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Impairment of Thymocyte Development by Dominant-Negative Kuzbanian (ADAM-10) Is Rescued by the Notch Ligand, Delta-1[1]

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Although Notch plays a crucial role in T cell development, regulation of Notch signaling in the thymus is not well understood. Kuzbanian, an ADAM protease, has been implicated in the cleavage of both Notch receptors and the Notch ligand, Delta. In this study we show that the expression of a dominant-negative form of Kuzbanian (dnKuz) leads to reduced TCRβ expression in double-negative thymocytes and to a partial block between the double-negative to double-positive stages of development. These defects were rescued by overexpression of Delta-1 on thymocytes. Mixed chimeras showed a cell-autonomous block by dnKuz, but non-cell-autonomous rescue by Delta-1. This suggests that dnKuz impairs Notch signaling in receiving cells, and increasing Delta-1 on sending cells overcomes this defect. Interestingly, the expression of an activated form of Notch-1 rescued some, but not all, the defects in dnKuz Tg mice. Our data suggest that multiple Notch-dependent steps in early thymocyte development require Kuzbanian, but differ in the involvement of other Notch signaling components. *The Journal of Immunology,* **2005, 174: 6732–6741.**

Notch signaling is important at several stages of T cell development (reviewed in Refs. 1–3). In particular, Notch signaling can influence the T cell/B cell fate decisions (4, 5), development of double-negative (DN)[4] thymocytes (6, 7), CD4 vs CD8 cell fate decisions (8, 9), differentiation of Th cells (10–12), and peripheral T cell responses (13–15). Within the DN thymocyte compartment, there is evidence for multiple, distinct functions for Notch, including a role in promoting TCRβ rearrangement (7) and a role in promoting cellular expansion in response to pre-TCR signals (6). Although these studies demonstrate the importance of Notch signaling for normal T cell development and function, the regulation of endogenous Notch signaling during each of these steps in T cell development is still not well understood.

In classical models of Notch signaling, regulated proteolysis of the Notch receptor is crucial for generation of a Notch signal, resulting in CSL-mediated transcription of Notch target genes (reviewed in Ref. 16). Kuzbanian (Kuz) and TACE are ADAM family proteases that have been described to cleave Notch receptors (17–21). The putative cleavage site for these proteases, S2, is located extracellularly, very close to the transmembrane domain of Notch. It is thought that upon binding to Notch ligand, the S2 cleavage site becomes exposed to ADAM protease-mediated cleavage. Although it is clear that ADAM family proteases are involved in Notch signaling, the specific roles of Kuz and TACE in Notch cleavage are controversial. Expression of a dominant-negative form of Kuzbanian (dnKuz) in *Drosophila* and *Xenopus* resulted in neurogenic phenotypes, consistent with a reduction in Notch signaling (21). Later reports suggested that TACE, not Kuz, was the protease involved in Notch cleavage (17). Kuz knockout mice (22) and TACE knockout mice (23) display very different phenotypes, suggesting that these proteases serve distinct roles. More recent studies have shown that some Notch ligands, Delta-1 and Jagged, also can be cleaved by ADAM proteases (24, 25). Thus, the specific role of ADAM proteases in Notch signaling is an open question.

Conventional Kuz knockout mice are early embryonic lethals (22), preventing their use in the study of thymocyte development. In addition, the related ADAM family protease, TACE, might serve a redundant function with Kuz and obscure a thymic phenotype. To circumvent both problems, we decided to interfere with Kuz activity in thymocytes by generating transgenic (Tg) mice that express dnKuz in the thymus. We show in this study that T cell development is blocked between DN and double-positive (DP) stages of T cell development in dnKuz Tg mice. This block correlates with premature down-regulation of CD25 expression and reduced TCRβ expression, similar to the effect of Notch-1 deletion (7). Overexpression of Delta-1 provided a nearly complete rescue of thymocyte development in dnKuz Tg mice. Results from mixed bone marrow chimeras show that the effect of dnKuz is cell autonomous, whereas the rescue by Delta-1 is non-cell autonomous. These results fit with the idea that dnKuz impairs Notch signaling in receiving cells when levels of Delta-1 are normal, and that increased Delta-1 expression on sending cells can overcome this defect. Interestingly, although the expression of an activated form of Notch-1 rescued the block in TCRβ expression, it did not restore DP development in dnKuz Tg mice. These data suggest that Kuz is involved in multiple steps in early thymic development, and that these individual steps may differ in their requirements for components of the Notch pathway.

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[4] Abbreviations used in this paper: ADAM, a disintegrin and a metalloprotease; CSL, CBF1, suppressor of hairless Lagl; DL1, Delta-1; DN, double negative ($CD4^-CD8^-$); dnKuz, dominant-negative Kuz; DP, double positive ($CD4^+CD8^+$); hHGH, human growth hormone; icTCRβ, intracellular TCRβ-chain; ISP, immature single positive; Kuz, Kuzbanian; NIC9, NotchIC-9; Notch-1-IC, intracellular activated form of Notch-1; PETR: Phycoerythrin-Texas Red; SP, single positive; TACE, TNF α-converting enzyme; Tg, transgenic.

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Materials and Methods

Mice

Lck-dnKuz Tg mice were generated by subcloning a murine dnKuz (21) into the p1017 vector (26). Linearized Tg DNA was injected into (CBA × B6)F_1 oocytes. Founder mice were backcrossed to the C57BL/6 background. Most data reported in this study were obtained from the fourth backcrossed generation. Animals were analyzed at 3–12 wk of age. Relative transgene copy number was assessed by Southern blotting (data not shown). Unless otherwise indicated, the data reported in this study are from the dnKuz-155 line. Lck-Delta-1 Tg (DL1 Tg) mice were prepared by subcloning a full-length mouse Delta-1 cDNA (27) into the p1017 vector (26) and were injected into (CBA × B6)F_1 oocytes. DL1 Tg mice were backcrossed to the C57BL/6 background four times. Overexpression of the DL1 Tg in thymocytes was confirmed by real-time PCR (data not shown). NotchIC-9 (NIC9) (8) Tg mice were bred in our facility. Vβ8.3-SGT Tg mice were a gift from P. Savage (Allison Laboratory, University of California, Berkeley, CA). All mice were housed under conventional conditions and were healthy at the time of analysis. Genotyping of mice was performed by PCR using genomic DNA obtained from ear skin. The primers used were: hHGH 5′ primer, 5′-GTC TAT TCC GAC ACC CTC CA-3′; hHGH 3′ primer, 5′-GGA TGC CTT CCT CTA GGT CC-3′; dnKuzHGH 5′ primer, 5′-GAG GCC CCG AGA GAG TTA TC3–3′; dnKuzHGH3′ primer, 5′ TGT GCC CAA AGG GAT TTT AG-3′; DLL-1HGH5′ primer, 5′ TGC ACT ATG GAC AGT TGC TT-3′; Vβ8.3 5′ primer, CCA GTA TCT CGA GCG GAT GG-3′; Vβ8.3 3′ primer, TGC ACT ACC CCC AGT CCC AC-3′; Notch-1IC forward, 5′-ATG GAC TAC AAA GAC GAT GAC G-3′; and Notch-1IC reverse, 5′ CCA TCT GGT CCT CGA ACA TTG.

