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Obligatory Role for Cooperative Signaling by Pre-TCR and Notch during Thymocyte Differentiation[1]

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The first checkpoint during T cell development, known as β selection, requires the successful rearrangement of the TCR-β gene locus. Notch signaling has been implicated in various stages during T lymphopoiesis. However, it is unclear whether Notch receptor-ligand interactions are necessary during β selection. Here, we show that pre-TCR signaling concurrent with Notch receptor and Delta-like-1 ligand interactions are required for the survival, proliferation, and differentiation of mouse $CD4^-CD8^-$ thymocytes to the $CD4^+CD8^+$ stage. Furthermore, we address the minimal signaling requirements underlying β selection and show a hierarchical positioning of key proximal signaling molecules. Collectively, our results demonstrate an essential role for Notch receptor-ligand interactions in enabling the autonomous signaling capacity of the pre-TCR complex. ***The Journal of Immunology*****, 2004, 172: 5230–5239.**

The generation of $\alpha\beta$-T lymphocytes from fetal liver- or adult bone marrow-derived progenitors occurs in the thymus. The earliest thymic precursors lack expression of both CD4 and CD8 coreceptors and are therefore referred to as double-negative (DN)[4] cells. This population can be further subdivided into four consecutive developmental stages by the ordered expression of CD44 and the IL-2 receptor α-chain (CD25) as follows: DN1 ($CD44^+CD25^-$); DN2 ($CD44^+CD25^+$); DN3 ($CD44^-CD25^+$); and DN4 ($CD44^-CD25^-$) (1). Recombinase-activating gene (RAG) 1/2-catalyzed rearrangement at the TCRβ locus is initiated as DN2 thymocytes progress to the DN3 stage (2–4). Only DN3 cells that have productively rearranged a TCR β-chain, which can assemble with the invariant pre-Tα and CD3 molecules to form the pre-TCR complex, are selected for further differentiation (5). This first critical checkpoint during thymocyte development is termed β selection (6). Expression of the pre-TCR promotes survival and proliferation of DN3 thymocytes, as well as differentiation to the CD4CD8 double-positive (DP) stage and cessation of further rearrangement at the TCRβ locus (5, 6). The critical role of pre-TCR formation is revealed through targeted disruption of *Rag1*, *Rag2*, or genes encoding components of the pre-TCR complex in mice, all of which result in a severe arrest in $\alpha\beta$ T cell development at the DN3 stage (5).

A notable feature of the DN to DP transition is the apparent ligand independence of the pre-TCR. This view is supported by the finding that the ectodomains of both pTα and TCRβ are dispensable for pre-TCR function (7, 8). The cell-autonomous nature of pre-TCR signaling has been attributed to the membrane localization of the receptor with signaling molecules in glycolipid-enriched microdomains and to the relatively low signaling threshold of pre-T cells (9, 10). Pre-TCR signaling alone, however, is not sufficient to drive T cell development. Indeed, isolated DN3 cells fail to differentiate in vitro in the absence of a supporting thymic microenvironment (11), revealing a requirement for thymic-derived signals for differentiation to the DP stage. Our recent finding that the expression of the Notch ligand Delta-like 1 (DL1) on a bone marrow stromal cell line is sufficient to support a normal program of T cell development (12), suggests that Notch receptor-ligand interactions may in part underlie the thymic dependence of β selection.

Signaling from the Notch family of transmembrane receptors has been shown to influence cell fate decisions in multiple developmental systems (13). Of the four mammalian Notch proteins, Notch 1, 2, and 3 are expressed in the thymus along with ligands Jagged 1 and 2 and Delta-like-1 and -4 (14–18). On ligand engagement, proteolytic cleavage events free the intracellular domain of Notch (Notch-IC) which translocates to the nucleus and modifies transcription of target genes through its association with CBF1. Recent studies have implicated Notch receptor-ligand interactions at various critical junctions in T cell development, most notably in the T vs B lineage choice (19, 20). Notch1 signals have also been suggested to promote the $\alpha\beta$ over the $\gamma\delta$ T lineage (21) and to influence the CD4 vs CD8 T lineage decision (22–24). The role of Notch signals beyond T cell commitment, however, remains controversial given that deletion of *Notch1* following the β selection stage failed to influence subsequent thymocyte development (25), whereas inactivation of Notch1 at the DN2/3 boundary affected $\alpha\beta$ but not $\gamma\delta$ T cell development due to an impairment in

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[4] Abbreviations used in this paper: DN, double-negative; RAG, recombinase-activating gene; DP, double-positive; DL1, Delta-like-1; GFP, green-fluorescent protein; FL, fetal liver; FTOC, fetal thymic organ culture; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; hrFlt3L, human recombinant Flt-3 ligand; mIL-7, murine IL-7; BIM, bisindolylmaleimide I; MEK, mitogen-activated protein/extracellular signal-related kinase kinase; Notch-IC, intracellular domain of Notch.

