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J Immunol 2002; 169:1817-1821;
doi: 10.4049/jimmunol.169.4.1817
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A Reassessment of the Effect of Activated Notch1 on CD4 and CD8 T Cell Development

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The Notch signaling pathway plays an important role in the early steps of T cell development and in the generation of T cell tumors, but its role in the CD4 vs CD8 lineage decision is controversial. Notch1 is not essential for CD4 or CD8 T cell development; however, there are suggestions that multiple Notch family members may act in a redundant fashion during thymic development. In theory, expressing a constitutively activated form of Notch in CD4+CD8+ thymocytes could provide clues about the normal role of Notch in developing CD4 and CD8 T cells. Unfortunately, two different studies of transgenic mice expressing activated forms of Notch1 (Notch1IC) led to conflicting conclusions. In this study, we re-examine the effect of the two Notch1IC transgenes on thymocyte development. We find that both Notch1IC transgenic lines display a decrease in CD4 single positive (SP) thymocytes and a corresponding increase in CD8 SP thymocytes. The enhanced development of CD8 SP thymocytes is dependent on either class I or II MHC. Thus, data from two different Notch1IC transgenic lines indicate that Notch activity promotes CD8 and inhibits CD4 SP development. We suggest that the discrepancies in previous reports of Notch1IC transgenic mice are due to differences in the propensity of the two different transgenic lines to develop tumors. The Journal of Immunology, 2002, 169: 1817–1821.

The TCR signaling pathway plays a crucial role in directing cell fate in the thymus, but it does not do so alone. Signals through the TCR must integrate with evolutionarily conserved developmental signaling pathways, such as the Wnt and Notch pathways to direct T cell fate. Initial reports that Notch1 is expressed in thymocytes, together with evidence that Notch activity can contribute to T cell tumors, prompted investigation into the role of Notch in T cell fate decisions. It is now clear that Notch plays an important role in early T cell fate decisions (1–3); however, its role in the development of CD4 and CD8 T cells from CD4+CD8+ precursors remains controversial (4).

The first indication that Notch activity could promote CD8 single positive (SP) development came from analysis of the effect of activated Notch1 on thymic development (5). Transgenic mice expressing an activated form of Notch1 under the control of the Lck proximal promoter display an increase in CD8 SP thymocytes. In addition, activated Notch1 promotes CD8 SP development in MHC class I-deficient mice, but not in mice lacking both class I and II MHC. These results indicated that constitutive Notch activity could divert thymocytes bearing class II-specific TCRs into the CD8 lineage. A model for normal development was proposed in which MHC class I promotes the CD8 fate by up-regulating Notch activity.

Recently, a role for Notch in CD8 SP development has been called into question by analysis of a conditional Notch1 mutation (6). Deletion of the Notch1 locus by a CD4-driven CRE transgene does not lead to any alterations in the numbers or proportions of CD4 and CD8 SP thymocytes. However, thymocytes also express Notch2, Notch3, and Notch4 (7, 8), raising the possibility that multiple Notch family members might contribute to CD4 or CD8 SP development in a redundant fashion. Indeed, kinetic analysis of the development of thymocytes deficient for Notch1 reveals a delay in the appearance of CD8 SP, suggesting that Notch1 makes a nonessential contribution to CD8 SP development (6).

Further support for the idea that Notch promotes CD8 SP development comes from experiments in which Notch activity is blocked in fetal thymic organ culture (9, 10). Additions of inhibitors of presenilin, a family of proteins that are essential for Notch activity, inhibit the development of CD8 SP thymocytes. In addition, Notch1 antisense RNA expression and Notch1 Abs prevent the developmental progression of CD8 SP in a two-step culture system for thymic development (11).