Western blotting

Thymocytes (10^6) were lysed in 1% Triton X-100 with protease inhibitors (Roche) for 20 min on ice, then centrifuged at top speed for 15 min at 4°C. SDS-reducing sample loading buffer was added to the lysate and boiled for 5 min. Samples were loaded onto a 10% SDS-PAGE separating gel with 5% stacking gel. Eight microliters of Rainbow Marker (Amersham Biosciences) was also loaded in one lane. Gels were run at a constant 25 mA, then transferred to ECL nitrocellulose at 200 mA for 2 h. The membranes were incubated with 0.2% Tween 20-TBS-10% milk overnight at 4°C, washed, incubated with peptide affinity-purified anti-Kuz C-terminal peptide-specific Ab (1/1000; produced for us by Zymed Laboratories) for 30 min at room temperature, washed, and incubated with goat anti-rabbit IgG-HRP (1/5000) for 30 min at room temperature, and washed. ECL reagent was added to the membrane for 1 min, and then the membrane was exposed to ECL Hyperfilm (Amersham Biosciences).

Flow cytometry

Thymi were harvested and crushed with the base of a 5-cc syringe. Bone marrow cells were flushed from the bones using a 25-cc needle. Cells were resuspended in medium 199–2% FCS medium. RBC were lysed with ammonium-chloride-potassium lysis buffer, and cell counts were obtained using trypan blue exclusion. Cells were pelleted and resuspended in FACS buffer, and 2.4G2 supernatant was added to all cells before staining. CD4-FITC, $\alpha\beta$TCR-FITC, CD8-FITC, CD8-PeCy5, CD25-PE, CD3-FITC, CD4-PETR, CD8-PETR, CD44-CyChrome, CD44-PeCy5, CD45 (clone 30-F11)-PeCy5, CD45.1-PE, CD45.2-FITC, CD45.1-FITC, Thy1.2-PE, CD3-biotin, CD19-biotin, CD11b-biotin, GR1-biotin, DX5-biotin, Vβ8.3-FITC, purified anti-$\alpha\beta$TCR, streptavidin-FITC, streptavidin-CyChrome, streptavidin-613, and streptavidin-PETR were purchased from BD Pharmingen, eBioscience, Caltag Laboratories, and Invitrogen Life Technologies. For intracellular staining, extracellular staining was performed first, then cells were fixed and permeabilized using the BD Pharmingen kit. $\alpha\beta$TCR-FITC was added to cells and incubated for 30 min, washed with 1× Perm/Wash buffer (BD Pharmingen), then resuspended in FACS buffer. FACS was performed with an ELITE FACS machine (Beckman Coulter). Data analysis was performed with FlowJo software (TreeStar).

Cell sorting of lineage-negative thymocytes

Lineage-negative thymocytes were enriched from whole thymocytes using anti-CD8β magnetic beads and collecting CD8β-negative cells using AutoMACS (Miltenyi Biotec). MACS-enriched CD8β-negative thymocytes were then stained with anti-CD3-biotin, anti-CD4-FITC, and anti-CD8-FITC, anti-CD11b-biotin, DX5-biotin, and/or Gr-1-biotin plus streptavidin-FITC. FITC-negative thymocytes were collected using a MoFlow cell sorter. Sorted populations were 95–100% pure.

Semiquantitative RT-PCR

Sorted Lin$^-$ thymocytes were pelleted and resuspended in TRIzol. RNA was isolated using standard protocols, and cDNA was generated using SuperScript II reverse transcriptase (Invitrogen Life Technologies). For PCR, the input amount of cDNA was normalized between controls and dnKuz samples using β-actin. The following primers were used: β-actin forward, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′; β-actin reverse, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′; and pre-Tα, Deltex and Hes-1 primers (previously reported) (28, 29). PCR products were Southern blotted, probed with gene-specific ^{32}P-labeled oligonucleotide probes, and exposed on PhosphorImager screens (BD Biosciences).

Preparation of mixed bone marrow chimeras

Bone marrow cells were harvested from the femurs and tibiae of WT B6 (Ly5.2/5.2 or Ly5.2/5.1), dnKuz (Ly5.1/5.2), DL1 Tg (Ly5.2/5.2), and dnKuz/DL1 double-Tg (Ly5.2/5.2) mice. WT B6 (Ly5.1/Ly5.1) recipient mice were lethally irradiated with 1000 rad from a cesium source. At least 4 h after irradiation, 15×10^6 bone marrow cells from WT, dnKuz Tg, DL1 Tg, or dnKuz/DL1 mice alone (single donor chimeras) or a mixture of WT plus dnKuz, WT plus dnKuz/DL1, or DL1 Tg plus dnKuz Tg (total of 15×10^6) bone marrow cells were transferred to these hosts via tail vein injection. Animals were given antibiotic-containing water and were housed in sterile microisolator cages. Animals were killed 6 wk after bone marrow transplant. FACS analysis of thymocytes and bone marrow cells was performed.

Real-time PCR

RNA was isolated using standard protocols and was treated with RNase-free DNase I (Roche) to remove any possible genomic DNA contamination. RT was performed using the TaqMan RT kit (Applied Biosystems) according to the manufacturer's suggestions. Real-time PCR was performed using the ABI TaqMan 5700 thermal cycler default cycling protocol. SYBR Green PCR Master Mix (2×; Applied Biosystems) was used. The following primers were used: Delta-1 forward, CAC TAT GGA CAG TTG CTT TGA AGA GT; and Delta-1 reverse, TGG CTC ATA GTA ATC CAA GAT AGA CG. GAPDH primer/probe sets were obtained from Applied Biosystems. PCRs were performed in triplicate, and the ratio of Delta-1 to GAPDH expression was calculated and averaged. The mean ratio across samples was then calculated and compared with the reference sample.

Statistical analysis

Mann-Whitney tests to test the difference in medians were used (InStat 3; GraphPad). Differences were considered statistically significant at $p < 0.05$.

Results

Thymocyte development is impaired in dnKuz Tg mice

To generate dnKuz Tg mice, a cDNA encoding a dominant negative form of Kuzbanian (21) was subcloned into the p1017 vector, which contains the Lck-proximal promoter (26). Six independent founders containing different transgene copy numbers were obtained and backcrossed to C57BL/6 mice. The dnKuz protein was expressed in all founders (Fig. 1*A* and our unpublished observations). A clear effect of the dnKuz transgene on thymus size was evident in the higher copy lines (Fig. 1*B*). The thymic cellularity in the highest copy dnKuz Tg mice (dnKuz-155; hereafter simply called dnKuz Tg) ranged between 10 and 40% of non-Tg littermate controls.

Examination of CD4 and CD8 expression on dnKuz Tg thymocytes revealed a partial block between the DN and DP stages, with an increase in the percentage of DN and a decrease in the percentage of DP thymocytes in dnKuz Tg mice (Fig. 1*C*). Calculation of the absolute numbers of thymocytes at all stages revealed a lower number of thymocytes within all developmental stages. Although statistically significant, the difference in the number of DN thymocytes in dnKuz Tg mice was not as striking as the difference at later stages (Fig. 1*D*). This may be due to the low activity of the Lck promoter at early DN stages (30). In addition, the numbers of $\gamma\delta$ cells in the thymus were significantly reduced in dnKuz mice (data not shown).

