common intrathymic T/DC precursor.
curator (7, 8). Nevertheless, this hypothesis has not been proven at the clonal level, and it remains possible that the populations transferred contain distinct DC and T cell precursors that cannot be distinguished phenotypically.

Further evidence that DC and T cell development might be closely linked is derived from gene-targeted mice. Mice homozygous for an Ikaros dominant-negative mutation (deletion of the DNA-binding domain) lack all cells of lymphoid origin, such as T, B, and NK cells, as well as lymphoid- and myeloid-derived DCs (9). More interestingly, mice homozygous for an Ikaros null allele (deletion of the CooH terminus of Ikaros) lack B and NK cells, as well as lymph nodes, but maintain a certain T cell differentiation potential (10). Although no myeloid DCs were found in such mice, lymphoid-related DCs were present in the thymus, suggesting a correlation between T cell and thymic DC development (11).

Notch gene family members have been shown to play crucial roles in binary cell fate decisions in many developmental systems (12). Notch proteins are conserved transmembrane receptors containing EGF repeats in their ectodomain that are implicated in ligand binding. The cytoplasmic domain harbors six ankyrin repeats and is involved in intracellular signaling (13). To date, four mammalian Notch homologues (Notch1–4) that interact with transmembrane-bound ligands such as Jagged1, Jagged2, Delta1, and Delta-like3 have been identified (14–24).

Several reports suggest a role for Notch family members in T cell development. Notch1, 2, and 3 as well as the ligands Jagged1 and 2 have been shown to interact with transmembrane-bound ligands such as Jagged1, Jagged2, Delta1, and Delta-like3 have been identified (14–24).

Several reports suggest a role for Notch1 family members in T cell development. Notch1, 2, and 3 as well as the ligands Jagged1 and 2 have been shown to be expressed in thymocytes and thymic stromal epithelium (25–27). Expression of transdominant active forms of Notch genes in transgenic mice has implicated Notch1 signaling in T cell development at the level of both CD4 versus CD8 and α/β versus γ/δ cell fate decisions (28, 29). Notch1 signaling may also regulate proliferation, survival, and/or apoptosis of developing T cells (30, 31).

We recently reported the generation of mice in which the Notch1 gene was conditionally inactivated. Lethally irradiated recipients reconstituted with BM cells derived from induced Notch1−/− mice were devoid of T cells in both the thymus and periphery. Instead of T cells, immature B cells accumulated in the thymus of such BM chimeras, suggesting a possible role for Notch1 in regulating the T versus B lineage decision (32). In other studies, transfer of retrovirally infected BM-derived hematopoietic precursor cells expressing the cytoplasmic domain of Notch1 has recently been shown to impair early B cell development and promote ectopic T cell development in the BM (33).

Taken together, these complementary reports support a role for Notch1 in regulating T versus B cell fate specification from a bipotent common lymphoid progenitor (CLP).

The putative ability of Notch1 to regulate the B/T lineage decision in a binary fashion is of considerable interest in relation to the developmental origin of thymic DCs. It is generally accepted that progenitor B cells differentiate from a CLP in the BM, whereas CLPs that reach the thymus can adopt a T cell fate under the influence of the thymic microenvironment (34). Since thymic DCs are believed to be derived from an intrathymic common T/DC precursor that is downstream of the T/B lineage choice (7, 35), it is of obvious interest to investigate whether thymic DCs can arise from Notch1-deficient BM precursors.

Here, we show that although induced Notch1−/− hematopoietic precursors are unable to generate T cells, they retain their full potential to generate all other myeloid and lymphoid lineages, including thymic DCs.

Materials and Methods

Generation of Mice with a loxP-flanked Notch1 Allele, and Activation of the Cre Recombinase. Notch1−/loxlox and Notch1−/lox/+ Mx-Cre mice were generated as described previously (32). These mice are positive for the common leukocyte antigen CD45.2. Activation of the Cre recombinase was performed as described previously (32). In brief, adult mice received five intraperitoneal injections of 250 μg polyI-polyC (Sigma Chemical Co.) at 2-d intervals. 2 d after the last injection, mice were killed, and the BM was prepared for BM transplantation by T cell depletion. Genomic DNA was prepared from a portion of the T cell-depleted BM cells for assessing Notch1 deletion efficiency by Southern blot, and was quantified using a PhosphorImager (Molecular Dynamics).

