Surface Expression of Notch1 on Thymocytes: Correlation with the Double-Negative to Double-Positive Transition

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Surface Expression of Notch1 on Thymocytes: Correlation with the Double-Negative to Double-Positive Transition

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Notch1 plays a critical role in regulating T lineage commitment during the differentiation of lymphoid precursors. The physiological relevance of Notch1 signaling during subsequent stages of T cell differentiation has been more controversial. This is due in part to conflicting data from studies examining the overexpression or targeted deletion of Notch1 and to difficulties in distinguishing between the activities of multiple Notch family members and their ligands, which are expressed in the thymus. We employed a polyclonal antiserum against the extracellular domain of Notch1 to study surface expression during thymopoiesis. We found high levels of Notch1 on the cell surface only on double negative (DN) stage 2 through the immature single-positive stage of thymocyte development, before the double-positive (DP) stage. The Notch signaling pathway, as read out by Deltex1 expression levels, is highly active in DN thymocytes. When an active Notch1 transgene, Notch1IC, is exogenously introduced into thymocytes of recombinase-activating gene 2-deficient mice, it promotes proliferation and development to the DP stage following anti-CD3 treatment without apparently affecting the intensity of pre-TCR signaling. In addition, a stromal cell line expressing the Notch ligand, Delta-like-1, promotes the in vitro expansion of wild-type DN3 thymocytes in vitro. Consistent with other recent reports, these data suggest a role for Notch1 during the DN to DP stage of thymocyte maturation and suggest a cellular mechanism by which Notch1IC oncogenes could contribute to thymoma development and maintenance. The Journal of Immunology, 2003, 171: 2296–2304.

As T cells develop in the thymus, TCR signals provide critical checkpoints as cells transit through the various stages of maturation. For example, a pre-TCR signal is necessary for the most immature thymocyte subset, termed double negative (DN),1 to develop into double-positive (DP) thymocytes, expressing both CD4 and CD8 (1). The assembly and surface expression of CD3, pre-Tα, and a functionally rearranged TCRβ-chain mediate this checkpoint, termed β selection. After successful pre-TCR signaling, DN thymocytes undergo many rounds of division and multiple phenotypic changes. In addition to genes that encode pre-TCR components, a number of other genes regulate maturation. These genes either affect pre-TCR signaling indirectly or are required for the numerous cellular changes seen during the DN to DP transition (2–5).

The Notch1 signaling pathway has been proposed to play a role during various stages of T cell development. Notch response genes such as Deltex1 are regulated during thymocyte development. Deltex1 is highly expressed in DN, down-regulated in DP cells, and up-regulated in mature CD8 and CD4 single-positive cells (SP) (6). Studies with transgenic mouse models and retrovirally transduced stem cells have shown that expression of the active intracellular portion of Notch1, Notch1IC, can modify the survival, proliferation, and maturation of thymocytes and potentially modulate TCR signaling (6–11). In addition, pharmacological inhibition of Notch signaling in thymic organ culture systems impairs thymocyte development (12, 13). Specific gene deletion of Notch1 at the earliest lymphoid precursors obviates further development into the T lineage (14, 15).

The Notch1 protein belongs to a family of receptors that upon interaction with ligand releases the intracellular fragment, Notch1IC, via a proteolytic cleavage event. Notch1IC translocates into the nucleus, binds C promoter binding factor 1, and activates transcription (16). Target genes induced by Notch1 in T cell lines include Hes family members, Deltex1, and pre-Tα (6, 17, 18). Multiple homologues of the Notch1 receptor and a number of different Notch ligands are expressed throughout the normal thymus. The four Notch family members share homology in their intracellular domains, and all have been shown to bind to C promoter binding factor 1. Therefore, intracellular signaling of the family members may converge on similar target genes. For example, overexpression of the active intracellular forms of Notch1, Notch2, and Notch3 is able to up-regulate Hes family members (19–21).