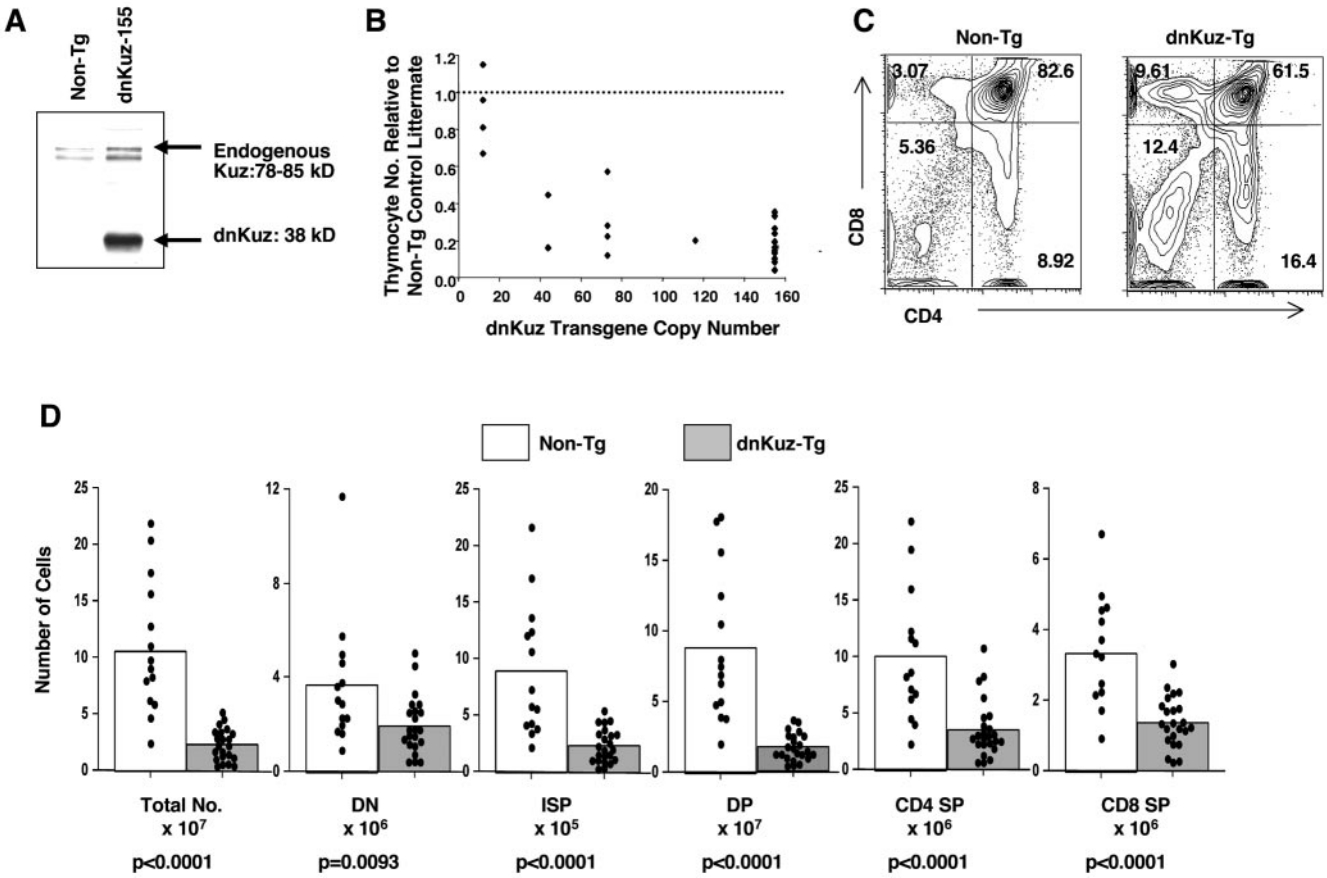

FIGURE 1. Thymocyte development is impaired in dnKuz Tg mice. *A*, Expression of the dnKuz protein in thymocytes of dnKuz Tg mice. Thymocytes (10^6) from non-Tg and dnKuz Tg littermates were lysed, and Western blotting was performed using an anti-Kuz, C terminus-specific antisera. A Western blot of dnKuz-155 (155 copies of the transgene) thymocytes is shown. *B*, Total thymocyte counts for dnKuz Tg mice were plotted as a ratio of the non-Tg littermate control (represented as 1.0 in the graph) in each experiment. *C*, CD4/CD8 flow cytometry profiles for thymocytes from non-Tg and dnKuz Tg mice are shown. Data shown are from one representative non-Tg and one representative dnKuz Tg mouse. *D*, Calculation of the absolute numbers of thymocytes within each developmental stage is shown. Bars represent the mean thymocyte number, and dots represent individual mice within each group (non-Tg, $n = 14$; dnKuz, $n = 24$). The median number of cells within each stage was significantly lower in dnKuz Tg mice. Statistical differences were determined using the Mann-Whitney *U* test for differences in medians between groups. Animals were analyzed at 4–8 wk of age.

To further investigate where the developmental block might be occurring, lineage-negative (Lin^-; $CD3^-CD4^-CD8^-CD11b^-DX5^-Gr1^-$) thymocytes were stained for CD25 and CD44 expression, delineating the DN1-DN4 stages (Fig. 2*A*). Calculation of the absolute number of DN1 cells showed a slight reduction in dnKuz Tg mice compared with controls, but this difference was not statistically significant (Fig. 2*B*). However, the absolute number of DN2 cells was significantly reduced in dnKuz Tg mice compared with non-Tg controls (Fig. 2*B*). Therefore, thymocyte development in dnKuz Tg mice may be blocked as early as the DN2 stage.

During the transition from DN3 to DN4 stages, CD25 expression is normally down-regulated (31). In dnKuz Tg mice, a $CD25^{int}$ $CD44^-$ population was present between DN3 and DN4 stages, suggesting that CD25 is prematurely down-regulated during the DN3 to DN4 transition (Fig. 2*A*). The presence of this $CD25^{int}CD44^-$ population complicates the assignment of thymocytes to the DN3 and DN4 stages, thus preventing an accurate accounting of the thymocytes within these stages in dnKuz Tg mice.

In addition to this aberrant CD25 expression, TCRβ expression was affected in dnKuz Tg mice. Fewer intracellular TCRβ-positive (icTCRβ^+) cells were present in the DN3 and DN4 stages compared with non-Tg controls (Fig. 2*C*). In both non-Tg and dnKuz Tg mice, the icTCRβ^+ cells were larger than icTCRβ^- cells (Fig. 2*C*). This suggests that the icTCRβ^+ thymocytes in both non-Tg and dnKuz Tg mice passed β selection and proliferated normally. To confirm that the lack of TCRβ expression was contributing to the DN to DP block in dnKuz Tg mice, the mice were crossed with Vβ8.3 SGT Tg mice (a gift from P. Savage), which express a rearranged Vβ8.3 transgene under control of the natural TCR regulatory elements (32). Coexpression of the rearranged TCRβ gene and the dnKuz transgene resulted in a reduced DN to DP ratio (Fig. 2, *D* and *E*). The total thymocyte number in dnKuz/Vβ8.3 Tg mice was slightly higher than that in dnKuz littermates (Fig. 2*E*), although this difference was not statistically significant ($p = 0.528$). These results imply that reduced TCRβ expression at the DN stage is partly responsible for the reduced number of DP thymocytes seen in dnKuz Tg mice.