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Vβ to DJβ rearrangement (26). Thus, the role of Notch receptor-ligand interactions during the DN to DP transition remains unclear.

Assessment of the requirement for Notch signaling during β selection using traditional mouse models is hampered by several factors. Foremost, the expression of multiple Notch receptors and ligands in the thymus complicates gene-targeting approaches in mice due to issues of functional redundancy among protein family members. Also, the recently described role for Notch1 in TCRβ rearrangement (26) precludes the analysis of β selection events in thymocytes deficient for this receptor. Moreover, studies based on transgenic overexpression of Notch-IC are unlikely to represent physiological activation as evidenced by the induction of T cell lymphomas in these mice (24, 27, 28).

To circumvent these problems, in the present study, we make use of the OP9-DL1 in vitro T cell differentiation system, which we recently described (12, 29). This provides an ideal system in which to examine β selection outcomes by inducing pre-TCR signaling in RAG-2-deficient DN cells in the presence and absence of productive Notch signaling triggered by interaction with DL1. Here, we demonstrate that Notch-receptor ligand interactions are required for the functional outcomes of β selection, including rescue from apoptosis, cellular expansion, and differentiation to the DP stage. This approach also allowed us to identify the minimal signaling requirements for the transition of DN thymocytes to the DP stage of T cell development. Taken together, our results suggest that Notch receptor-ligand interactions occurring within the thymus enable the autonomous signaling capacity of the pre-TCR complex and thus underlie the thymic dependency for β selection and subsequent $\alpha\beta$ T cell differentiation.

Materials and Methods

Mice

RAG-2-deficient mice (30) were bred and maintained in our animal facility under specific pathogen-free conditions. Timed-pregnant Swiss.NIH mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). All animal procedures were approved by the Sunnybrook and Women's College Health Science Centre Animal Care Committee (Toronto, Ontario, Canada).

Cell lines

OP9-DL1 and OP9-control cells were generated from the OP9 bone marrow stromal cell line as previously described (12). Briefly, OP9 cells were transduced with either empty retroviral vector MigR-1(20) or MigR-1 engineered to express DL1 and green-fluorescent protein (GFP) from a single bistronic message. Monolayers of OP9 cells were cultured in OP9 medium (αMEM supplemented with 20% FBS (HyClone, Logan, UT) and 2.2 g/L sodium bicarbonate). The ecotropic retroviral packaging cell line, GP+E.86 (31) (obtained from P. Ohashi, University of Toronto, Toronto, Ontario, Canada), was maintained in complete DMEM supplemented with 10% FBS.

Flow cytometry and cell sorting

FITC-, PE-, Cy-Chrome-, or APC-conjugated mAb specific for murine CD4, CD8α, CD25, CD44, and CD45 were purchased from BD Biosciences (San Diego, CA). Staining of cells was conducted as previously described (32). Flow cytometry was performed using a FACSCalibur instrument and CELLQuest Pro software (both from BD Biosciences). For analysis, the data were live gated based on forward and side scatter and propidium iodide exclusion. Cells were sorted using a FACSDiVa (BD Biosciences); sorted cells were $\geq$99% pure, as determined by postsort analysis.

Fetal cell isolation and in vitro T cell differentiation

Fetal thymus and fetal liver (FL) were harvested on day 14 or 15 of gestation. Single-cell suspensions were generated by disruption through a 40-μm pore size nylon mesh using a syringe plunger and washed once in OP9 medium. CD24$^{low/-}$ FL cells, enriched for hematopoietic progenitor cells, were obtained by Ab- and complement-mediated lysis, as previously described (32). Briefly, cell suspensions were incubated at 37°C for 30 min in a total of 10 ml of complete medium containing 4 ml of J11d.2 (anti-CD24) culture supernatant and a 1/10 dilution of Low-Tox rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada). Viable cells were recovered by density gradient centrifugation over Lympholyte-M (Cedarlane Laboratories) and washed once in OP9 medium before plating onto subconfluent OP9-DL1 monolayers for T lineage differentiation. Human recombinant Flt-3 ligand (hrFlt3L; 5 ng/ml; Peprotech, Rocky Hill, NJ) and 1 ng/ml murine IL-7 (mIL-7; Peprotech) were added to cocultures to supplement the endogenous levels of these cytokines generated by OP9 monolayers (33).

OP9 cell cocultures

All cocultures were performed in the presence of 1 ng/ml mIL-7 and 5 ng/ml hrFlt3L. Anti-CD3ϵ (clone 145-2C11, purified from hybridoma culture supernatants) or hamster IgG (BD Biosciences), were added to OP9-control and OP9-DL1 cocultures in suspension at the times and concentrations indicated in the figure legends.