Although all of these studies are consistent with a role for Notch signaling in promoting CD8 SP development, the effect of Notch activity on CD4 T cell development is less clear. The original description of Notch1 (Notch1IC) transgenic mice reported a decrease in the development of CD4 SP, but a recent study came to a different conclusion (12). In this study, which examined a slightly different version of the Notch1IC transgene, an increase in both CD8 SP and CD4 SP thymocytes was reported. Moreover, the Notch1IC transgene appeared to drive the development of CD4 and CD8 SP thymocytes in the absence of class I and II MHC. In addition to the apparent differences in CD4 and CD8 SP development, the modified Notch1IC transgene promoted early tumorigenesis. Thus, there was the possibility that these tumors might obscure effects of Notch on CD4 vs CD8 lineage commitment.

To clarify the effect of activated Notch1 on the development of CD4 and CD8 SP, we examined young mice before tumors appear. This analysis indicates that the two Notch1IC transgenic lines have very similar effects on thymic development, leading to extra CD8 SP thymocytes at the expense of CD4 SP. Activated Notch1 allows CD8 SP to develop in class I-deficient mice, but does not permit the development of CD4 SP thymocytes in class II MHC-deficient mice. Moreover, the enhanced development of CD8 SP thymocytes...
by activated Notch1 depends on the expression of either class I or II MHC. Thus, analysis of both versions of Notch1IC transgenic mice indicates that Notch activity favors CD8 SP and inhibits CD4 SP development. These results are consistent with a model in which Notch signaling regulates CD4 vs CD8 lineage determination.

Materials and Methods

Mice

C57BL/6j (B6) were obtained from the National Cancer Institute (Frederick, MD) and MHC-deficient, C57BL/6j-KOβ-J,−M-KOβ−/− N17 (line 80; Ref. 13) from a National Institutes of Allergy and Infectious Diseases (NIAID; Bethesda, MD) breeding contract to Taconic Farms (Germantown, NY). Notch1IC mice (5) had been maintained in a NIAID facility since 1996. Notch1IC-transactivation domain (TAD) mice (12) were kindly provided by M. Bevan (University of Washington, Seattle, WA). Mice were bred, maintained, and used in NIAID Research Animal Facilities according to American Association of Laboratory Animal Care specifications and on protocols approved by NIAID Animal Care and Use Committee. Transgenic mice were genotyped for Notch1IC transgenics by PCR and for MHC by flow cytometry using standard protocols.

Cell preparation, Abs, and flow cytometry

Thymocytes were prepared in single-cell suspensions, stained with labeled Abs, and analyzed by flow cytometry as described (14, 15). Cells were pretreated with a culture supernatant containing anti-FcRγIII/IIIR, 2.4G2, to block FcR binding by labeled Abs. PBLs were used for some MHC typing. After staining with labeled Abs, samples were depleted of RBCs with ACK lysing buffer (pH 7.4) and analyzed by flow cytometry. Labeled mAbs included: anti-TCRβ-FITC (H57-597), -CD4-PE (RM4-5), -CD8α-allophycocyanin (53-6-7), -CD25-FITC (7D4), -CD44-PE (IM7), and -H-2K b-FITC (AF6-88.5) obtained from BD PharMingen (San Diego, CA); anti-Aβ-FITC (28-16-85) and -CD45R/B220-PE (RA3-6B2) obtained from Cahag Laboratories (Burlingame, CA). Flow cytometry was performed on a FACScan (Hewlett Packard, Mountain View, CA). Dead cells were excluded by light scatter and propidium iodide gating. A total of 150,000 events were collected for three- and four-color analyses.

Results

Two independent analyses of mice expressing activated forms of Notch1 produced very different results and conclusions (5, 12). Although the Notch1IC transgenic constructs described in the two reports both contain intracellular portions of the mouse Notch1 gene expressed under the control of the Lck proximal promoter, the Notch1IC described by Deftos et al. (12) contained an extended C-terminal sequence (Fig. 1). This region of Notch1 contains a TAD that contributes to the oncogenic activity of Notch1IC (16). The complete TAD is included in the construct of Deftos et al. (12), but is partially deleted in the construct of Robey et al. (5). In this study, we designate the Notch transgenic mice of Deftos et al. (12) as Notch1IC-TAD to indicate this difference.