The mammalian Notch1 receptor was originally identified in a subset of human T cell acute lymphoblastic leukemias (T-ALL), where a chromosomal translocation resulted in a Notch1 truncation, ostensibly Notch1IC, brought under the control of a T cell-specific promoter (22). In mice this has been recapitulated by the expression of intracellular fragments of both human and mouse Notch1 and mouse Notch3 (23–25). Tumor development with these constructs, even when expressed in all hemopoietic cells, occurs exclusively in immature thymocytes (23, 24). In addition, in vivo mouse mammary tumor virus insertional mutagenesis studies identified intracellular Notch1 constructs as putative collaborators.
with c-Myc transformation in the majority of the isolated tumors (26). However, the cellular mechanisms that are targeted by Notch1IC signaling during thymomagenesis have not been well characterized.

Notch1 has been clearly implicated in promoting T lineage choice from common lymphoid progenitors, although its role in later development is less clear (15, 27). Inducible gene ablation of Notch1 via Lck-Cre-mediated deletion suggests its role at least through the DN stages (14). However, ablation of Notch1 at the late DN stages via CD4/Cre-mediated gene deletion allows normal thymocyte development (28). On the other hand, overexpression of Notch1IC, pharmacological inhibition of Notch signaling, and the pattern of Notch target gene expression suggest a role at the later stages of DP to SP development (6, 10, 12, 13). In light of the conflicting data on the physiological role of Notch1 during different stages of thymocyte development, we sought to determine at what stages of development is biologically accessible Notch1 receptor expressed on thymocytes and to correlate it with Notch target gene expression. Notch ligand family members Delta and Jagged, have been shown to be expressed throughout the thymus, and all are able to interact with Notch1 (29, 30). This suggests that what stages of development is biologically accessible Notch1 receptor expressed on thymocytes and to correlate it with Notch target gene expression. Notch ligand family members Delta and Jagged, have been shown to be expressed throughout the thymus, and all are able to interact with Notch1 (29, 30). This suggests that

Materials and Methods

Cell lines

Human 293 HEK cells were transfected with full-length murine Notch1 (FLN1/293) inserted into the pCDNA3 vector (Stratagene, La Jolla, CA) or with vector alone. Clones were selected in DMEM with 10% BSA and 1 mg/ml G418, and the expression of murine Notch1 was confirmed by Western analysis with Abs to both intracellular and extracellular portions of mNotch1.

Ab and cell staining

The anti-Notch1ec reagent is an immunoaffinity-purified rabbit IgG raised against the rat Notch1 epidermal growth factor (EGF) 10-EGF22, residues 381–853 (31) (Upstate Biotechnology, Lake Placid, NY), which cross-reacts with mouse Notch1. Cells were stained using a tertiary protocol with a biotinylated goat anti-rabbit Ab, followed by a streptavidin-conjugated fluorochrome. Purified normal rabbit IgG (Upstate Biotechnology) was also included as a staining control. Fluorochrome-conjugated Abs for CD4, CD8, CD24 (heat shock Ag), CD44, CD25, CD69, B220, CD3 (BD Pharmingen [San Diego, CA] and eBioscience [San Diego, CA]) were used to phenotypically characterize and sort cell populations by FACS analysis.

GST fusion protein corresponding to the murine extracellular region of genomic sequence: hypoxanthine phosphoribosyl-transferase (HPRT): forward, 5′-TGG AAA GAA TGG TGT TGA A; reverse, 5′-AGC TTG CAA CCT TAA CCA TTT TG; probe, 5′-FAM-CAT TTG TTC TCT CCT GTG ACC TAG TAC AGC-TAMRA; Notch1: forward, 5′-GAG ACC AAG AAG TTC CGG TTT G; reverse, 5′-CCT CAC TTC GTC CAC TCT CAA GG; probe, 5′-FAM-CAA TCG TTG AGC ACC AGA TGG CTT CCT AC A-TAMRA; Deltex1: forward, 5′-GTA GGA TGT GGG TTC GAG GT; reverse, 5′-CCC TCA TAG CCA GAT GCT GTG; probe, 5′-FAM-CGC CTG ATG AGG ACT GTA CCA TTT GCA T-TAMRA; and pre-Ta: forward, 5′-CTG CTT CTG GGC GTC AGG T; reverse, 5′-TGG GTC CTT CCA TCT ACC AGC AGT; probe, 5′-FAM-CTC TTC CGT CTC TCC CCA TTT CCA GTA CCA GCA-AGT; and TaqMan 2′X universal PCR Mastermix (PE Applied Biosystems). No template controls and no reverse transcription controls were included for each primer/probe set and cDNA set, respectively. Transcript levels were normalized to HPRT levels before determining the relative fold difference in the gene of interest. RT-PCR analysis of the Notch1IC transgene was performed as described previously (6).