Generation of Mixed BM Chimeras. Mice were generated using a 1:2 mixture (10 × 106 and 20 × 106) of T cell-depleted BM cells from polyI-polyC-treated C57BL/6 (CD45.1) and CD45.2−/− Notch1−lox/lox or induced Notch1−lox/lox mice. 10-wk-old hosts (C57BL/6, CD45.1; purchased from The Jackson Laboratory) were given 1,000 rads γ-irradiation 24 h before receiving the BM transplant. Radiation chimeras were maintained on antibiotic water and analyzed after 3 mo.

Flow Cytometry and Antibodies. Four-color FACS staining was performed as described elsewhere (36) using the following mAbs: anti-CD44-FITC or -biotin, anti-CD45.2-FITC or -biotin, anti-CD123–FITC or -PE, anti-CD3e–FITC or –allophycocyanin (APC), anti-Gr1–APC, anti-CD11b–PE, anti-CD44–CyChrome, anti-CD8α–CyChrome or –APC, anti-CD4–CyChrome, anti-CD25–CyChrome or –APC, and anti-NK1.1–PE (PharMingen). Anti-F4/80–biotin was purified and conjugated in this laboratory. Biotinylated antibodies were revealed with either Streptavidin–CyChrome (PharMingen) or Streptavidin–APC (Molecular Probes). All FACS® analysis was performed using four colors on a FACSCalibur™ flow cytometer (Becton Dickinson), and was analyzed using CELLQuest™ software (Becton Dickinson). Dead cells were excluded by live gating of forward scatter and side scatter, and 100,000–200,000 cells were analyzed in each file.

Isolation of DCs from the Thymus and Spleen. DC-enriched cell suspensions were prepared as described previously (37). In brief, organs were cut into small pieces and digested with collagenase A (0.5 mg/ml Boehringer Mannheim) and DNase I (40 μg/ml Boehringer Mannheim) in RPMI 1640 medium supplemented with 5% FCS for 10 min at 37°C with continuous agitation. Digested fragments were filtered through a sieve, and the cell suspension was washed twice in PBS supplemented with 5% FCS and 5 mmol/liter EDTA containing 5 μg/ml DNase I. The cells were then resuspended in cold isosomatic Optiprep™ solution (Nyegaard Diagnostics), pH 7.2, 1.061 g/cm3, containing 5 mmol/liter EDTA to dissociate DC–thymocyte complexes, and the low density fraction was obtained by centrifugation at 1,700 g.
Results

Experimental Strategy. As the block in thymocyte development after induced inactivation of Notch1 is at a very early stage (32), Notch1 may also be involved in thymic DC differentiation, affecting a putative intrathymic common T/DC precursor. Since the deletion efficiency of Notch1 (mediated by the IFN-α-inducible MxCre transgene) in the thymus is only ~40%, but is close to 100% in the BM, we decided to investigate this question using BM chimeras. To this end, CD45.2−Notch1\textsuperscript{lox/lox} MxCre\textsuperscript{+−} (Notch1\textsuperscript{−/−}), CD45.2\textsuperscript{+} Notch1\textsuperscript{lox/lox} (control), and CD45.1\textsuperscript{+} wt mice were treated with the IFN-α inducer polyI-polyC five times at 2-d intervals to delete an essential portion of the Notch1 gene (Fig. 1 A). 2 d after the last injection, BM was harvested and prepared for transfer. To assess the Notch1 deletion efficiency, genomic DNA was prepared from a sample of induced Notch1\textsuperscript{−/−} or control BM and analyzed by Southern blot. The deletion efficiency in Notch1\textsuperscript{−/−} BM was close to 100%, as expected (Fig. 1 B). Lethally irradiated wt hosts (CD45.1\textsuperscript{−}) were reconstituted with either CD45.2\textsuperscript{−} Notch1\textsuperscript{−/−} BM or CD45.2\textsuperscript{+} control BM, each mixed in a 2:1 ratio with CD45.1\textsuperscript{−} wt BM (hereafter described as Notch1\textsuperscript{−/−} or control chimeras, respectively), and analyzed 3 mo later for the different myeloid and lymphoid lineages derived from each donor population.