Expression of Notch target genes in dnKuz Tg mice

If Notch signaling were inhibited by dnKuz, we might expect the expression of Notch target genes to be reduced in dnKuz Tg thymocytes. To test this, the expression of Deltex-1, Hes-1, and pre-Tα, three reported Notch target genes (33–35), was examined by semiquantitative RT-PCR. Lineage-negative DN thymocytes were isolated from dnKuz Tg and non-Tg littermates, and RNA was isolated from these sorted populations. The expression of pre-Tα in dnKuz Tg mice was similar to that in controls (Fig. 3). In contrast, the expression of Deltex-1 and, to a lesser extent, Hes-1, was

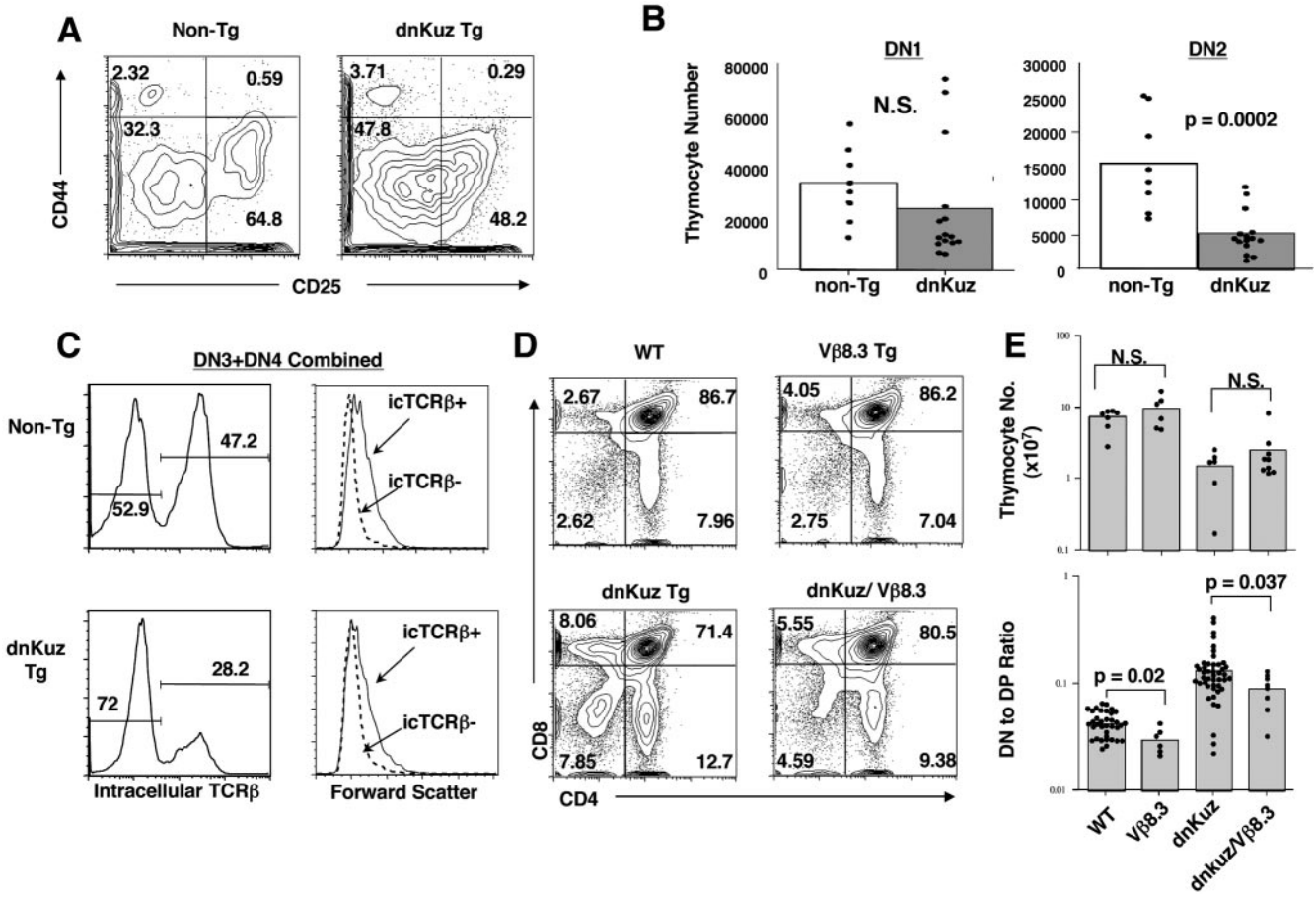

FIGURE 2. The block in thymic development in dnKuz Tg mice correlates with aberrant CD25 expression and a defect in TCRβ expression. *A*, Lineage-negative thymocytes from non-Tg and dnKuz Tg mice were stained with anti-CD44 and anti-CD25 to delineate the DN1 through DN4 stages of T cell development. The dnKuz Tg mice display a peculiar CD25/CD44 profile, where DN3 and DN4 stages are not clearly defined due to the presence of a $CD25^{int}$ population. *B*, Absolute numbers of DN1 and DN2 thymocytes in non-Tg and dnKuz Tg mice were calculated. *C*, Lineage-negative $CD44^-$ thymocytes (DN3 and DN4 combined) from non-Tg and dnKuz Tg mice were gated together, and intracellular TCRβ expression was examined in these grouped cells. Forward scatter data on the $icTCR\beta^-$ (dashed lines) and $icTCR\beta^+$ (solid lines) in both non-Tg and dnKuz Tg DN3/DN4 thymocytes are shown. *D*, The dnKuz Tg mice were crossed with mice expressing a rearranged Vβ8.3 transgene. Animals were analyzed at 4–12 wk of age. CD4/CD8 profiles of one representative WT ($n = 8$), Vβ8.3 ($n = 6$), dnKuz ($n = 6$), and dnKuz/Vβ8.3 ($n = 9$) Tg littermates are shown. E. *Top panel*, Total thymocyte numbers for WT ($n = 8$), Vβ8.3 ($n = 6$), dnKuz ($n = 6$), and dnKuz/Vβ8.3 ($n = 9$) Tg littermates are shown. Bars represent the mean thymocyte number, and dots represent individual mice within each group. *Bottom panel*, The DN to DP ratio for WT ($n = 39$), Vβ8.3 ($n = 6$), dnKuz ($n = 50$), and dnKuz/Vβ8.3 ($n = 9$) are shown. Bars represent the mean DN to DP ratio. Mann-Whitney tests for the difference in medians were used to test statistical significance between groups.

reduced in dnKuz Tg mice. These results are consistent with reduced Notch signaling in DN thymocytes in dnKuz Tg mice.

Overexpression of Delta-1 rescues thymocyte development in dnKuz Tg mice

In many respects, the block in T cell development observed in dnKuz Tg mice is similar to that observed in conditional Notch-1 knockout mice, in which Cre is driven by the Lck promoter (7). This similarity suggested that Notch signaling was impaired in the thymocytes of dnKuz Tg mice. Kuzbanian has been implicated in the cleavage of both Notch and its ligand, Delta-1 (24, 36, 37). Regardless of whether dnKuz impairs the cleavage of Notch or Delta, we would expect that overexpression of Delta might rescue the resulting Notch signaling defect by increasing the number of

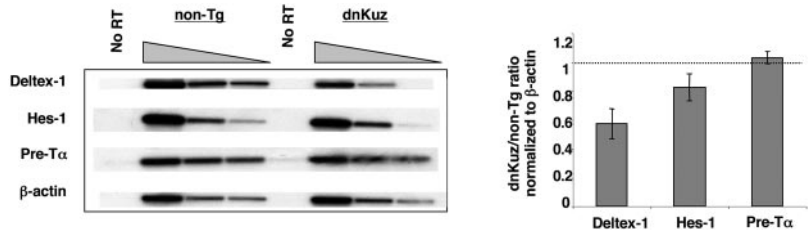

FIGURE 3. Examination of Notch target gene expression. Semiquantitative RT-PCR for Deltex-1, Hes-1, and pre-Tα genes was performed. Lineage-negative thymocytes were used as a source of RNA. A bar graph depicting the ratio of Deltex-1, Hes-1, or pre-Tα in dnKuz Tg mice compared with controls is shown. All values were normalized to β-actin. Bars represent the average ratio for six independent reactions. The expression of dnKuz protein was confirmed by Western blotting of sorted $CD3^-CD4^-CD8^-$ thymocytes (data not shown).

receptor-ligand interactions and therefore compensating for the reduced efficiency of Notch signaling. To test this idea, dnKuz Tg mice were crossed with mice that overexpress full-length Delta-1 in thymocytes under control of the Lck promoter (Fig. 4). Delta-1 Tg (DL-1Tg) mice express 13 times more Delta-1 mRNA in the thymus compared with WT thymus (data not shown) and do not display any differences in thymocyte cellularity compared with WT controls (Fig. 4*C*). Importantly, the DN to DP block was rescued in dnKuz/DL-1 double-Tg mice, as indicated by the DN to DP ratio (Fig. 4*A*, *top panels*, and Fig. 4*B*) and the overall thymic cellularity (Fig. 4*C*). In addition, the CD25/CD44 profiles of DN thymocytes and the expression of icTCRβ in dnKuz/DL1 mice were similar to those in DL1 controls (Fig. 4*A*, *middle* and *lower panels*, and Fig. 4*D*). These data indicate that overexpression of Delta-1 can substantially rescue the developmental block induced by dnKuz in the thymus.