Previous analysis of Notch1IC-TAD transgenic mice examined 5- to 12-wk-old mice (12). This study noted that mice develop tumors at 4 wk of age, raising the possibility that such tumors could obscure the effects of activated Notch on CD4 and CD8 SP development. To minimize these complications, we examined development in young Notch1IC-TAD mice, 10–21 days after birth. Similar to what was reported previously for Notch1IC mice, the development of CD8 SP thymocytes (CD4<sup>+</sup>CD8<sup>+</sup>TCR<sub>β</sub>high) in young Notch1IC-TAD mice is increased (Fig. 2), and the vast majority of these CD8 SP thymocytes are heat-stable Ag<sup>low</sup> (data not shown). However, contrary to the Deftos et al. (12) conclusion that Notch1IC promotes the development of CD4 as well as CD8 SP thymocytes, our analysis of young Notch1IC-TAD mice reveals that the absolute number of CD4 SP thymocytes is diminished (Fig. 2).

Analysis of both the percentage and absolute number of thymocyte subsets shows a decrease in CD4 SP and an increase in CD8 SP thymocytes. For comparison, we examined the two Notch1IC transgenic lines at a similar age (Fig. 3). Both sets of mice give indistinguishable results in this analysis. In contrast, our examination of older (>5 wk) Notch1IC-TAD mice showed a large mouse-to-mouse variation in the proportion of thymic subsets, and in some cases a reduced CD4<sup>+</sup>CD8<sup>+</sup> population (data not shown), consistent with the published data (12). In addition, a significant proportion of these older Notch1IC-TAD transgenic mice had tumors that appear to be of thymic origin, based on their expression of CD4 and CD8 and their thymic location (data not shown). The greater mouse-to-mouse variation seen in >5-wk-old Notch1IC-TAD transgenic mice compared with <3-wk-old mice, together with indications that the majority of older Notch1IC-TAD transgenic mice are about to succumb to tumors (data not shown), suggests that analysis of thymocyte populations in 3 wk provides a

![FIGURE 1](http://www.jimmunol.org/) Comparison of two versions of activated Notch1 transgenic constructs. Schematic representations are shown of the Notch1IC transgenes reported in Refs. 5 and 12. Both transgenes consist of intracellular portions of the mouse Notch1 gene under the control of the Lck proximal promoter (30). A. The Notch1IC mice described by Deftos et al. (12) contain a complete RAM domain, ankry repeat region, and an extended C-terminal region that includes a full TAD (20) (aa 1750–2293). This transgene is designated as Notch1IC-TAD. B. The Notch1IC mice described by Robey et al. (5) contain a portion of the TAD domain (aa 1751–2444) and is designated as Notch1IC.
FIGURE 3. Both versions of Notch1IC transgenic mice display decreased CD4 SP and increased CD8 SP development compared with nontransgenic littersmates. Thymocytes from young Notch1IC transgenic mice were analyzed for expression of CD4, CD8, and TCRβ by three-color flow cytometry. Absolute number (A and B) and the percentage (C and D) of CD4+ CD8+ TCRβ<sup>high</sup> (CD4 SP) or CD4<sup>+</sup> CD8<sup>+</sup> TCRβ<sup>high</sup> (CD8 SP) thymocytes are displayed for transgenic (Tg<sup>+</sup>) and nontransgenic (Tg<sup>-</sup>) littersmates of Notch1IC (A and C) or Notch1IC-TAD (B and D) mice. Each bar represents the mean (with SE bars or dots for values from individual mice) of 3–4 mice per group. Data are derived from single litters analyzed on days 17 (A and C) and 18 (B and D) after birth.

more reliable indicator of the effect of NotchIC on the CD4/CD8 lineage decision.

Another apparent discrepancy between the two reports of Notch1IC transgenic mice is the requirement for MHC for positive selection. We previously found that positive selection on either class I or II MHC is required for CD8 SP development in mice expressing Notch1IC (5, 17). In contrast, the Notch1IC-TAD transgene was reported to drive the development of both CD8 and CD4 SP in the absence of positive selection (12). This conclusion was based on the appearance of CD4 and CD8 SP thymocytes in radiation chimeras in which bone marrow from Notch1IC-TAD transgenic mice was used to reconstitute MHC-deficient hosts.

