

# The requirement for Notch signaling at the $\beta$ -selection checkpoint in vivo is absolute and independent of the pre-T cell receptor

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**Genetic inactivation of Notch signaling in CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) thymocytes was previously shown to impair T cell receptor (TCR) gene rearrangement and to cause a partial block in CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) thymocyte development in mice. In contrast, in vitro cultures suggested that Notch was absolutely required for the generation of DP thymocytes independent of pre-TCR expression and activity. To resolve the respective role of Notch and the pre-TCR, we inhibited Notch-mediated transcriptional activation in vivo with a green fluorescent protein-tagged dominant-negative Mastermind-like 1 (DNMAML) that allowed us to track single cells incapable of Notch signaling. DNMAML expression in DN cells led to decreased production of DP thymocytes but only to a modest decrease in intracellular TCR $\beta$  expression. DNMAML attenuated the pre-TCR-associated increase in cell size and CD27 expression. TCR $\beta$  or TCR $\alpha\beta$  transgenes failed to rescue DNMAML-related defects. Intrathymic injections of DNMAML<sup>-</sup> or DNMAML<sup>+</sup> DN thymocytes revealed a complete DN/DP transition block, with production of DNMAML<sup>+</sup> DP thymocytes only from cells undergoing late Notch inactivation. These findings indicate that the Notch requirement during the  $\beta$ -selection checkpoint in vivo is absolute and independent of the pre-TCR, and it depends on transcriptional activation by Notch via the CSL/RBP-J-MAML complex.**

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Notch regulates multiple stages of T cell development (1, 2). Canonical Notch signaling involves release of the intracellular (i.c.) Notch (ICN) domain into the cytoplasm and ICN migration to the nucleus. ICN forms a complex with the transcription factor CSL/RBP-J, creating a binding interface that recruits transcriptional coactivators of the Mastermind-like (MAML) family (3). Disruption of Notch1 signaling leads to B cell accumulation in the thymus and a block in T cell development (4). Notch is required to generate the earliest T lineage progenitors in the thymus (5, 6). Subsequent differentiation depends on continuous Notch signaling (7–11). In particular, Notch is important for developmental progression from

the CD4<sup>-</sup>CD8<sup>-</sup> double-negative (DN) to the CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) stage at the  $\beta$ -selection checkpoint.

Several studies have identified a role for Notch during  $\beta$ -selection, although they have provided conflicting information as to the mechanism and importance of this effect. In vivo, Lck-Cre (LC)-mediated inactivation of Notch1 or CSL/RBP-J at the CD25<sup>+</sup>CD44<sup>-</sup>DN3 stage decreased but did not abolish the generation of DP cells (7, 9). This was associated with accumulation of DN3 cells that were abnormally bright for CD25 and CD25<sup>-</sup>CD44<sup>-</sup>DN4-like cells with reduced expression of i.c. TCR $\beta$ . DN3 and DN4 cells in Notch1-deficient mice had impaired V-DJ $\beta$  rearrangement (7). In addition, Notch could induce *Ptcra* transcripts (encoding pre-TCR $\alpha$ ) (12). These findings suggested that Notch might act at the

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The online version of this article contains supplemental material.

$\beta$ -selection checkpoint by regulating pre-TCR expression. In contrast, *in vitro* studies have suggested that the requirement for Notch was independent of the pre-TCR (10). Instead, Notch was shown to influence cellular metabolism and survival through a molecular pathway involving activated Akt (11).

The basis for the discrepancy between the *in vivo* and *in vitro* data is unclear. One possibility is that the requirement for the metabolic effects of Notch is less stringent *in vivo* than *in vitro*. This could create a situation in which effects of Notch on pre-TCR expression are limiting only *in vivo*. Alternatively, pre-TCR-independent effects of Notch could be critical *in vivo*, and the apparent leakiness of the DN-DP transition block could be related to the precise timing of Notch inactivation.