Notch1\textsuperscript{−/−} BM Precursors Fail to Generate Thymic or Peripheral T Cells. The distribution of the two allelic markers CD45.1 and CD45.2, which denote the donor origin of the cells, showed an equivalent contribution of leukocytes from each donor population in the blood of both control and Notch1-deleted chimeras (Fig. 2 A). In the control chimeras, B220\textsuperscript{+} (B) and CD3\textsuperscript{+} (T) cells derived from both CD45.1\textsuperscript{+} and CD45.2\textsuperscript{+} BM were present, as expected. However, in the Notch1-deleted chimeras, although B220\textsuperscript{+} B cells were derived from both donor populations, >99% of CD3\textsuperscript{+} T cells were derived from the CD45.1\textsuperscript{+} wt BM, and <0.3% from the CD45.2\textsuperscript{+} Notch1-deficient BM (Fig. 2 A).

In the thymus of control chimeras, reconstitution was similar to that observed in blood and other peripheral organs with an equal contribution from both CD45.1\textsuperscript{+} and CD45.2\textsuperscript{+} BM donor populations. In contrast, in the Notch1-deleted chimeric thymus, 98% of cells were derived from CD45.1\textsuperscript{+} wt BM (Fig. 2 B), confirming that Notch1-deficient precursors are unable to generate T cells in a competitive situation (32).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Inducible targeting of the Notch1 gene. (A) Schematic representation of the murine Notch1 gene. The Notch1 protein contains a signal peptide, 36 EGF repeats followed by a cysteine-rich domain (LN) in the extracellular domain, a transmembrane domain (TM), cytoplasmic ankyrin repeats (Cdc10), and a PEST sequence. The genomic organization of the Notch1 locus derived from control mice is partially shown (1); the exon coding for the leader peptide (filled square) is flanked by two loxP sequences (gray triangles), followed by the exon coding for the first EGF repeat (gray box). After induction of the Cre recombinase, the genomic portion harboring the exon coding for the leader peptide is deleted (2). Arrows indicate EcoRI fragments that differ in size between the genomic locus derived from control mice and the locus after deleting the loxP-flanked gene segment (induced Notch1\textsuperscript{−/−}). (B) Southern blot analysis of EcoRI-digested genomic DNA derived from the BM used for setting up the mixed chimeric mice. The probe indicated in A reveals a 5.8-kb fragment for the allele derived from control animals (1), whereas deletion of the flanked loxP segment gives rise to a 2.3-kb fragment (2). Deletion efficiency was calculated after PhosphorImager analysis to be >98%.

for 10 min. This low density fraction was washed twice in PBS-EDTA-FCS. DCs were identified in the low density cell fraction as MHC class II+CD11c+B220−F4/80− cells.

Isolation of Langerhans Cells from the Skin. Langerhans cell (LC) preparation was performed as described previously (37, 38). In brief, ears were rinsed with 70% ethanol, split using forceps into dorsal and ventral halves, and incubated with 0.5% trypsin (Sigma Chemical Co.) in PBS containing 5% FCS for 30 min at 37°C to allow the separation of the epidermal sheets from dermis. Subsequently, epidermal sheets were cultured for 24 h in 24-well tissue culture plates in the presence of 100 ng/ml GM-CSF (provided by Immunex Corp., Seattle, WA). LCs, together with keratinocytes, were released into the culture medium. Epidermal cell suspensions were obtained by filtering the culture medium through a sieve, and were washed in PBS with 5% FCS. LCs were identified in the epidermal cell suspension as MHC class II+, CD11c+, and B220− cells. Analyses of other hematopoietic lineages such as granulocytes, macrophages, B cells, and NK cells were performed on BM and spleens from individual mice.

![Diagram A](https://example.com/diagramA.png)

**A**

Notch1

![Diagram B](https://example.com/diagramB.png)

**B**

Control

induced Notch1−/−

Figure 2. CD8+ and CD4+ T cell and BM Precursor Failure in Notch1−/− Chimeras. (A) Flow cytometry analysis to be...
Notch1<sup>−/−</sup> BM precursors are capable of generating peripheral DCs and LCs. To investigate whether Notch1 deficiency could also affect the generation of myeloid-derived DCs, we analyzed splenic DCs in the same chimeric mice. As shown in Fig. 4, both lymphoid-related DCs (characterized by the expression of CD8<sup>a</sup>) and myeloid-related DCs (which are CD8<sup>a</sup>−) were detected in similar numbers in the spleens of control and Notch1<sup>−/−</sup> BM chimeras. This result indicates that Notch1 deficiency does not affect the generation of either myeloid- or lymphoid-related splenic DCs.