To further explore the role of MHC in T cell development in Notch1IC transgenic mice, we crossed Notch1IC-TAD transgenic mice to MHC-deficient mice and examined thymic subsets in mice from the second back-cross. Again, the analysis was confined to young (10–21 days old) mice to avoid the complicating effects of tumors. As shown in Fig. 4, CD8 SP thymocytes develop in class I- and II-deficient mice, but not in mice deficient for both class I and II MHC. Moreover, there is no increase in CD4 SP thymocytes in class II-deficient mice. These results are in accord with previous analyses of Notch1IC transgenic mice (5, 17), and suggest that constitutive Notch activity can direct thymocytes bearing class I and II-specific TCRs to the CD8 lineage, but does not override the requirement for positive selection. We suspect that the CD4 and CD8 SP cells seen in MHC-deficient mice reconstituted with Notch1IC-TAD bone marrow may represent tumors that arose in the Notch1IC-TAD mice that were used as bone marrow donors in these studies.

It was reported that many Notch1IC-TAD mice develop tumors at 4 wk of age (12). Indeed, we observe that the majority of Notch1IC-TAD transgenic mice are moribund or die by 80 days after birth (Fig. 5A). Biopsy of several moribund Notch1IC-TAD transgenic mice revealed the presence of thymic tumor masses (data not shown). In contrast, Notch1IC mice develop tumors later (2–6 mo) and with a much lower frequency. Also noted was that the tumors of Notch1IC-TAD transgenic mice express CD4, CD8, and TCRβ expression by three-color flow cytometry. A, Representative histograms obtained from analysis of thymocytes for CD4 and CD8 using software gating to display only data from TCRβ<sup>hi</sup> cells. Numbers inside the quadrants indicate the percentage of total thymocytes in each subpopulation. B, Data compiled from analyses of several litters. Bars represent the mean percentage (with SE bars) of CD4<sup>+</sup> CD8<sup>+</sup> TCRβ<sup>hi</sup> (CD4 SP) or CD4<sup>-</sup> CD8<sup>+</sup> TCRβ<sup>hi</sup> (CD8 SP) thymocytes of transgenic (Tg<sup>+</sup>) and nontransgenic (Tg<sup>-</sup>) littersmates. Number of mice analyzed in each group: wild type, Tg<sup>-</sup>, n = 9, Tg<sup>+</sup>, n = 10; class I<sup>+</sup>, Tg<sup>-</sup>, n = 11, Tg<sup>+</sup>, n = 11; class II<sup>+</sup>, Tg<sup>-</sup>, n = 6, Tg<sup>+</sup>, n = 10; MHC<sup>-</sup>, Tg<sup>-</sup>, n = 10, Tg<sup>+</sup>, n = 9.

FIGURE 4. Requirement for MHC class I or II for CD8 SP development in Notch1IC-TAD transgenic mice. Mice bearing the Notch1IC-TAD transgene were crossed with MHC-deficient (MHC<sup>-</sup>) (β<sub>2</sub>-microglobulin-deficient (class I<sup>+</sup>) and A<sup>2</sup>-deficient (class II<sup>+</sup>)) B6 mice. At 10–21 days after birth, thymocytes from offspring of the second back-cross were analyzed for CD4, CD8, and TCRβ expression by three-color flow cytometry. A, Representative histograms obtained from analysis of thymocytes for CD4 and CD8 using software gating to display only data from TCRβ<sup>hi</sup> cells. Numbers inside the quadrants indicate the percentage of total thymocytes in each subpopulation. B, Data compiled from analyses of several litters. Bars represent the mean percentage (with SE bars) of CD4<sup>+</sup> CD8<sup>+</sup> TCRβ<sup>hi</sup> (CD4 SP) or CD4<sup>-</sup> CD8<sup>+</sup> TCRβ<sup>hi</sup> (CD8 SP) thymocytes of transgenic (Tg<sup>+</sup>) and nontransgenic (Tg<sup>-</sup>) littersmates. Number of mice analyzed in each group: wild type, Tg<sup>-</sup>, n = 9, Tg<sup>+</sup>, n = 10; class I<sup>+</sup>, Tg<sup>-</sup>, n = 11, Tg<sup>+</sup>, n = 11; class II<sup>+</sup>, Tg<sup>-</sup>, n = 6, Tg<sup>+</sup>, n = 10; MHC<sup>-</sup>, Tg<sup>-</sup>, n = 10, Tg<sup>+</sup>, n = 9.