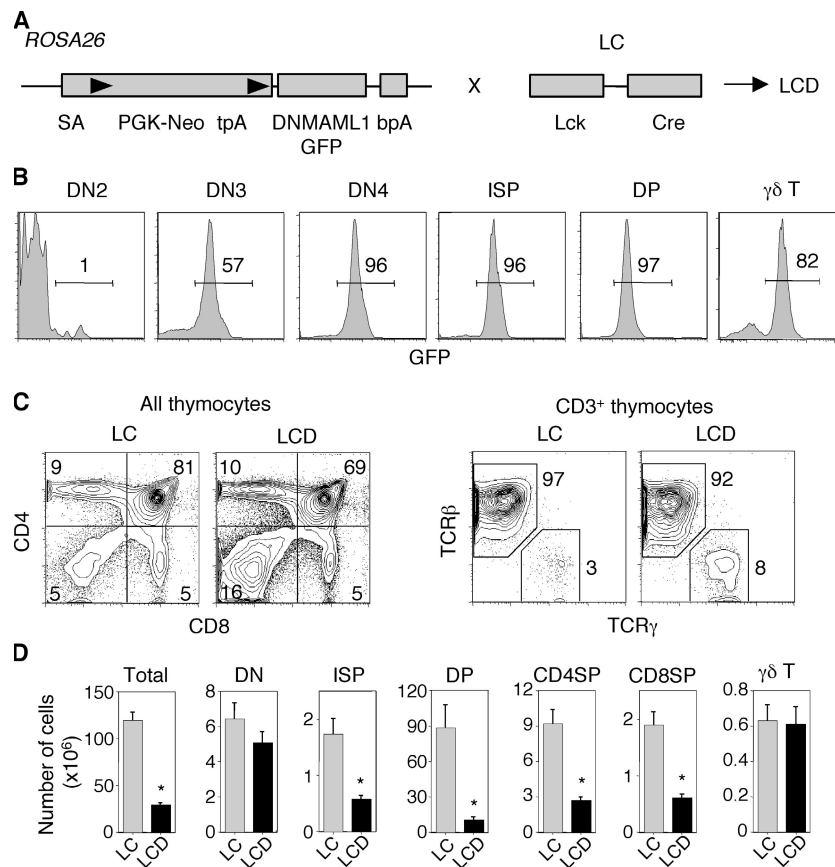
To address these questions, we generated mice expressing a conditional allele of the pan-Notch inhibitor dominant-negative MAML1 (DNMAML<sup>f/+</sup>) (13, 14). The DNMAML allele is linked to a GFP sequence, providing the opportunity to track single Notch-deprived cells. We bred DNMAML<sup>f/+</sup> mice with LC transgenic (tg) mice. Our results demonstrate

an absolute requirement for a CSL/RBP-J-dependent, pre-TCR-independent effect of Notch at the  $\beta$ -selection checkpoint *in vivo*.

**RESULTS AND DISCUSSION**

**Impaired  $\alpha\beta$  but not  $\gamma\delta$  T cell development in LC x DNMAML<sup>f/+</sup> (LCD) mice**

To inhibit signaling from all Notch receptors (Notch1–4), we generated DNMAML<sup>f/+</sup> mice (14) (Fig. 1 A). These mice harbor a cassette encoding the GFP-tagged Notch inhibitor DNMAML downstream of a floxed sequence that prevents transcription of DNMAML. Cre recombinase results in DNMAML expression from the ubiquitously active ROSA26 promoter. Notch-deprived cells can be tracked through GFP detection. DNMAML<sup>f/+</sup> mice were crossed to LC tg mice to generate LCD mice and compared with control LC littermates. GFP expression was detected in a small percentage of CD44<sup>+</sup>CD25<sup>+</sup> DN2 cells (Fig. 1 B). In subsequent stages, DNMAML was induced in >50% of CD44<sup>-</sup>CD25<sup>+</sup> DN3 cells and >95% of CD44<sup>-</sup>CD25<sup>-</sup> DN4, TCR $\beta$ -CD8<sup>+</sup> immature single-positive and CD4<sup>+</sup>CD8<sup>+</sup> DP cells.



**Figure 1. Characterization of LCD mice.** (A) Structure of the ROSA26 DNMAML<sup>f/+</sup> locus. Triangles represent loxP sites. SA, splice acceptor; tpA, trimer of SV40 polyadenylation sequence; bpA, bovine growth hormone polyadenylation sequence. (B) GFP expression in LCD thymocyte subsets. (C) Flow cytometric analysis of LC and LCD thymi. Numbers in B and C