Histology

Analyses of 6- to 8-wk-old B6 mouse thymuses used an indirect enzyme immunohistochemical procedure (33). Briefly, frozen sections of tissue were serially incubated with biotinylated and detected with streptavidin-peroxidase conjugate. Immunostaining was visualized by reaction with diaminobenzidine. Results were then counterstained with hematoxylin.

Cell cycle analysis

Cells were stained with the DNA-binding dye 7-amino actinomycin D to determine DNA content (BD Biosciences). Briefly, cells were stained for cell surface Ags, permeabilized, fixed, and stained with 0.25 μg of 7-amino actinomycin D/1 × 10⁶ cells before FACS analysis. Cells were gated on the appropriate thymic subsets as defined by CD8 and CD4 cell surface expression and were analyzed for the percentage of cells with >2 N DNA content. B6 DP thymocytes, which are mostly in G0, were used to define 2 N gates.

To induce DN to DP development in RAG2-deficient mice, 100 μg of 2C11, anti-CD3ε, mAb was delivered in one i.p. injection. Mouse thymi were harvested and analyzed at various time points after injection, as indicated. 2C11 mAb was immunoaffinity purified from ascites and dialyzed against sterile PBS.

Transcript expression analysis

Sorted subsets. Thymocytes were isolated from 4- to 6-wk-old B6 mice and sorted on a FACSVantage (BD Biosciences, San Diego, CA) into phenotypic thymocyte subsets; unsorted thymocytes, DN (CD3⁻, CD8⁻, CD4⁻, B220⁺), DP (CD8⁺, CD4⁺), CD4⁺ (CD4⁺, CD3⁻), CD8 (CD8⁺, CD3⁻), and DN subsets as previously described (32); unsorted DN (CD3⁻, CD8⁻, CD4⁺, B220⁻), DN1 (DN and CD4⁻, CD25⁻), DN2 (DN and CD4⁺, CD25⁺), DN3 (DN and CD4⁻, CD25⁺), and DN4 (DN and CD4⁺, CD25⁻). RNA and cDNA were prepared using RNA STAT 60 (Tel-Test, Friendswood, TX) and SuperScript II (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

The cDNA samples were analyzed in triplicate by real-time PCR with sequence-specific probes on an ABI 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Primers and probes were designed to span exon/intron borders to prevent amplification of genomic sequence: hypoxanthine phosphoribosyl-transferase (HPRT): forward, 5′-TGG AAA GAA TGG TGT TGA A; reverse, 5′-AGC TTG CAA CCT TAA CCA TTT TG; probe, 5′-FAM-CAT TTG TTC TCT CCT GTG ACC TAG TAC AGC-TAMRA; Notch1: forward, 5′-GAG ACC AAG AAG TTC CGG TTT G; reverse, 5′-CCT CAC TTC GTC CAC TCT CAA GG; probe, 5′-FAM-CAA TCG TTG AGC ACC AGA TGG CTT CCT AC A-TAMRA; Deltex1: forward, 5′-GTA GGA TGT GGG TTC GAG GT; reverse, 5′-CCC TCA TAG CCA GAT GCT GTG; probe, 5′-FAM-CGC CTG ATG AGG ACT GTA CCA TTT GCA T-TAMRA; and pre-Ta: forward, 5′-CTG CTT CTG GGC GTC AGG T; reverse, 5′-TGG GTC CTT CCA TCT ACC AGC AGT; probe, 5′-FAM-CTC TTC CGT CTC TCC CCA TTT CCA GTA CCA GCA-AGT; and TaqMan 2′X universal PCR Mastermix (PE Applied Biosystems). No template controls and no reverse transcription controls were included for each primer/probe set and cDNA set, respectively. Transcript levels were normalized to HPRT levels before determining the relative fold difference in the gene of interest. RT-PCR analysis of the Notch1IC transgene was performed as described previously (6).
Extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation

Thymocytes were pooled from 3- to 5-week-old Notch1IC × Rag2−/− or Rag2−/− mice. Cells were stimulated with 5 ng/ml PMA at 37°C for 1 h, washed, and lysed with TBS, 1% Triton 0.2 mM Na2VO4, 50 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotenin, and 1 mM PMSF. Supernatant from 2.5 × 10⁷ thymocyte equivalents was resolved by SDS-PAGE on a 10% gel and transferred to OptiBlot nitrocellulose membrane (Schleicher & Schuell, Keene, NH) according to the manufacturer’s protocol. The blot was subsequently stripped in 100 mM 2-ME, 2% SDS, and 62.5 mM Tris (pH 6.8) at 50°C for 30 min; rebloked; and immunoblotted for total ERK (ERK2; Santa Cruz Antibodies, Santa Cruz, CA) as a loading control. Proteins were detected by HRP-conjugated secondary Abs (Santa Cruz Antibodies) and ECL according to the manufacturer’s protocol (Amersham Pharmacia Biotech, Piscataway, NJ).

OP9 monolayer cultures

DP and DN3 thymocytes were isolated from 4- to 6-week-old mice. CD8+CD4+CD69−/− (DP) thymocytes were isolated by FACS sorting. For isolation of DN3 thymocytes, the total DN population was enriched by immunomagnetic separation using biotinylated Abs to CD4 and CD8 (Dynal, Oslo, Norway) according to the manufacturer’s instructions, and the DN3 population was sorted on a FACS vantage. Total DN (CD4+CD8−) thymocytes were negatively gated for lineage markers (CD4, CD8, CD3, B220, Mac1, Gr1, and Ter114), and the DN3 fraction was identified as CD44+CD25−. Sorted thymocyte subsets (1 × 10⁷) were plated on monolayers of OP9 or OP9 cells expressing the Notch ligand, Delta-like 1 (OP9-D1) (18) in 24-well plates in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 15% FCS, penicillin/streptomycin, 1-glutamine, and 5 ng/ml of rIL-7 and Flt3L (Peprotech, Rocky Hill, NJ). The growth and viability of the thymocytes were assessed by Trypan Blue staining.

Results

Anti-N1ec Ab specifically stains surface mouse Notch1

To date there has been little characterization of Notch1 surface expression in mammalian systems, including the thymus. We first sought to identify and characterize an Ab that is able to specifically stain surface Notch1. We used a rabbit immunoaffinity-purified IgG against the extracellular portion of rat Notch1, corresponding to EGF10-EGF22 anti-N1ec, for immunohistochemistry and FACS staining (Fig. 1A). Specificity for surface mouse Notch1 was determined by three assays. Firstly, 293 cells stably expressing full-length murine Notch1, FLN1/293, were generated. The transfected cells stained positively using anti-N1ec Ab (Fig. 1B). Secondly, anti-N1ec was preadsorbed on FLN1/293 or control cDNA3/293 monolayers, and specific staining of Notch1 on the FLN1/293 and thymocytes was compared. Preadsorption on FLN1/293, but not on control cDNA3/293 removed all staining activity (data not shown). Finally, a GST fusion protein containing the murine residues corresponding to the immunogen (i.e., EGF10−22), mN1ec–GST, was able to block staining on FLN1/293 cells and thymocytes when preincubated with anti-N1ec relative to GST controls (Figs. 1C, 2, and 3).

Early thymocytes express Notch1 at the cell surface

The expression of endogenous Notch1 during thymocyte development has not been well characterized. The majority of published reports have examined mRNA, and these results have been inconsistent. The expression of Notch1 at the cell surface requires several post-transcriptional modifications, including proteolytic processing, assembly in the endoplasmic reticulum, and transport to the cell surface before ligand-induced signaling. To address which cells may be capable of receiving a Notch1 signal, we assayed for the expression of Notch1 in the thymus by immunohistochemistry.

Using anti-N1ec to stain C57BL/6 thymus sections, we found that specific Notch1 staining is localized primarily to the outer cortex and subcapsular zone of the thymus (Fig. 2). This staining is blocked in the presence of mN1ec–GST fusion protein. The expression appears to be intercellular and suggests cell surface expression of Notch1. In the medulla, where mature SP thymocytes are thought to reside before export, Notch1 staining is less apparent (Fig. 2). This pattern of staining is consistent with expression by early thymocytes. T-cell progenitors are thought to enter the thymus at the cortical-medullary junction and migrate to the subcapsular zone during DN development. Specifically at the subcapsular zone late DN populations, DN3 and DN4 thymocytes, are present (35).