DnKuz acts cell autonomously to block the DN to DP transition

In the classical model of Notch signaling, Notch ligands expressed on sending cells are thought to induce Notch signaling in the receiving cells (38). Kuzbanian might be involved in the cleavage of both Notch receptors (19–21) and the Notch ligand, Delta-1 (24, 25, 36, 37). Therefore, if dnKuz affects Delta-1 expression or function, the effect of dnKuz on thymocyte development might be cell

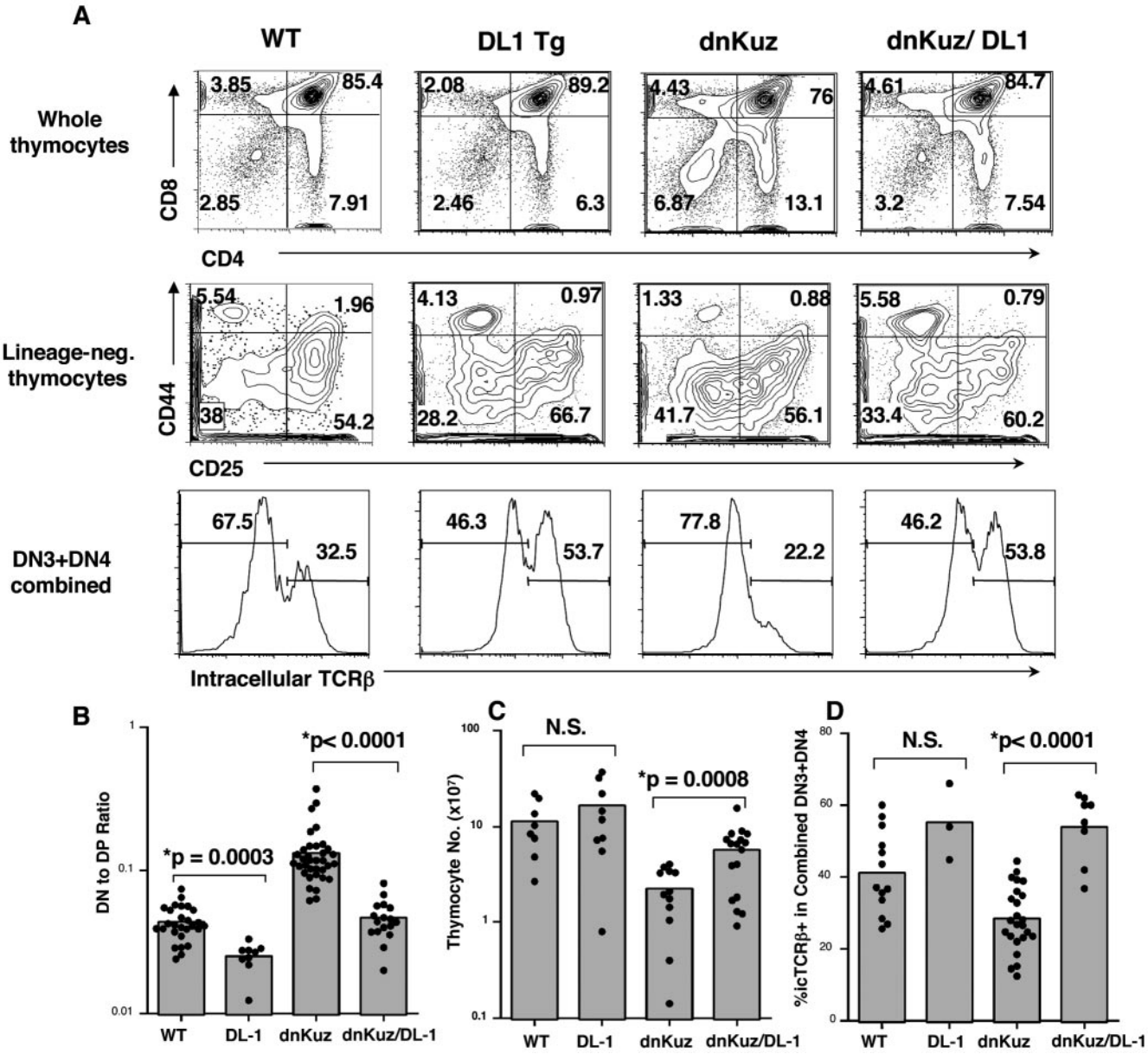

FIGURE 4. Overexpression of Delta-1 can rescue thymocyte development in dnKuz Tg mice. *A*, CD4/CD8 FACS profiles of whole thymocytes (*top panels*) CD25/CD44 FACS profiles of Lin$^-$ thymocytes (*middle panels*), and intracellular TCRβ staining profiles of DN3/DN4 thymocytes (*bottom panels*) are shown from one representative WT, Delta-1 (DL1 Tg), dnKuz, and dnKuz/Delta-1 (dnKuz/DL1) Tg mouse. *B*, The DN to DP ratio was calculated for WT ($n = 27$), DL1 Tg ($n = 9$), dnKuz Tg ($n = 35$), and dnKuz/DL1 ($n = 17$) double-Tg mice. Bars represent the mean DN to DP ratio, and dots represent individual mice within each group. *C*, Total thymocyte numbers in WT ($n = 8$), DL1 Tg ($n = 9$), dnKuz Tg ($n = 12$), and dnKuz/DL1 mice ($n = 17$) littermates. Bars represent the mean thymocyte number, and dots represent individual mice within each group. *D*, The mean percentages of icTCRβ^+ cells within combined DN3 and DN4 (Lin$^-$ CD44$^-$) thymocytes are shown for WT ($n = 13$), DL1 Tg ($n = 3$), dnKuz ($n = 27$), and dnKuz/DL1 ($n = 9$) mice. Bars represent the mean percentage within each group, and dots represent individual mice. Statistical significance was calculated using the Mann-Whitney *U* test for the difference in medians between groups. Animals were analyzed at 4–12 wk of age.

nonautonomous. In contrast, if dnKuz affects the cleavage of Notch receptors, the effect of dnKuz would be cell autonomous. To examine these possibilities, we prepared mixed (WT + dnKuz) bone marrow chimeras. Thymocyte development in the mixed chimera was compared with that in chimeras in which WT or dnKuz bone marrow was transferred alone. Thymocytes derived from the WT donor developed normally in both the WT→WT and the (WT+dnKuz)→WT chimeras (Fig. 5*A*, *first* and *third rows*). In contrast, thymocytes derived from the dnKuz donors comprised only 5% of the total thymocytes in the (WT+dnKuz) mixed chimeras (Fig. 5*B*, *top graph*) despite the fact that equal numbers of donor cells were injected, and bone marrow engraftments by WT-derived and dnKuz-derived bone marrow were similar (our unpublished observations). Interestingly, the DN to DP block was more pronounced in the dnKuz thymocytes developing in the mixed chimera compared with chimeras receiving only dnKuz bone marrow (Fig. 5*A*, *second* and *fourth rows*, and Fig. 5*B*, *bottom graph*) and intact dnKuz Tg mice (Fig. 1*C*). Some possible explanations for this enhanced block will be addressed in *Discussion*. These experiments demonstrate that the effect of the dnKuz transgene is cell autonomous and indicate that the dnKuz transgene impairs the ability of thymocytes to receive a Notch signal.