Skin DCs, known as LCs, are immature DCs that differentiate into mature DCs after antigenic stimulation and migrate to the T cell areas of the draining lymph nodes (1). To investigate if the generation of LCs was affected by the loss of Notch1, LCs were isolated from the epidermis of Notch1<sup>−/−</sup> BM chimeras. The bars represent average values of pooled thymi derived from eight BM chimeras from each group. In the bar diagram, gray bars correspond to wt (CD45.1), and stippled bars correspond to control (Notch1<sup>lox/lox</sup>, CD45.2) or induced Notch1<sup>−/−</sup> (CD45.2) derived cell populations. On the right is shown the distribution of CD44<sup>+</sup> and B220<sup>+</sup> cells in the double-negative (CD4<sup>+</sup>CD8<sup>+</sup>) compartment of mixed BM chimeras constructed with wt (CD45.1) and induced Notch1<sup>−/−</sup> (CD45.2) BM cells.
mouse ears from both control and Notch1−/− chimeric mice. As shown in Fig. 5, MHC class II-expressing LCs derived from Notch1−/− BM were present to a similar extent as LCs derived from control BM. Therefore, development of this form of immature DC likewise appears to be unaffected by the absence of Notch1.

Notch1 deficiency does not influence granulocyte, macrophage, NK cell, or B cell development. To investigate whether the absence of Notch1 affects other myeloid or lymphoid lineages, control or Notch1-deficient chimeric BM was analyzed for the presence of granulocytes, macrophages, and immature B cells by staining for the expression of Gr1, Mac-1, or B220, together with the corre-

Figure 3. Induced Notch1−/− BM generates thymic DCs in mixed BM chimeras. (A) Mixed chimeric mice were analyzed 3 mo after reconstitution with a 1:2 mixture of wt (CD45.1) and control (Notch1lox/lox, CD45.2) or induced Notch1−/− (CD45.2) BM-derived populations. Thymi of these chimeric mice were pooled, and the DC fraction was enriched as described in Materials and Methods. The DC-enriched fraction was stained using anti-F4/80, anti-CD11c, anti-MHC class II, and anti-CD8α antibodies. The graph shows a FACS® analysis of CD11c versus MHC class II gated on F4/80+ cells, as well as histograms for the expression of CD8α gated on CD11c+ MHC class II+ cells. (B) Absolute cell numbers of thymic DCs derived from control and Notch1−/− chimeric mice. The bars represent average values per thymus of two independent experiments, and the triangles represent the average number of DCs per thymus from pooled thymi. n = 4 for wt (CD45.1) plus control (CD45.2), and n = 8 and 7 for wt (CD45.1) plus Notch1−/− (CD45.2) chimeras.
Notch1 is expressed on BM CD34

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Discussion

The data presented in this report demonstrate that Notch1 deficiency dissociates the development of T cells from all other known myeloid and lymphoid lineages. Thus, although T cell development from Notch1−/− precursors is arrested at a very early stage, the development of B cells, NK cells, macrophages, granulocytes, and DCs (including both thymic and splenic DCs, as well as LCs) is unaffected by Notch1 deficiency, even in the situation of competitive repopulation in mixed BM chimeras. Collectively, these results reveal an obligatory and selective role of Notch1 in T cell fate determination.

Implications for Myeloid Differentiation.