To identify the population of thymocytes expressing surface Notch1, we used anti-N1ec in FACs analysis of cells isolated from normal C57BL/6 mice. We detected Notch1 surface expression in the majority of DN3 and on a subset of DP and CD8 SP cells (Fig. 3B). Staining of CD8 SP cells was surprising given the lack of staining in the thymus medulla (Fig. 2). This apparent discrepancy was resolved when we analyzed Notch1 surface expression on immature CD8 SPs (ISPs), which express high levels of CD24 and are an intermediate step between DN and DP stage, vs staining on mature, CD24low, CD3highCD8 SP thymocytes. The data in Fig. 3C show that only the immature, pre-DP CD8+ T cells stain at the surface for Notch1.

We further examined Notch1 surface expression on the DN subpopulations by costaining for CD44 and CD25. We found surface expression of Notch1 up-regulated at the DN2 stage, when cells become fully committed to the T lineage, and remained throughout the DN and ISP compartments until down-regulated at the DP stage (Fig. 3, B and C, and Fig. 4B) (36). The early expression in DN thymocytes was further confirmed by real-time cDNA analysis.
of DN, DP, and mature CD4 and CD8 SP subsets (Fig. 3D). We found that the Notch1 transcript is highly expressed in the DN subset relative to the levels in DP and SP subsets.

While surface Notch1 expression is necessary for conventional activation of the Notch1 signaling pathway, it does not indicate which cells have actually received a Notch1 signal. To determine at what stage of T cell maturation the Notch1 pathway is normally activated, we examined the expression of Deltex1 mRNA in DN thymic subsets. Previous data from thymoma cell lines and transgenic mice showed Deltex1 is highly induced by active Notch1IC (7). We found that Deltex1 expression is increased at the DN3 stage before being down-regulated at the DN4 stage (Fig. 4C) consistent with a recent PCR analysis of Deltex1 expression (37). The decrease in Deltex1 expression relative to the high surface Notch1 expression in DN4 subsets suggests that Deltex1 expression may not fully reflect activation of the Notch pathway. It is clear that not all Notch-responsive genes, such as Hes1 and Meltrin β, are regulated to the same extent (6). This may be due to additional control by other transcriptional regulators and other Notch family members. These data suggest a role for Notch1 and activation of the Notch signaling pathway during immature thymocyte development, specifically during the late DN transition. This is consistent with a previous report that intracellular Notch1 expression is highest in DN thymocytes (38).

**Notch1 activation during the DN-to-DP transition increases thymic cellularity and cell cycling**

Given the expression pattern of Notch1 at the cell surface and the evidence for activation of the pathway, as indicated by target gene expression, we investigated the function of Notch1 during early thymocyte development. During the DN to DP transition there are several distinguishing hallmarks of thymocyte development. Upon functional rearrangement of the TCRβ, a pre-TCR signal is delivered, allowing transition from the DN3 to the DP stage and a coincident proliferative expansion. We asked what role the expression of an active Notch1 transgene would play during this developmental stage.