Delta-1/dnKuz interactions in trans *partially rescue the developmental block in dnKuz-derived thymocytes*

The observation that dnKuz mutation is cell autonomous (Fig. 5) together with the rescue in development seen by overexpression of Delta-1 on dnKuz thymocytes (Fig. 4) imply that Delta-1 might rescue thymocyte development by increasing Kuz/Delta-1 interactions between thymocytes (i.e., in *trans*). To test this prediction, we prepared mixed (WT + dnKuz/DL1) bone marrow chimeras, in which the Delta-1 and dnKuz transgenes were expressed on the same thymocyte population (i.e., in *cis*-), and mixed (DL1 + dnKuz) bone marrow chimeras, in which Delta-1 and dnKuz transgenes were expressed on different thymocyte populations (i.e., in *trans*). In the (WT + dnKuz/DL1) mixed chimeras, dnKuz/DL1-derived thymocytes comprised only 1.5% of the total thymocytes and displayed a DN to DP ratio of 0.91 (Fig. 5*B*). In contrast, in the (DL1 + dnKuz) mixed chimeras, dnKuz-derived thymocytes comprised 15% of the total thymocytes and displayed a DN to DP ratio of 0.1 (Fig. 5*B*). Although dnKuz thymocytes from (DL1 + dnKuz) mixed chimeras still showed some developmental impairment, the block was much less severe than that seen in control mixed chimeras (i.e., (WT + dnKuz) and (WT + dnKuz/DL1) mixed chimeras). Taken together, these data indicate that dnKuz expression in thymocytes impairs Notch signaling, and that overexpression of Delta-1 on neighboring thymocytes can partially compensate for this impairment.

An activated form of Notch-1 rescues TCRβ expression, but does not promote the DN to DP transition in dnKuz Tg mice

If Kuzbanian acts to cleave Notch receptors and promotes Notch signaling, we would expect that introduction of a cleaved, ligand-independent, activated form of Notch would rescue thymocyte development in dnKuz Tg mice. The dnKuz Tg mice were crossed with NIC9 Tg mice, which express an intracellular, activated form

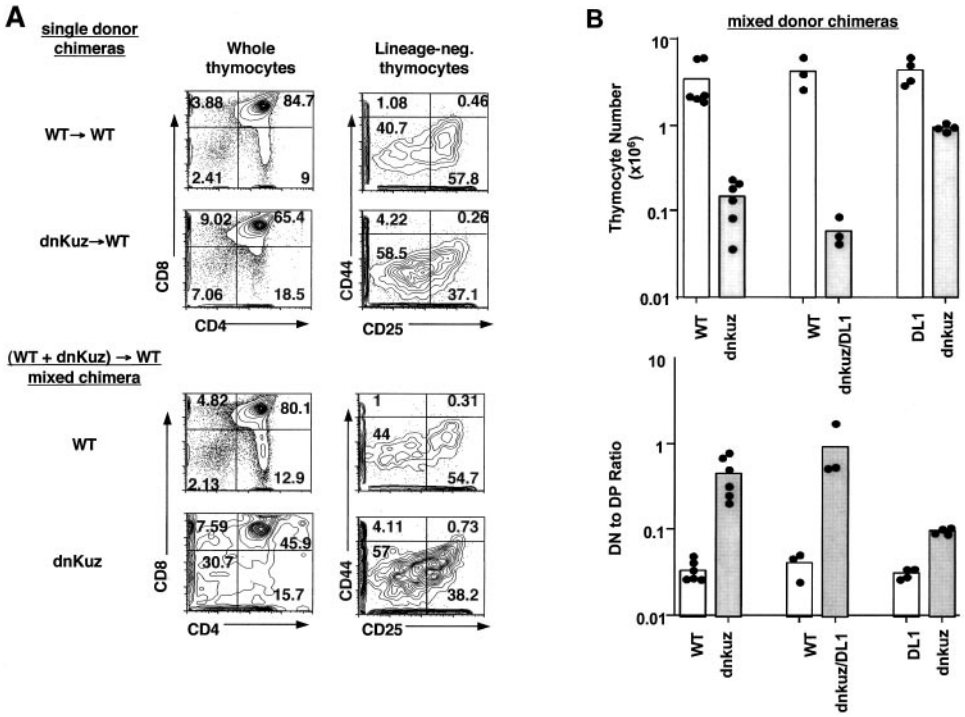

FIGURE 5. The effect of dnKuz is cell autonomous and can be partially rescued by overexpression of Delta-1 in *trans*. Bone marrow from single donors or a 50:50 mixture of bone marrow from WT, dnKuz, DL1 Tg, or dnKuz/DL1 double-Tg mice was administered to lethally irradiated WT (Ly5.1/5.1) hosts. In all cases, the genotypes of the two donors were either Ly5.2/5.2 or Ly5.2/5.1; thus, cells derived from both donors and any residual host cells could be distinguished. Chimeras were analyzed at 6 wk after bone marrow transplantation. No residual host cells were observed in the chimeras (our unpublished observations). *A*, Thymocyte profiles from donor-derived whole thymocytes and Lin$^-$ thymocytes are shown from one representative WT or dnKuz single donor chimera of four (*top* two rows), and one representative (WT plus dnKuz) mixed chimera of six (*bottom* two rows). For the mixed chimera, data for WT-derived and dnKuz-derived thymocytes are separated (third and fourth rows, respectively). *B*, The total number of thymocytes (*top* graph) and the DN to DP ratio (*bottom* graph) in mixed bone marrow chimeras are shown. For mixed bone marrow chimeras, values are given for each donor population. Single donor chimeras displayed thymocyte development similar to that of intact WT, dnKuz Tg, DL-1 Tg, and dnKuz/DL-1 Tg mice (our unpublished observations and Figs. 1 and 4). Bars represent the mean, and dots represent individual mice within each group.

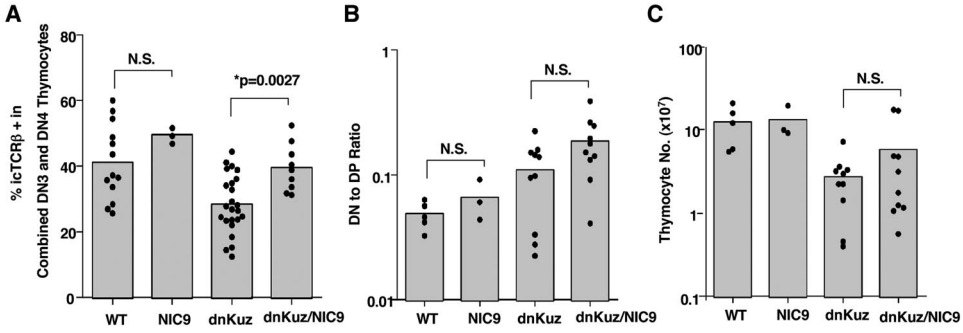

FIGURE 6. The expression of activated Notch-1 rescues TCRβ expression, but does not completely overcome the DN to DP block in dnKuz Tg thymocytes. *A*, The mean percentages of intracellular TCRβ^+ cells within combined DN3 and DN4 (Lin^- $CD44^-$) thymocytes are shown for WT (n = 13), NIC9 (n = 3), dnKuz (n = 27), and dnKuz/NIC9 (n = 9) mice. Animals were analyzed at 3–6 wk of age. Bars represent the mean percentage within each group, and dots represent individual mice. Statistical difference was calculated using the Mann-Whitney U test for difference of medians between groups. *B*, The DN to DP ratio of WT, NIC9, dnKuz, and dnKuz/NIC9 mice are shown. Bars represent the mean DN to DP ratio, and dots represent individual mice within each group. *C*, Thymocyte number among WT, NIC9, dnKuz, and dnKuz/NIC9 mice are shown. Bars represent the mean thymocyte number, and dots represent individual mice within each group. No statistical difference between the dnKuz and dnKuz/NIC9 mice was observed (p = 0.7394, by Mann-Whitney U test).