The finding that Notch1 is expressed on BM CD34+ progenitors (39), together with the observation that Notch ligands are expressed on BM stromal cells, initially led to the suggestion of a role for the Notch pathway in myeloid and/or erythroid differentiation (40, 41). Further evidence for Notch function in myelopoiesis comes from studies using 32D cells, which are progenitor cells that can differentiate into granulocytes in the presence of a cytokine cocktail. Expression of a dominant active form of Notch or activation of full-length Notch1 by Jagged1 (a ligand for the Notch receptors) was found to inhibit differentiation of 32D cells in response to the cytokine cocktail (40, 42). Our data indicating that Notch1 is dispensable for the development of DCs, granulocytes, and macrophages in vivo appear to conflict with previous results suggesting that constitutive Notch1 activity perturbs myeloid differentiation (40, 43). This discrepancy may be due either to the experimental protocol used for studying myeloid differentiation, or simply to the fact that we cannot exclude the possibility that redundant signaling from other Notch gene family members may rescue the development of myeloid lineages. Nevertheless, our results are in agreement with a recent report in which retroviral infection of BM cells with a dominant active form of Notch1 did not affect myeloid differentiation, although it had profound effects on lymphoid differentiation (33).

Implications for B/T Cell Fate Determination.

Two independent lines of evidence implicate Notch1 in a binary lineage decision of a CLP to develop into a B cell or a T cell. First, as shown here and elsewhere (32), Notch1−/− BM precursors are unable to develop into T cells in the thymus of lethally irradiated wt recipients. However, instead of T cells, immature B cells of Notch1−/− origin (with a phenotype indistinguishable from those normally found in BM) accumulate in the irradiated wt thymus. In reciprocal experiments, Pui et al. (33) have shown that transferred BM cells overexpressing the constitutively active Notch1 intracellular domain do not develop into B cells in the BM of lethally irradiated recipients. Instead, immature T cells (mainly of the CD4+CD8+ phenotype) accumulate in the BM of these chimeric mice.

Taken together, these complementary “loss-of-function” and “gain-of-function” studies clearly indicate that varying levels of Notch1 expression not only control the development of T and B cells, but in addition are capable of promoting ectopic development of either lineage at the expense of the other. By analogy with the role of Notch genes in invertebrate systems (12), the simplest interpretation of these data would be that Notch1 provides a critical signal that determines a binary (T or B) cell fate decision by a CLP. Since the absence of Notch1 blocks T cell development and promotes ectopic B cell development, whereas Notch1 overexpression promotes ectopic T cell development at the expense of B cells, we would hypothesize that B cell development from the CLP represents the default pathway or primary cell fate that occurs in the absence of Notch1 signaling. According to this scenario, T cell development would be the secondary fate of a CLP, and would require Notch1 signaling.

Implications for the Existence of a Common Intrathymic T/DC Precursor.

Two types of experiments have led to the
widely held hypothesis (Fig. 7 A) that thymic DCs are a lymphoid lineage derived from a common intrathymic precursor that also gives rise to T cells: (a) intrathymic transfer of sorted populations of CD44+/CD4−/CD25− progenitor T cells gives rise to both thymic DCs and T cells in sublethally irradiated recipients (7, 8); and (b) the development of thymic DCs and T cells could not be clearly dissociated in gene-targeted mice deficient for either Ikaros or RelB transcription factors (10, 11, 44). However, both of these lines of evidence are ultimately based on correlations, and it remains possible that thymic DCs and T cells are derived from independent precursors that cannot be phenotypically distinguished.

The clear-cut dissociation of intrathymic T cell and DC development in chimeric mice reconstituted with Notch1−/− BM cells provides a serious challenge to the hypothesis that thymic DCs represent a lymphoid lineage derived from a common T/DC precursor. Indeed, as shown here and elsewhere (32), T cell development from Notch1−/− BM precursors is blocked at a very early stage (before expression of CD25), and preliminary results suggest that the few remaining CD44+ intrathymic cells of Notch1−/− origin do not express typical markers of CLPs such as Sca-1 and CD117 (data not shown). Nevertheless, thymic DC development is totally unaffected (in terms of both phenotype and absolute cell numbers) by Notch1 deficiency. Taken together with the evidence that Notch1 is dispensable for the development of all myeloid and lymphoid lineages with the exception of T cells, Moreove, taken together with a recent report by Rodewald et al. (45), they seriously challenge the widely held hypothesis that thymic DCs and T cells share a common intrathymic precursor.

In conclusion, our data demonstrate that Notch1 is dispensable for the development of all myeloid and lymphoid lineages with the exception of T cells. Moreover, taken together with a recent report by Rodewald et al. (45), they seriously challenge the widely held hypothesis that thymic DCs and T cells share a common intrathymic precursor.

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