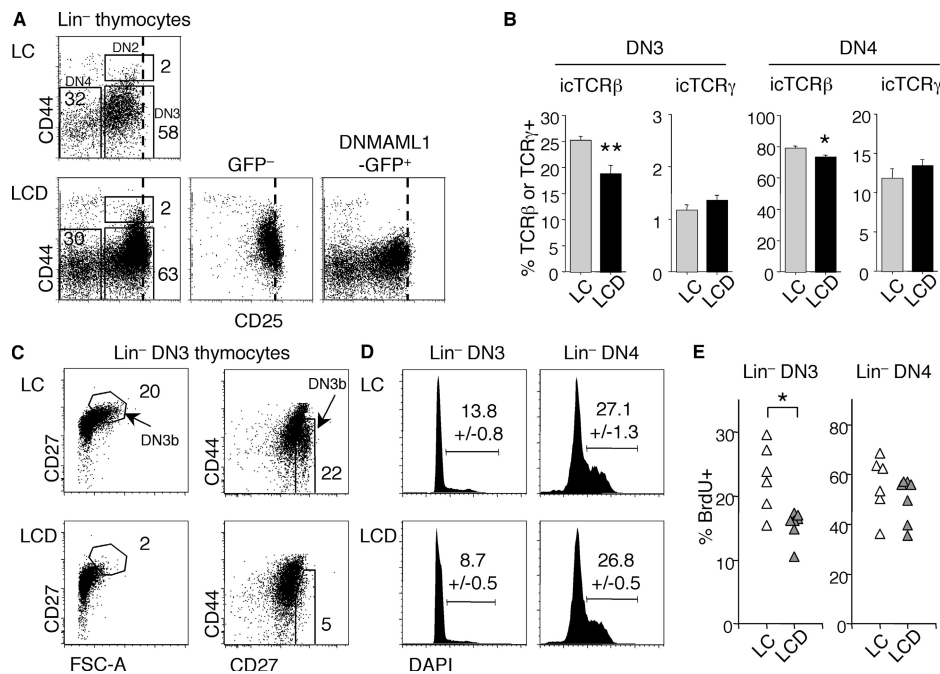
represent the percentage of cells in the indicated areas. (D) Absolute number of thymocyte subsets in LC and LCD mice. Numeric data represent means  $\pm$  SEM ( $n = 8$ ). \*,  $P < 0.01$  according to the Student's  $t$  test.  $\gamma\delta$  T, all CD3<sup>+</sup> TCR $\gamma$ <sup>+</sup> T cells (including CD8 $\alpha$ <sup>+</sup>).

LCD thymi had impaired  $\alpha\beta$  T cell development, as shown by a decreased percentage of DP and an increased percentage of DN cells (Fig. 1 C), translating into a four to fivefold decrease in thymic cellularity (Fig. 1 D). The absolute number of DN cells was preserved, whereas numbers of immature single-positive, DP, CD4<sup>+</sup> single positive (SP) and mature CD8<sup>+</sup> SP cells were decreased, which was consistent with a block at the DN-DP transition. Among CD3<sup>+</sup> thymocytes, the percentage of TCR $\gamma$ <sup>+</sup> cells was increased in LCD mice (Fig. 1 C), an effect secondary to decreased TCR $\beta$ <sup>+</sup> cells because the absolute number of TCR $\gamma$ <sup>+</sup> cells was maintained (Fig. 1 D). This was observed despite expression of DNMA1 in  $\sim$ 80% of LCD  $\gamma\delta$  thymocytes (Fig. 1 B). Regarding  $\alpha\beta$  T cell development, our observations in LCD mice were consistent with findings reported after LC-mediated inactivation of Notch1 or CSL/RBP-J (7, 9). In contrast, the preservation of  $\gamma\delta$  thymocytes in LCD mice was similar to LC x Notch1<sup>fl/fl</sup> mice (7) but differed from the increase in absolute  $\gamma\delta$  cell numbers in LC x CSL/RBP-J<sup>fl/fl</sup> mice (9). This cannot be explained by Notch2–4 activity after *Notch1* deletion because signaling from all four Notch receptors is inhibited both by DNMA1 (14) and the absence of CSL/RBP-J. A potential explanation is that Notch-independent CSL/RBP-J-mediated transcriptional repression plays a role during  $\gamma\delta$  development. This repressor function would be lost in the absence of CSL/RBP-J but is unaffected

by DNMA1. Alternatively, subtle differences in genetic background or timing of Notch inactivation may account for the discrepant results.

### Characterization of LCD CD4<sup>-</sup>CD8<sup>-</sup> DN thymocytes

We studied the phenotype of Lin<sup>-</sup> DN thymocytes in LC and LCD mice (Fig. 2), using GFP to better define the effects of Notch deprivation. The proportions of DN2, DN3, and DN4 cells were similar in LC and LCD thymi (Fig. 2 A). LCD DN3 cells expressed higher levels of CD25 than LC DN3 cells (Fig. 2 A). Although the difference was modest, it was statistically significant (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20061020/DC1>). In addition to DN3 cells, abnormal CD25<sup>hi</sup> DN2 cells were also present in LCD mice. These findings were reminiscent of the CD25<sup>hi</sup> DN2-DN3 population observed after LC-mediated inactivation of Notch1 or CSL/RBP-J (7, 9). This population was hypothesized to represent Notch-deprived DN2-DN3 cells that failed to undergo  $\beta$ -selection, as in mice lacking pre-TCR components (7). However, all CD25<sup>hi</sup> DN2-DN3 cells in LCD mice were GFP<sup>-</sup>, whereas GFP<sup>+</sup> DN3 cells expressed lower levels of CD25 than the GFP<sup>-</sup> DN3 cohort (Fig. 2 A). These findings indicated that CD25<sup>hi</sup> DN2-DN3 cells emerged in LCD mice not as a direct consequence of Notch deprivation but because of a non-cell autonomous effect; e.g., an abnormality in intra-thymic niches occupied by DNMA1<sup>+</sup> DN cells.