Notch1IC-TAD transgenic mice are moribund or die by 80 days after birth (Fig. 5A). Biopsy of several moribund Notch1IC-TAD transgenic mice revealed the presence of thymic tumor masses (data not shown). In contrast, Notch1IC mice develop tumors later (2–6 mo) and with a much lower frequency. Also noted was that the tumors of Notch1IC-TAD transgenic mice express CD4, CD8, and TCRβ expression by three-color flow cytometry. A, Representative histograms obtained from analysis of thymocytes for CD4 and CD8 using software gating to display only data from TCRβ<sup>hi</sup> cells. Numbers inside the quadrants indicate the percentage of total thymocytes in each subpopulation. B, Data compiled from analyses of several litters. Bars represent the mean percentage (with SE bars) of CD4<sup>+</sup> CD8<sup>+</sup> TCRβ<sup>hi</sup> (CD4 SP) or CD4<sup>-</sup> CD8<sup>+</sup> TCRβ<sup>hi</sup> (CD8 SP) thymocytes of transgenic (Tg<sup>+</sup>) and nontransgenic (Tg<sup>-</sup>) littersmates. Number of mice analyzed in each group: wild type, Tg<sup>-</sup>, n = 9, Tg<sup>+</sup>, n = 10; class I<sup>+</sup>, Tg<sup>-</sup>, n = 11, Tg<sup>+</sup>, n = 11; class II<sup>+</sup>, Tg<sup>-</sup>, n = 6, Tg<sup>+</sup>, n = 10; MHC<sup>-</sup>, Tg<sup>-</sup>, n = 10, Tg<sup>+</sup>, n = 9.

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transgenic mice develop tumors by 4 wk, and Deftos et al. (12) analyzed mice at 5–12 wk of age. The presence of tumors could distort the results in a variety of ways. For example, transformation could lead to changes in the expression of surface molecules, and/or could cause the abnormal proliferation or expansion of subsets of thymocytes. Alternatively, the presence of tumors could indirectly distort thymocyte populations, because the stress associated with illness could lead to loss of CD4+CD8+ thymocytes, causing an increase in the proportion of SP thymocytes. Because the current study examines young mice before the onset of tumors, it is likely to provide a more accurate picture of the effect on activated Notch1 on T cell development. Confounding effects of tumors might also explain the lack of an obvious CD4/CD8 phenotype in transgenic mice expressing an activated form of Notch1C3 in thymocytes (18). Retroviral expression of Notch1IC has been reported to lead to diminished CD4 SP and CD8 SP development (19). This inhibition of both CD4 and CD8 SP development in this system may be due to higher levels of expression of activated Notch1 or the continued expression of activated Notch1 in SP thymocytes.

The differences between the two versions of Notch1IC focuses attention on the portion of the Notch1 intracellular domain from aa 2294–2444, residues that are included in the Notch1IC transgenic construct of Deftos et al. (12), but are missing from the construct of Robey et al. (5). This region of Notch1 forms part of a domain referred to as TAD, because it has been shown to contribute to the transactivation function of Notch1C (20). Although it appears that the TAD domain of Notch1 can contribute to Notch signaling, it does not appear to be essential. Expression of a minimal ankyrin repeat region of Drosophila Notch or the related Caenorhabditis elegans proteins, glp-1 and lin-12, is sufficient to confer an activated Notch phenotype (21). In addition, the transgenic version of Notch1IC that lacks the full TAD domain still induces the up-regulation of the Notch target gene, hairy enhancer of split homolog 1 (HES1; Refs. 17 and 22). Finally, a similarly truncated form of Notch1 overrides the block in Notch signaling due to ectopic expression of lunatic fringe (3). Thus, it appears that the TAD domain provides an accessory function to Notch that may contribute to the level of Notch signaling, but is not essential for Notch activity.