The lack of RAG recombinase machinery prevents TCR rearrangement and thus precludes β-selection. However, dysregulation of other genes that affect survival and cell cycle, TCR signaling, or transcriptional activation, such as p53, Lck, β-catenin, and FADD, permit development to the DP stage independent of pre-TCR signaling. (39–42) To determine whether Notch1 activation alone would cause DN to DP development in the absence of a pre-TCR signal, we analyzed thymi from mice expressing an active form of Notch1IC under the thymus-specific Lck-proximal promoter on a RAG2-deficient background, Notch1IC × RAG2−/−. Activated Notch1IC is unable to overcome the RAG developmental block, as thymus cellularity is similar to RAG2−/− controls, and the cells remain at the DN3 stage (Fig. 5A). To check that the Notch1IC transgene is expressed at this early stage, transgene-specific primers were used in semiquantitative RT-PCR assay to show the presence of the Notch1IC transcript. As expected from the Lck-proximal promoter, Notch1IC is expressed in the RAG2-deficient background, although at lower levels than in the RAG-sufficient thymocytes, which are predominately DP cells (Fig. 5B). This result is consistent with the failure of Notch1IC retrovirally introduced into RAG-deficient hemopoietic stem cells to develop into DP cells (43). Additionally, the Notch1IC × RAG2−/− mice or retrovirally transduced hemopoietic stem cells do not generate...
Notch1 activation has been implicated in both preventing apoptosis and promoting proliferation (7–9). To assess which cellular mechanism may explain the increased cell numbers found in the anti-CD3-treated, Notch1IC × RAG2−/− mice, we examined annexin V staining as a measure of apoptosis and DNA content to determine the percentage of cycling cells. Annexin V staining of thymocytes after anti-CD3 treatment revealed little difference between anti-CD3-treated Notch1IC × RAG2−/− mice and controls (data not shown). However, the percentage of cycling cells with >2 N DNA content, was increased in the DN, ISP, and DP populations from the Notch1IC × RAG2−/− mice (Fig. 6D). These data suggest that constitutive Notch1 activation promotes the increase in cell number by enhancing cell proliferation.

**Pre-TCR signals are not affected by Notch1 activation**

To determine whether the increase in cellularity and the fraction of cycling cells in anti-CD3-treated Notch1IC × RAG2−/− mice is due to an effect of Notch1 activation on pre-TCR signaling, we analyzed three components of the pre-TCR signaling cascade. Because the pre-TCR gene is a target of Notch1 activation, we analyzed the expression of pre-TCR and surface CD3 levels to determine whether the difference in response could be attributed to differences in the levels of the TCR components. We saw no increase in basal levels of surface CD3 by FACS analysis in Notch1IC × RAG2−/− mice compared with littermate controls (data not shown). Similarly, using real-time PCR to measure transcripts, we found no significant increase in pre-TCR expression in Notch1IC × RAG2−/− thymocytes (Fig. 7A). We also performed a kinetic analysis of ERK phosphorylation in response to PMA stimulation to determine whether proximal phosphorylation events of the TCR pathway were modulated by an active Notch1 signal. This kinetic analysis allowed us to determine whether changes in the absolute levels or in the duration of signals were affected by anti-CD3 Ab over time or complete filling of the thymic compartment (data not shown).

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enforced activation of Notch1. We found that ERK phosphorylation is induced and sustained after stimulation with the same kinetics in Notch1IC × RAG2KO and control RAG2−/− thymocytes (Fig. 7B).

Up-regulation of CD69 at the cell surface is an early marker of TCR stimulation and reflects the intensity of TCR signaling received by the T cell (45, 46). Mice injected with anti-CD3 were analyzed for CD69 up-regulation on thymocytes after 24 h. CD69 levels were up-regulated in both controls and Notch1IC × RAG2−/− mice, and we observed no significant difference in mean fluorescence of CD69 staining or the percentage of CD69-positive cells, suggesting that there was no difference in the perceived level of TCR signal due to the Notch1IC transgene (Fig. 7C). These results indicate that both proximal and distal points of the pre-TCR signaling cascade are unaffected by concurrent activation of the Notch1 pathway, suggesting that Notch1IC affects proliferation by augmenting a parallel pathway to the pre-TCR cascade or via other downstream effectors of the pre-TCR cascade outside the ERK signaling branch.

Delta-like-1-dependent proliferation of DN in vitro

To examine the role of Notch signaling in a more physiological setting, i.e., without an active NotchIC transgene, we turned to the recent description of T lymphopoiesis in vitro driven by a stromal cell line expressing the Notch ligand, Dll1 (34). The OP9 stromal cell line can support B cell development from fetal liver or bone marrow precursors, and Schmidt and Zuniga-Pflucker (34) showed that following expression of full-length Dll1 in OP9, T, but not B, cell development ensued. To determine whether Notch-Notch ligand signaling would enhance the expansion of DN cells from...
Notch2, and Notch3 plus all four ligands of the Notch receptors to express in the thymus, it has been described that Notch1 activity is important during SP development. However, deletion of Notch1 at the early DP stage via the CD4-Cre transgene reveals no aberrations in mature SP cells (6, 10, 11). However, deletion of Notch1 at the DN3 stage via an Lck-Cre transgene shows impairment in thymic phenotypes, mostly on the development of DP to SP development, decreased DP development, and an overall decreased CD69low DP thymocytes showed no difference in response when plated on control or Dl1-expressing OP9 monolayers. The results presented in Fig. 8 complement our previous conclusions on the role of Notch signaling at the DN to DP transition. Thus, DN3 cells plated on OP9-Dl1 expanded dramatically, while following plating on control OP9 monolayers, they only maintained the starting number. Also in line with our Notch1 expression analysis, CD69low DP thymocytes showed no difference in response when plated on control or Dl1-expressing OP9 monolayers.