**Figure 2. Lin<sup>-</sup> thymocyte subsets in LCD mice.** (A) Flow cytometric analysis of LC/LCD Lin<sup>-</sup> thymocytes. Boxed regions identify CD44<sup>+</sup>CD25<sup>+</sup> DN2, CD44<sup>-</sup>CD25<sup>+</sup> DN3, and CD44<sup>+</sup>CD25<sup>-</sup> DN4 cells. CD25 expression in DN2-DN3 cells was higher in LCD than in LC mice (dashed line). Numbers represent the percentage of cells in the indicated areas. (B) Expression of i.c. TCR $\beta$  (LC,  $n = 11$ ; LCD,  $n = 12$ ) and TCR $\gamma$  (LC,  $n = 8$ ; LCD,  $n = 9$ ) in DN3 and DN4 cells from LC and LCD mice. (C) Decreased DN3b population

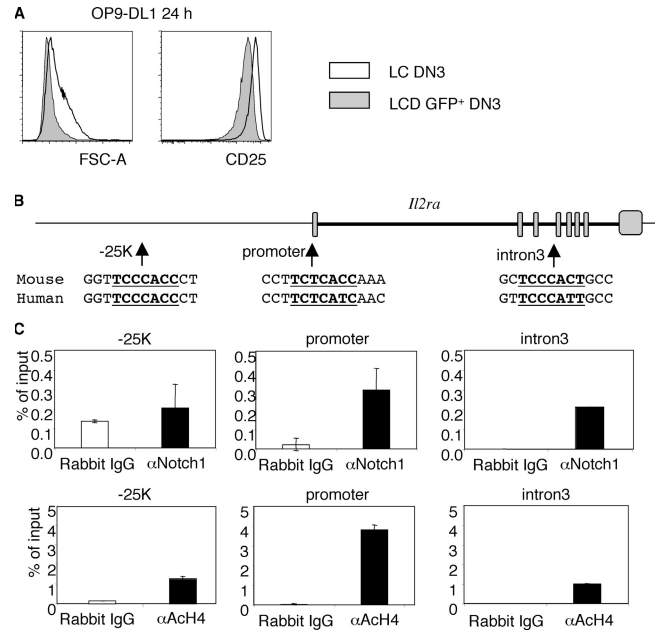
in LCD versus LC DN3 cells. CD27<sup>hi</sup>FSC<sup>hi</sup> or CD27<sup>hi</sup>CD44<sup>lo</sup> DN3 cells have been defined as DN3b thymocytes (reference 15). Plots are gated on total (GFP<sup>-</sup> and GFP<sup>+</sup>) Lin<sup>-</sup> DN3 cells. (D) Cell cycle analysis of LC/LCD Lin<sup>-</sup> DN3 and DN4 cells ( $n = 7$  and  $8$ , respectively). Numbers in C and D represent the percentage of cells in the indicated areas. (E) BrdU incorporation after a 3-h labeling ( $n = 6$  and  $7$ , respectively). Numeric data represent means  $\pm$  SEM. \*,  $P < 0.05$ ; or \*\*,  $P < 0.01$  according to the Student's  $t$  test.

We next assessed LC/LCD DN3 and DN4 cells for i.c. TCR $\beta$  and TCR $\gamma$  (Fig. 2 B and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20061020/DC1>). The percentage of i.c. TCR $\beta^+$  DN3 and DN4 cells showed a modest, though statistically significant, decrease in LCD as compared with LC mice. The percentage of i.c. TCR $\gamma^+$  thymocytes was not significantly different.