of Notch-1 (Notch-1IC) under control of the Lck promoter (8, 39). A higher expression of intracellular TCRβ expression was observed in dnKuz/NIC9 mice compared with dnKuz Tg mice (Fig. 6*A*). However, despite this increased TCRβ expression, the block between the DN to DP stages was still present in dnKuz/NIC9 double-Tg mice (Fig. 6*B*), and thymocyte cellularity between dnKuz and dnKuz/NIC9 Tg mice was not significantly different (Fig. 6*C*). These data can be interpreted in light of earlier studies showing that multiple Notch-dependent steps are involved in the DN to DP transition. In an early step, Notch promotes TCRβ expression to allow for the formation of the pre-TCR, and in a later step promotes cellular expansion in response to pre-TCR signaling (6). Our data suggest that although dnKuz blocks both these steps, activated Notch-1 can substitute for the first Notch-dependent step, but not the second. The possible implications of this will be discussed below.

Discussion

The importance of Notch signaling in T cell fate decisions and development has been well established (reviewed in Refs. 1, 3, and 40); however, the regulation of endogenous Notch signaling in thymocytes is not well understood. It is known that Notch receptors and Notch ligands can be cleaved by ADAM and presenilin-type proteases (16, 21, 24, 25, 36, 37, 41), raising the possibility that controlled proteolytic cleavage might be a mechanism in which endogenous Notch signaling is regulated. To examine the role of Kuz in Notch signaling during thymocyte development, we created Tg mice that express a dominant-negative form of Kuz in the thymus.

The block in thymocyte development in dnKuz Tg mice is similar in many ways to that in the Lck-conditional Notch-1 knockout (Notch-$1^{-/-}$) mouse (7). Both dnKuz and Notch-$1^{-/-}$ mice display blocks in the DN to DP transition, and this block correlates with a defect in TCRβ expression at the DN3 stage. In addition, CD25 appears to be prematurely down-regulated in DN thymocytes from dnKuz and Notch-$1^{-/-}$ mice (7). This is consistent with the up-regulation of CD25 seen in thymocytes and mature T cells in response to activated Notch (9, 13) and suggests that CD25 expression is regulated by Notch-1.

A connection between Kuzbanian and Notch signaling during thymocyte development is also suggested by the expression of some Notch target genes. The expression of Deltex-1 and, to a lesser extent, Hes-1 was reduced in dnKuz Tg mice, consistent with an inhibition of Notch signaling. The modest effect of the dnKuz mutation on Hes-1 expression may be a reflection of Notch-independent expression of this gene as well as the transient nature of Hes-1 induction in response to Notch signaling (42, 43). These considerations may also explain the observation that Hes-1 expression was not significantly altered in RBP-Jκ mutant mice, in which CSL-dependent Notch signaling is abolished (44). In addition, although pre-Tα was suggested to be a Notch target gene based on in vitro reporter assays by Reizis and Leder (34), analysis of conditional Notch-1 knockout mice did not reveal any difference in its expression (7). Similarly, we did not observe any change in the expression of pre-Tα in dnKuz Tg mice. Therefore, pre-Tα may not be a major target of Notch in vivo, and it is likely that other mechanisms are involved in the regulation of its expression.

There are, however, some slight differences between the Notch-$1^{-/-}$ and dnKuz mice. DN3 thymocytes from Notch-$1^{-/-}$ mice were reported to have decreased rearrangement of the TCRβ gene, suggesting that reduced TCRβ expression in these cells results from an impairment of gene rearrangements. In contrast, we did not observe a consistent decrease in TCRβ gene rearrangements in dnKuz Tg mice (our unpublished observations). Although this may reflect a difference in phenotype, it is also possible that this difference is due to limitations of this assay, which in our hands is not sufficiently quantitative to reliably detect differences of $<$10-fold. Thus, it is unclear whether the primary effect of the dnKuz mutation is on rearrangement or expression of the TCRβ gene. However, our observation that expression of a TCRβ transgene in dnKuz mice rescues thymocyte development (Fig. 2, *D* and *E*) strongly suggests that a defect in TCRβ expression is contributing to the block.

Another difference between the Notch-$1^{-/-}$ and dnKuz mice is found in the thymocyte populations that are affected by the different mutations. Specifically, in the Notch-$1^{-/-}$ mice, no differences in the absolute numbers of DN thymocytes or $\gamma\delta$ T cells were observed (7), whereas in dnKuz mice, both these populations were reduced (Figs. 1 and 2 and data not shown). This discrepancy might be explained by an earlier inhibition of Notch signaling in the dnKuz mice compared with the Notch-$1^{-/-}$ mice. For example, in dnKuz mice, the dnKuz transgene might be expressed as early as the DN1 stage (30), and the dnKuz could begin inhibiting Notch signaling as soon as it is expressed. In the Lck-Notch-$1^{-/-}$ mice, in contrast, deletion of the Notch gene is not complete until the DN3 stage (7). At this stage and beyond, common defects in

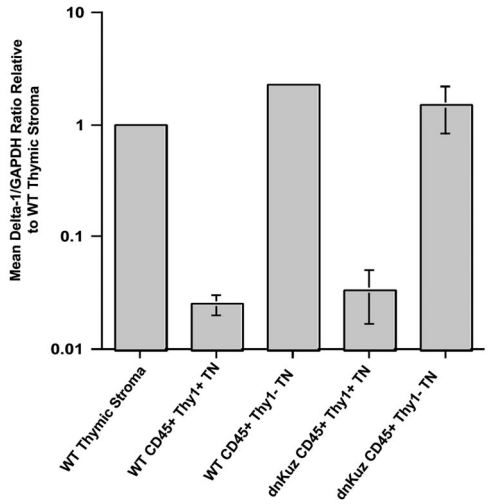

FIGURE 7. Expression of Delta-1 on early stage thymocytes in WT and dnKuz Tg mice. RNA was isolated from FACS-sorted $CD45^+Thy1^+CD3^-CD4^-CD8^-$ (TN) and $CD45^+Thy1^-$ TN thymocytes from WT and dnKuz Tg mice and analyzed by real-time PCR for expression of Delta. This sorting scheme was used to allow for exclusion of CD45-negative thymic stromal cells, but permit comparison of multiple hemopoietic lineages, such as thymic B cells, dendritic cells ($CD45^+Thy1^-CD3^-CD4^-CD8^-$), and early thymic progenitors ($CD45^+Thy1^+CD3^-CD4^-CD8^-$). Real-time PCR analysis of Delta-1 and GAPDH expression in these populations was performed in triplicate, and the ratio of Delta-1 expression to GAPDH expression was calculated. The mean Delta-1/GAPDH ratio for each sample set was then calculated and compared with the Delta-1/GAPDH ratio from enriched WT thymic stroma as a reference sample. Enriched WT thymic stroma consisted of thymic tissue remaining after filtering thymocytes through nylon mesh. Data shown are combined from two independent experiments (six individual replicates in total). Error bars represent the SD. The SD for WT $CD45^+Thy1^-$ TN equals 0.127, but is not visible on the bar graph.

thymocyte development are observed in both dnKuz and Notch-$1^{-/-}$ mice.