Discussion

Overexpression of Notch1IC in cells of the T lineage by the use of transgenes or retroviral transduction has lead to a variety of alterations in thymic phenotypes, mostly on the development of DP to mature SP cells (6, 10, 11). However, deletion of Notch1 at the early DP stage via the CD4-Cre transgene reveals no aberrations in the differentiation of SP cells (28). In contrast, deletion of Notch1 at the DN2/DN3 stage via an Lck-Cre transgene shows impairment in further development, decreased DP development, and an overall decrease in cellularity in thymic subsets beyond the DN stage (14). Because immunoblot or mRNA analyses have shown Notch1, Notch2, and Notch3 plus all four ligands of the Notch receptors to be expressed in the thymus, it has been difficult to establish the physiological relevance of the Notch1 protein specifically. Difficulties in interpretation arise due to genetic complementation and redundancy or due to the activation of multiple Notch pathways by overexpression of the highly conserved intracellular region of Notch1IC. Here we present expression analysis of endogenous Notch1 at the cell surface that reads out the level of biologically accessible Notch1. We also correlate Notch1 surface expression with the expression levels of transcriptional targets. These results suggest that Notch1 plays a role during DN development and is less involved in the maturation of SP thymocytes.

Our staining results are in general agreement with previous work by Hasserjian et al. (38). In that work, intracellular staining of BALB/c mouse thymocytes using an Ab against the cytoplasmic portion of human Notch1 suggested that all thymus subsets express Notch1, compared with the staining with an anti-GST-negative control. They reported highest levels in DN subsets and lower levels in DP and SP subsets.

We have previously shown that Notch target genes are up-regulated in both CD4 and CD8 SP compared with the levels in their DP precursors (6, 7). Thus, it remains possible that other Notch family members are important for DP to SP development. This possibility is supported by data showing that low levels of prese-nilin inhibitors can block SP development in fetal thymic organ cultures (12, 13). In these studies the inhibitors block γ-secretase/ presenilin-dependent cleavage, a step shared by all Notch family members for ligand-dependent signaling of the Notch receptors. Finally, multiple ligands that interact with all the Notch receptors are expressed throughout the thymus (data not shown), allowing the potential for ligand-dependent Notch signaling at a variety of developmental stages. These findings suggest that the effect of Notch1IC on SP development may be interpreted as activating other Notch pathways or target genes that may be physiologically important during SP development.

In a recent study Wolfer et al. (14) reported that deletion of Notch1 at the DN2 stage via the expression of Lck-Cre resulted in an impairment in the rate of VDJ recombination at the TCRβ locus. They also noted an accumulation of aberrant DN4 thymocytes that lacked a functionally rearranged TCRβ-chain. To explain the latter observation they hypothesized that signaling through Notch1 is required to eliminate DN thymocytes that have failed TCRβ selection (14). In line with this, our results show that Notch1 is highly expressed at the cell surface from the DN2 to ISP stage, and the levels of Deltex1 mRNA are highest at the DN3 stage. Breeding the Notch1IC transgenic mice to RAG2−/− mice allowed us to ask what effect constitutive Notch signaling would have on early thymocytes. We found no evidence that enforced Notch signaling increased the turnover rate or apoptosis of RAG2−/− thymocytes that do not express a functional β-chain. Staining RAG2−/− controls vs Notch1IC × RAG2−/− thymocytes for DNA content or for apoptotic cells showed no difference between the two (data not shown).