We then examined LC/LCD DN3 cells for phenotypic changes associated with  $\beta$ -selection (Fig. 2, C–E). DN3 cells exposed to pre-TCR signals exhibit active cell cycling, increased cell size, and CD27 up-regulation (DN3b population) (15). FSC<sup>hi</sup>CD27<sup>hi</sup> or CD44<sup>lo</sup>CD27<sup>hi</sup> DN3b cells were reduced in LCD as compared with LC thymi (Fig. 2 C). When assessed for DNA content, a smaller proportion of DN3 cells were in the S-G2M phases of the cell cycle (Fig. 2 D) and fewer cells incorporated BrdU in LCD as compared with LC DN3 cells (Fig. 2 E). In contrast, no significant proliferation defect was detected in DN4 cells. The proportion of i.c. TCR $\beta^+$  cells was reduced among BrdU<sup>+</sup> LCD as compared with LC DN3 cells (LC, 68  $\pm$  3% vs. LCD, 43  $\pm$  4%; mean  $\pm$  SEM;  $P < 0.01$ ), a finding that was consistent with reports of impaired proliferation, predominantly in  $\alpha\beta$  lineage cells in vitro (16, 17). Collectively, these findings indicate that Notch-deprived DN3 cells, although detected phenotypically as CD25<sup>lo</sup> cells at the DN3–DN4 transition, failed to undergo the typical changes associated with  $\beta$ -selection. We recently reported that Notch directly up-regulates *c-myc* transcription in primary DN3 cells and T cell leukemia cell lines, suggesting that abrogation of the Notch–*c-myc* axis contributes to the  $\beta$ -selection defects in LCD mice (18).

In vitro studies using Rag-deficient DN3 cells on OP9–DL1 stroma found that Notch signaling had important roles in cell metabolism and survival. In contrast, we did not detect consistent abnormalities in Annexin V staining and cellular bioenergetics, as measured by tetramethylrhodamine ethyl ester labeling in LCD thymi (unpublished data). This does not rule out Notch-related changes in these parameters because compromised/dying thymocytes are rapidly eliminated and have been notoriously difficult to detect in vivo (19).

The in vivo DNAML-related defects were reminiscent of in vitro findings using OP9 cells in which the main consequences of Notch deprivation were growth arrest, decreased cell size, and eventual cell death (10, 11). To better understand changes induced by the loss of Notch signaling, we assessed cell size and CD25 expression in cultures of LC DN3 or LCD GFP<sup>+</sup> DN3 thymocytes with OP9–DL1 cells (Fig. 3 A). After 24 h, LCD GFP<sup>+</sup> DN3 cells exhibited decreased cell size and CD25 expression when compared with LC DN3 cells. Therefore, these changes resulted from a transcriptional effect of Notch signaling mediated by the ICN–CSL–RBP–J–MAML complex. The rapid modulation of CD25 expression suggested that *Cd25* (*Il2ra*) is a direct transcriptional Notch target in thymocytes (20). A chromatin precipitation (ChIP) assay showed that Notch1 associated with two conserved CSL/RBP–J binding sites in the *Il2ra*



**Figure 3. Loss of canonical Notch signaling results in decreased cell size and CD25 expression.** (A) Sorted LC or LCD GFP<sup>+</sup> DN3 thymocytes were cultured with OP9–DL1 cells expressing the Notch ligand Delta-like 1. Flow cytometric analysis was performed after 24 h. (B) Structure of the *Il2ra* (*Cd25*) locus showing three conserved putative CSL/RBP–J binding sites. (C) ChIP assay from purified DN3 cells using anti-Notch1 ( $\alpha$ Notch1), antiacetylated histone 4 ( $\alpha$ ACh4), or control rabbit antibodies, followed by quantitative PCR. Input DNA (aliquot of preimmunoprecipitation–sheared chromatin) was used for normalization. Numeric data represent means  $\pm$  SEM.

locus; one is located immediately upstream of the transcription start site and the other in intron 3 (Fig. 3 B). These findings confirm that *Il2ra* is a Notch transcriptional target in developing thymocytes.

Collectively, these observations suggest a scenario in which Notch-deprived DN3 cells down-regulate CD25 and fail to proliferate. Thus, some CD44<sup>lo</sup>CD25<sup>lo</sup> DN3–DN4 thymocytes in LCD mice might represent Notch-deprived DN3 cells that have aberrantly down-regulated CD25. However, these cells did not accumulate to a large extent in LCD mice, as shown by the nearly normal frequency of i.c. TCR $\beta^+$  cells among LCD DN4 thymocytes (Fig. 2 B). The minimal accumulation of Notch-deprived DN cells in LCD mice might be related to their growth arrest (Fig. 2 D) and/or rapid elimination by apoptosis, which was consistent with in vitro work showing poor survival of cells lacking both Notch and pre-TCR signaling (16). In comparison to LCD mice, the accumulation of aberrant CD25<sup>lo</sup> DN3–DN4 cells appeared more prominent in LC x Notch1<sup>f/f</sup> and, to a lesser extent, in LC x CSL/RBP–J<sup>f/f</sup> mice, even if overall impairment of T cell development was similar (7, 9). We hypothesize that residual Notch signaling through Notch2–4 receptors in LC x Notch1<sup>f/f</sup> mice, or preformed CSL/RBP–J protein in LC x CSL/RBP–J<sup>f/f</sup> mice, contributed to enhanced accumulation

of aberrant DN3-DN4 cells without allowing normal progression through  $\beta$ -selection.