Although our results indicate that dnKuz impairs Notch signaling in the receiving cell, we also see clear evidence for an impact of neighboring thymocytes on the phenotype of the mutation. In particular, the effect of the dnKuz mutation is exaggerated when dnKuz thymocytes develop in the presence of wild-type thymocytes. This is reflected in the greater block seen in (WT plus dnKuz) mixed chimera compared with intact dnKuz mice and in the partial rescue seen in (DL1 plus dnKuz) mixed chimeras compared with intact dnKuz/DL1 Tg mice. This enhanced block in development in the mixed chimeras might reflect a general inability of the dnKuz-derived thymocytes to compete with WT-derived thymocytes in the thymus. For example, a decrease in overall proliferative ability might contribute to the reduced competitive fitness of the dnKuz-derived thymocytes. A more interesting possibility is that the enhanced block in development might be due to a lateral signaling mechanism. During lateral signaling, Notch ligand expression is up-regulated in cells that receive lower Notch signals (38, 45). If a lateral signaling mechanism operates during thymic development, reduced Notch signaling in dnKuz-expressing cells could cause these cells to up-regulate ligand expression. Increased levels of Notch ligand could partially relieve the block to Notch signaling in intact dnKuz Tg mice, a situation in which all thymocytes express dnKuz. In mixed chimeras, neighboring WT thymocytes would express normal levels of Notch ligand. This normal level of Notch ligand would be insufficient to bypass the block in Notch signaling the dnKuz-expressing thymocytes, resulting in a more severe developmental block. Although we did not observe any impact of the dnKuz transgene on the levels of Delta1 mRNA (Fig. 7), additional detailed analysis of the expression levels of Delta-1 and other Notch ligands is necessary to investigate the existence of lateral signaling mechanisms controlling Notch in thymocytes.

Our results raise the question of whether Notch ligands on thymocytes and stromal cells contribute to Notch signaling in thymocytes. The relatively low level of Delta-1 expression on thymocytes compared with thymic stromal cells (Fig. 7) (46, 47) suggests that stromal cells may be a more important source of Notch ligands. However, Notch/Delta-1 interactions between thymocytes could result in an equivalent level of signal as Notch/Delta-1 interactions between thymocytes and stromal cells, if one considers that thymocytes comprise >90% of the cells in the thymus. Moreover, our current experiments demonstrate that the expression of Delta-1 on thymocytes can induce Notch signals in adjacent thymocytes (Fig. 5). Comparison of the developmental signals derived from interactions between thymocytes vs signals derived from interactions between thymocytes and stromal cells is a topic of current investigation in our laboratory.

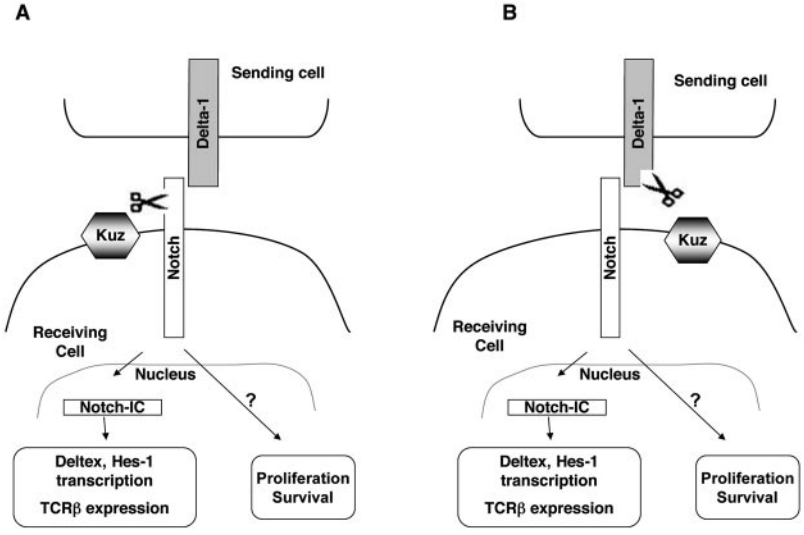

FIGURE 8. A working model to explain the interactions between Kuz and Delta-1 during T cell development. *A*, Upon binding of Notch receptor to the Notch ligand, Delta-1, Kuz cleaves the Notch-1 receptor extracellularly, which begins a proteolytic cleavage cascade resulting in the transcription of Notch target genes, such as Deltex-1 and Hes-1, and promotion of TCRβ expression. Other Notch components might be necessary for the survival and proliferation of early thymocytes. *B*, The data are also consistent with a model in which Delta-1 is the substrate for Kuz, but the Kuz activity is restricted to the Delta on an adjacent cell that is engaged in active Notch signaling with the Kuz-expressing cell (see *Discussion*).

We propose a model to explain our results, in which Kuz promotes Notch signaling in thymocytes by increasing their sensitivity to ligand expressed on a neighboring cell. The mixed bone marrow chimera experiments presented in Fig. 5 show that the effect of dnKuz is cell autonomous, yet the rescue by Delta-1 is non-cell autonomous. This suggests that dnKuz impairs Notch signaling in thymocytes, such that Delta-1 expression in the thymus becomes limiting, and overexpression of Delta-1 on thymocytes can compensate for this impairment. The requirement for Kuz activity in the receiving cell fits with a model in which Kuz cleaves Notch in response to Delta binding, helping to initiate Notch signaling and leading to the transcription of Notch target genes and TCRβ rearrangement and/or expression (Fig. 8*A*). Overexpression of Delta-1 would overcome the effect of dnKuz, perhaps by providing a strong agonist signal through Notch, and thus overriding the block in Notch processing. The cell-autonomous nature of dnKuz mutation is also consistent with an alternative model in which Delta-1 is the substrate for Kuz, but Kuz activity is restricted to Delta on an adjacent (sending) cell that is engaged in active Notch signaling with the Kuz-expressing cell (the receiving cell; Fig. 8*B*). Kuz-mediated cleavage of Delta-1 on an adjacent thymocytes might promote transendocytosis, a mechanism that is important for the promotion of Notch signaling (48, 49). In this model, overexpression of Delta-1 in *trans* would overcome the effect of dnKuz by providing more substrate for endogenous Kuz.

Given the strong suggestions of a link between Kuz and the regulation of Notch signaling and the documented importance for Notch-1 during T cell development (4, 5, 7, 8), it is surprising that Notch-1-IC expression only partly rescued the dnKuz phenotype. This result may be best understood in light of recent work indicating a complex cooperation between Notch and pre-TCR signaling in which Notch is required both to promote TCRβ expression at the DN3 stage and to allow proliferation and survival of thymocytes that pass β selection (6). Our results indicate that dnKuz inhibits both of these Notch-dependent steps, but that Notch-1-IC can restore the first, but not the second, step. Although the explanation for the failure of Notch-1-IC to restore DP development in dnKuz mice is not clear, several possible explanations could be considered. One could imagine that the timing and/or levels of Notch signaling at the DN stage need to be precisely controlled, and that presence of high levels of Notch activity throughout this stage interfere with a process required for normal DN to DP transition. It is also worth noting that although Notch-1-IC provides a potent Notch signal, it may not trigger all the same downstream effects triggered by endogenous Notch. Indeed, some of the effects of Notch-1-IC could be mediated by increased signaling through endogenous Notch and might thus still require Notch proteolysis. The up-regulation of endogenous Notch in response to activated Notch is consistent with this idea (8, 50).

Notch signaling can be regulated in a variety of ways, can trigger a diverse set of cellular responses, and can be deployed at successive developmental stages. These complexities pose a major challenge to a full understanding of the role of Notch signaling in T cell development. Our results provide the first indication that ADAM family proteases regulate the sensitivity of DN thymocytes to Notch signaling and, as such, represent an important piece to this complex puzzle.

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Disclosures

The authors have no financial conflict of interest.

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