It is noteworthy that the TAD region has also been identified as contributing to the oncogenic activity of Notch1IC (16). This finding provides a possible explanation for the more rapid development of tumors in Notch1IC-TAD compared with Notch1IC transgenic mice that do not contain the full TAD domain. However, we cannot exclude the possibility that transgene copy number or integration site could contribute to the difference in oncogenic activity of the two transgenes.

The issue of whether Notch activity inhibits or promotes CD4 SP development is related to the issue of whether Notch1IC exerts its effect on CD4/CD8 T cell development by altering lineage commitment or survival. Although the Notch signaling pathway is generally thought of as regulating cell fate decisions, in some experimental settings Notch can have pro- or anti-apoptotic effects (23–25). For example, thymocytes of Notch1IC transgenic mice show increased resistance to dexamethasone-induced apoptosis compared with thymocytes from nontransgenic mice. This observation led to the suggestion that Notch activity might promote the survival of CD4 and CD8 SP thymocytes during positive selection. However, mutations of the glucocorticoid receptor that abolish dexamethasone-mediated death of thymocytes have no apparent effect on positive or negative
selection (26), raising questions about the relevance of the dexamethasone-induced death assay. Indeed, kinetic analysis of thymic development in Notch1IC transgenic mice revealed no alterations in thymocyte life span (5), indicating that Notch activity does not alter thymocyte survival in the presence of endogenous glucocorticoids.

Finally, a recent report of the combined effects of constitutive Notch1 and Bcl2 argue against a model in which Notch promotes the CD8 T cell fate by providing a survival signal (26). Although neither a Notch1IC transgene or a constitutive Bcl2 transgene alone can allow the development of SP thymocytes in TCRα−/− mice, the combination of both transgenes leads to the appearance of phenotypically and functionally mature CD8 SP thymocytes. The effect of Notch1IC in this setting is unlikely to be mediated by survival because Bcl2 alone provides a potent survival signal. In addition, CD8 SP, but not CD4 SP, was observed in TCRα−/− mice expressing both Notch1IC and Bcl2 transgenes. Thus, the effects of activated Notch1 on CD4/CD8 T cell development cannot be readily explained by effects on survival, but are most consistent with models in which Notch activity regulates CD4/CD8 lineage commitment.

A separate line of evidence used to support the notion that Notch activity promotes both CD4 and CD8 SP development comes from analysis of Notch-induced genes (12). A number of genes that are expressed at higher levels in Notch1IC transgenic mice compared with nontransgenic mice are also expressed at higher levels in CD4 and CD8 SP thymocytes compared with DP thymocytes. If these genes represent direct targets of the Notch signaling pathway, their expression in CD4 and CD8 SP thymocytes could be taken as evidence for Notch signaling in both lineages. However, it is unclear whether these genes are direct targets of Notch signaling; therefore, their induction may not provide a reliable measure of Notch activity. Mature CD4 and CD8 T cells display very similar patterns of gene expression, and there is likely to be a large number of “T cell maturation genes” that are turned on in both lineages. If Notch activity induces CD8 maturation, it would turn on this set of T cell maturation genes, but signals that lead to CD4 maturation would turn on this same set of genes. Changes in gene expression due to Notch signaling may involve a limited set of genes and may lead to very transient induction or repression of gene expression.

Of the Notch-induced genes examined, the one most likely to be a direct target of the Notch signaling pathway is HES1. HES1 is related to the Drosophila Notch target genes encoded by the enhancer of split complex. The HES1 promoter is regulated by the enhancer of split 1 complex and is found in all cell types. Mice lacking major histocompatibility complex class I and II molecules. Proc. Natl. Acad. Sci. USA 90:3913.


Acknowledgments

We thank Michael Bevan for his gift of Notch1IC transgenic mice. We thank our colleagues Cal Esgio for flow cytometry, Errin O. Matechak for data analysis, and David Raulet and members of the Robey laboratory for comments on the manuscript.

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