#### No rescue of Notch-deprived DN cells by *Tcrb* or *TcrA/b* transgenes

As in the absence of Notch1 and CSL/RBP-J (7, 9), i.e. TCR $\beta$  expression was decreased in LCD DN3 cells (Fig. 2 B). To assess if this was functionally relevant in vivo, we crossed LCD mice to *Tcrb* tg mice (Fig. 4, A and B). DNMA1L did not affect transgene expression (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20061020/DC1>). The *Tcrb* transgene failed to restore cellularity of LCD thymi back to normal numbers (Fig. 4 A), despite a twofold increase in LCD/*Tcrb* tg mice. The percentage of LCD DP cells was decreased with and without the transgene (Fig. 4 B), although the decrease was slightly less pronounced with *Tcrb*. However, we consistently observed a higher percentage of GFP<sup>-</sup> cells among DP thymocytes of LCD/*Tcrb* tg than in non-tg LCD mice. This suggested that the transgene accelerated the transition through DN2-DN4 stages of development, allowing more DP cells to arise without being exposed to DNMA1L. Together, these results indicated that a *Tcrb*

transgene did not rescue Notch inactivation at the  $\beta$ -selection checkpoint in vivo.

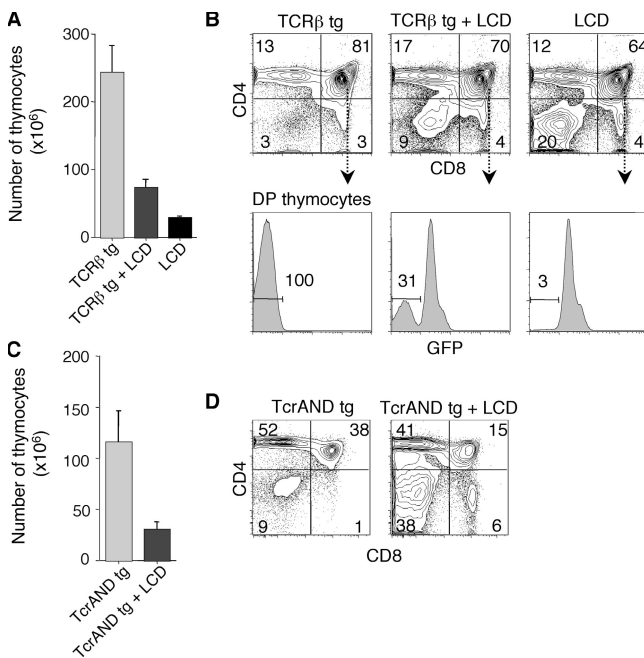
In addition to its effect on TCR $\beta$ , Notch might regulate pT $\alpha$  expression (12). To assess if this played a limiting role in vivo, we crossed LCD mice to TcrAND tg mice (Fig. 4, C and D). These mice express *TcrA/b* transgenes that can substitute for pre-TCR function (21), though with reduced efficiency (22). Expression of the *TcrA/b* transgenes did not restore thymic cellularity (Fig. 4 C). Accordingly, the percentage of DP cells was reduced in LCD/TcrAND as compared with TcrAND mice (Fig. 4 D). Similar results were observed with DO11.10 tg mice (unpublished data).

These results show that Notch deprivation in vivo cannot be rescued by restoring pre-TCR function. Instead, they suggest that Notch and the pre-TCR act in parallel pathways. An important future task will be to characterize the interactions between Notch, pre-TCR signals, and other partners that are active during  $\beta$ -selection, such as E proteins. Of note, recent work indicates that Notch and E proteins cooperate during T lineage commitment (23).