Enforced Notch1 signaling also did not alter the phenotype of the RAG2−/− thymus; cellularity was unchanged, and most cells remained arrested at the DN3 stage (Fig. 5). This establishes a hierarchy of Notch signaling relative to pre-TCR signaling. For example, constitutive activation of Lck and β-catenin in RAG-deficient mice results in DN to DP transition, suggesting that development in these mice is pre-TCR-independent and that Lck and β-catenin are proposed downstream targets after initiation of the pre-TCR signaling cascade (41). Conversely, the DN3 block seen in Notch1IC × RAG2−/− suggests that Notch1 signaling at this stage is upstream or separate from the pre-TCR signaling cascade. Previously, two groups using Notch1IC and Notch3IC have shown that rapid leukemia development requires pre-TCR signaling. From these experiments it is unclear whether Notch1IC requires a specific stage development, i.e., after β-selection, to induce transformation or whether Notch1IC plays a role in affecting pre-TCR signaling, which leads to transformation. The developmental timeline in our experiments evaluates developmental and cellular changes from a few hours to a few days after β-selection, i.e., proximal to the pre-TCR signal (25, 43).

Mimicking pre-TCR signaling by injection of anti-CD3 Ab allowed us to examine whether the Notch1IC transgene would have an effect on the kinetics of the DN to DP transition that follows β-selection. Notch1IC increases the rate of development of DN3 cells to the ISP and then the DP compartment. Thus, in addition to the role for Notch1 signaling in driving TCRβ rearrangement (14), we propose that Notch signaling may play a positive role following pre-TCR signaling. One explanation for the enhanced development of Notch1IC × RAG2−/− thymocytes following anti-CD3 injection could have been that the DN3 cells in these mice express a higher level of CD3 and would therefore receive a stronger signal from the anti-CD3 Ab. Relevant to this is the fact that pre-Tα has been shown to be a target of Notch signaling in thymomas and thymocytes (7). Even in the absence of a TCRβ-chain, it is conceivable that increased levels of pre-Tα could up-regulate the surface expression of CD3. Our expression analysis, however,
showed no heightened expression of pre-Tα nor any increase in detectable levels of surface CD3 (data not shown) in the Notch1IC transgenic thymocytes. Furthermore, by studying the degree and kinetics of ERK phosphorylation and CD69 up-regulation in stimulated thymocytes from RAG2−/− and Notch1IC × RAG2−/− mice, we found no evidence that enforced Notch signaling modulated the pre-TCR signaling cascade. Finally, the comparison of the in vitro growth of DN3 thymocytes from control B6 mice on OP9 vs OP9-D11 stromal cells strongly supports a role for Notch signals in the DN to DP expansion (Fig. 8).

The effect of Notch1IC on proliferation may shed light on the cellular mechanisms that Notch1IC targets as an oncogene in the maintenance of thymoma growth and tumorigenesis (47). Recently, Aster’s group (48) has shown that pharmacological inhibition of the Notch pathway in Notch1IC-induced T-ALL lines results in cell cycle arrest and induction of apoptosis. This suggests that constitutive Notch1 activation is required for the growth potential of Notch1IC-induced thymomas in addition to any effects Notch1IC may have on differentiation of the tumor.

Physiological Notch signaling as well as oncogenic Notch1IC signaling may be important in up-regulating genes that promote proliferation, such as the bHLH gene, Hes1. Hes1 is up-regulated in DN subsets, and deletion of Hes1 results in an autonomous defect in the ability of immature thymocytes to proliferate (49, 50). In vivo Hes1 deficiency blocks development at the DN2 and DN3 stages, which correlates with Notch1 pathway activity (Fig. 4C). Although the molecular mechanism for Hes1 involvement in proliferation is not well characterized, there is evidence that Hes1 inhibits E protein activity by binding E47 and preventing transcriptional repression. In line with this, the loss of E47 activity results in early DN proliferation and tumorigenesis (51). We hypothesize that enforced expression of Notch1IC results in an increase in Hes1-dependent proliferation at the DN3 stage. Additionally, Notch1IC may augment thymocyte proliferation by inhibiting E protein activity in a manner independent of Hes1 (52). It will be a challenge for the future to determine the molecular mechanism by which Notch1IC induces the development and perpetuates the growth of T-ALL.

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