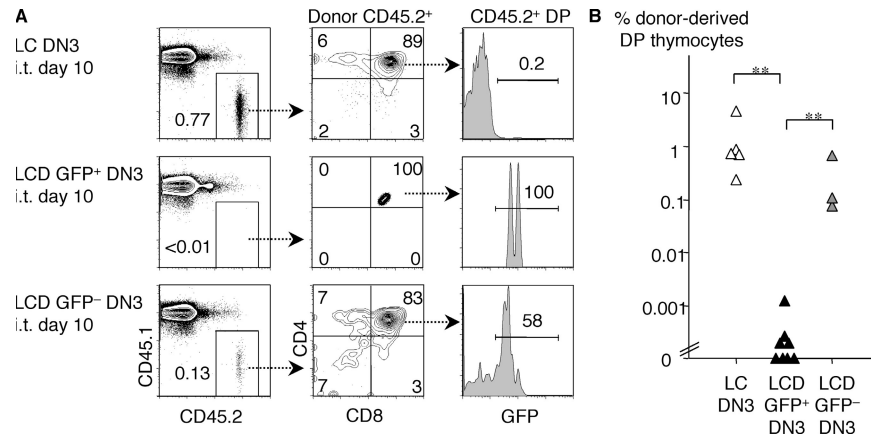
#### Intrathymic injections reveal an absolute DN to DP differentiation block in the absence of Notch signaling in vivo

In vitro experiments suggested an absolute requirement for Notch at the DN-DP transition (10, 11). However, the generation of LCD DP cells was reduced but not abolished in vivo. This could be explained by a less stringent requirement for Notch signaling in vivo or by the precise kinetics of LC-mediated excision. To differentiate between these possibilities, we purified GFP<sup>-</sup> and GFP<sup>+</sup> DN3 cells from LCD mice and performed intrathymic injections (Fig. 5). Control LC DN3 cells gave rise to donor-derived DP/SP T cells 10 d after injection (Fig. 5 A, top). In contrast, Notch-deprived GFP<sup>+</sup> LCD DN3 cells gave rise to no or barely detectable progeny (Fig. 5 A, middle). There was at least a 3-log reduction in donor-derived cells in the absence of Notch signaling (Fig. 5 B). When GFP<sup>-</sup> LCD DN3 cells were injected, significant numbers of donor-derived DP/SP cells were observed at day 10 (Fig. 5 A, bottom), and >50% of these cells were GFP<sup>+</sup> as a result of DNMA1L induction in vivo between injection and analysis. These results indicate that the requirement for Notch during  $\beta$ -selection is as stringent in vivo as in vitro. Furthermore, the apparently partial differentiation block observed in vivo results from late Notch inactivation in a fraction of DN3-DN4 cells.

In conclusion, our observations bring new insights into the role of Notch at the  $\beta$ -selection checkpoint in vivo. By tracking Notch-deprived cells, we demonstrated an absolute requirement for Notch signaling during  $\beta$ -selection that was not rescued by restoring pre-TCR function. Our results are consistent with a predominant and limiting effect of Notch on proliferation and cell survival, although we cannot rule out additional effects on differentiation. Because the effect was observed on interference with the Notch transcriptional activation complex, future work to identify relevant downstream



**Figure 4. *Tcrb* and *TcrA/b* transgenes do not rescue DN to DP differentiation in the absence of Notch signaling in vivo.** LC and LCD were bred to *Tcrb* or *TcrA/b* (TcrAND) tg mice. F1 progeny were compared with parental tg mice. (A) Total thymocyte numbers in *Tcrb* tg ( $n = 7$ ), *Tcrb* tg x LCD ( $n = 7$ ), and LCD mice ( $n = 8$ ). Numeric data represent means  $\pm$  SEM. (B) CD4/CD8 expression in *Tcrb* tg, *Tcrb* tg x LCD, and LCD thymi (top) and GFP expression in DP cells (bottom). Numbers represent the percentage of cells in the indicated areas. (C) Total thymocyte numbers in TcrAND tg and TcrAND tg x LCD mice ( $n = 5$ ). Numeric data represent means  $\pm$  SEM. (D) CD4/CD8 expression in TcrAND tg and TcrAND tg x LCD thymi. Numbers represent the percentage of cells in the indicated areas.



**Figure 5. Intrathymic injections reveal an absolute requirement for Notch signaling during  $\beta$ -selection in vivo.** (A)  $\text{Lin}^- \text{CD}25^+ \text{CD}44^- \text{DN}3$  cells from donor  $\text{CD}45.2^+$  LC or LCD mice were injected intrathymically into sublethally irradiated B6- $\text{CD}45.1^+$  mice. (left) Percentage of donor-derived  $\text{CD}45.2^+$  cells after 10 d. (middle)  $\text{CD}4/\text{CD}8$  expression on

donor-derived cells. (right) GFP expression on donor-derived DP cells. A representative example is shown (LC DN3,  $n = 5$ ; LCD GFP<sup>+</sup> DN3,  $n = 8$ ; LCD GFP<sup>-</sup> DN3,  $n = 3$ ). Numbers represent the percentage of cells in the indicated areas. (B) Percentage of donor-derived DP cells for each recipient. \*\*,  $P < 0.05$  according to the Student's  $t$  test.

mediators of Notch will have to focus on transcriptional targets of Notch signaling.

#### MATERIALS AND METHODS

**Mice.** DNMA1L<sup>f/+</sup> mice were generated as previously described (14). The DNMA1L-GFP construct encodes amino acids 13–74 of MAML1 fused to GFP (13, 14). DNMA1L<sup>f/+</sup> mice were crossed to LC tg mice (Taconic). LCD mice were compared with LC littermates. B6. $\text{CD}45.1$  and *Tcrb* tg mice expressing a TCR $\beta$  chain from the DO11.10 hybridoma were obtained from Taconic. Tg(TcrAND) and Tg(DO11.10) *Tcr $\alpha$ /Tcrb* tg mice were purchased from the Jackson Laboratory. Experimental protocols were approved by the University of Pennsylvania Office of Regulatory Affairs.

**Antibodies.** The following antibodies were from obtained from BD Biosciences or eBioscience: PE anti-CD25 (PC61), CD27 (LG.7F9), TCR $\beta$  (H57-597), TCR $\gamma$  (GL3), CD4 (RM4-5), and CD3 (145-2C11); APC anti-CD4, TCR $\beta$ , CD44 (IM7), and BrdU; biotinylated anti-CD45.2 (104), CD8 (53-6.7), TCR $\beta$ , TCR $\gamma$ , CD4 (GK1.5), CD3, NK1.1 (PK136), B220 (RA3-6B2), CD19 (1D3), CD11b (M1/70), Gr1 (RB6-8C5), and CD11c (HL3); APC-Cy7 anti-CD25; and PE-Cy5.5 anti-CD44 and PE-Cy7 anti-CD45.1 (A20). Biotinylated antibodies were revealed with streptavidin-PerCP (BD Biosciences), Pacific Blue (Invitrogen), or PE-Texas red (Caltag). Lineage<sup>+</sup> cells were defined with anti-CD8, TCR $\beta$ , TCR $\gamma$ , NK1.1, CD3, B220, CD19, CD11b, Gr1, and CD11c.

**Flow cytometry and cell sorting.** Cells were stained in PBS/2% FCS. i.c. staining was performed with fixation/permeabilization or BrdU labeling kits (Becton Dickinson). 0.5 mg BrdU was administered i.p. 3 and 1 h before death. Cells were sorted on a FACS DiVa (Beckton Dickinson) or a MoFlo (DakoCytomation). Analysis was performed on a FACS Calibur or LSR II (Becton Dickinson). DAPI was used to exclude dead cells or assess DNA content in fixed cells. Files were analyzed with software (FlowJo; Tree Star, Inc.).

**Intrathymic injections.**  $1-2 \times 10^5$  sorted DN3 cells were injected intrathymically in anesthetized B6. $\text{CD}45.1$  recipients given 500 rad 2–6 h before injection.

**OP9 cultures.** OP9-DL1 cells were provided by J.C. Zuniga-Pflucker (University of Toronto, Toronto, Canada) and used as previously described (24). Progenitors were seeded into 24-well plates containing a stromal monolayer with 1 ng/ml mIL-7 (PeproTech).

**ChIP.** ChIP was performed from Rag-2<sup>-/-</sup> DN3 cells using Notch1 TAD domain-specific antiserum (25), anti-acetylated histone 4 (Upstate Biotechnology), or rabbit IgG (Santa Cruz Biotechnology, Inc.), as previously described (18). Quantitative PCR was performed with SYBR green (Applied Biosystems) and the following *Il2ra*-specific primers: 25K, 5' CAGTCATTGGTTGGCCACTCT 3' and 5' GGACCTCCATGCAGACATCA 3'; promoter, 5' TGTGTGAGTCTTCTGGGGGAGAA 3' and 5' CTAGGAGGTGTGGGCAGTGTTT 3'; and intron 3, 5' TGCAGCATGGGTCAAA-TGAA 3' and 5' AGGTCTCCCCAGGAAAAGTCAC 5'.

**Online supplemental material.** Fig. S1 depicts CD25 median fluorescence intensity in  $\text{Lin}^- \text{CD}44 \text{DN}3/\text{DN}4$  LCD as compared with LC thymocytes. Fig. S2 shows expression of i.c. TCR $\beta$  and i.c. TCR $\gamma$  in  $\text{Lin}^- \text{DN}3\text{-DN}4$  LC and LCD thymocytes (a representative example is presented). Fig. S3 shows expression of i.c. TCR $\beta$  in the presence or absence of a *Tcrb* transgene in LC and LCD DN thymocyte subsets.

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