For inhibitor assays, FL-derived RAG-2$^{-/-}$ DN cells were harvested on day 8 of coculture with OP9-DL1 and replated onto fresh OP9-DL1 monolayers at 10^6 cells/well in 6-well plates. Equal volumes of DMSO or inhibitor (serially diluted in DMSO) were added to the coculture medium. Cells were preincubated with the indicated concentrations of PD98059, bisindolylmaleimide I (BIM), γ-secretase inhibitor X, or SB203580 (Calbiochem, San Diego, CA) for 2 h before the addition of 14 μg/ml anti-CD3ϵ mAb. Developmental progression was assessed after 4 days by flow cytometry. In cases in which cellularity is indicated, cell counts were performed by trypan blue exclusion.

For CFSE labeling, day 8 OP9-DL1 coculture-derived RAG-2$^{-/-}$ DN cells were incubated with 10 μM CFSE (Molecular Probes, Eugene, OR) at 5×10^6 cells/ml of PBS, 0.1% BSA for 10 min at 37°C. Cells were washed three times with OP9 medium and plated onto OP9-DL1 monolayers at 10^6 cells/well in a six-well plate. Loaded cells recovered for 1 h before the addition of 10 μg/ml anti-CD3ϵ Ab to one-half of the wells. Cell division of CFSE-labeled cells was analyzed by flow cytometry on the indicated days of culture.

Retroviral gene transfer

Retroviral constructs were generated by subcloning the cDNAs of interest into a MSCV-based retroviral vector, 5′ of the internal ribosomal entry site, permitting the bicistronic expression of the given genes and GFP. Constructs encoding constitutively active LckF505 (34), PKCαCAT (35), and FynF528 (36) were cloned into the MigR-1 vector (20); RasV12 (37) was cloned into the MIEV vector (38). A TCRβ cDNA (Vβ1-DJβ2.1-Cβ2) was cloned into the MIG2 vector (MIGR modified to include additional restriction sites). Stable retrovirus-producing cell lines were generated from GP+E.86 packaging cells, as previously described (39). RAG-2$^{-/-}$ FL-derived DN cells were harvested on day 7 of coculture with OP9-DL1 cells and infected by overnight coculture with subconfluent packaging cell monolayers. RAG-2$^{-/-}$ cells were seeded at a density of 10^7 cells/10-cm tissue culture dish of virus-producing cells in 8 ml of OP9 culture medium supplemented with 6.7 μg/ml hexadimethrine bromide (Sigma-Aldrich, St. Louis, MI), 1 ng/ml mIL-7, and 5 ng/ml hrFlt3L. After 16–20 h, transduced CD44$^-$CD25$^+$ DN3 T cells expressing GFP were purified by FACS and subsequently cultured on OP9-control and OP9-DL1 cells in the presence of 1 ng/ml mIL-7 and 5 ng/ml hrFlt3L. Developmental progression was assessed by flow cytometry on the indicated day of culture.

Cell stimulation and immunoblotting

RAG-2$^{-/-}$ DN cells were harvested on day 12 of OP9-DL1 coculture, washed twice with ice cold DMEM supplemented with 0.1% BSA (DMEM-BSA), and incubated on ice with saturating concentrations of biotinylated anti-CD3ϵ (145-2C11; BD-Bioscience) for 30 min in DMEM-BSA. The cells were washed and resuspended at a concentration of 5×10^7 cells/ml in DMEM-BSA containing 25 μg/ml avidin (Sigma-Aldrich). Cells were stimulated at 37°C for the time periods indicated in the figure. Control cells (unstimulated) were treated identically with the exception of avidin addition. The stimulation was stopped with the addition of ice cold PBS supplemented with 1 mM Na_3VO_4 and 1 mM NaF. Cells lysates were prepared as previously described (40), resolved by SDS-PAGE through 10% acrylamide gels, and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Baie d'Urfé, Quebec, Canada). Immunoblotting was performed with Abs specific for phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) and phospho-p42/44 MAPK (extracellular signal-regulated kinase; ERK) (Cell Signaling Technology, Beverly, MA). A β-actin-specific Ab (Sigma-Aldrich) was used to determine protein loading.

RT-PCR

Total RNA was isolated using the Trizol reagent (Invitrogen, Burlington, Ontario, Canada). Oligo(dT)$_{20}$-primed cDNA was generated with the Omniscript RT kit (Qiagen, Mississauga, Ontario, Canada). All PCR were conducted using the same serially diluted cDNA samples normalized to a β-actin-specific signal. Gene-specific primer sequences were as follows (5′→3′): β-actin forward GTG GGC CGC TCT AGG CAC CAA; β-actin reverse CTC TTT GAT GTC ACG CAC GAT TTC; CD3ϵ forward ACT TGC CAG GAC GAT GCC GAG A; CD3ϵ reverse TGC GGA TGG GCT CAT AGT CTG G; TCR Cα forward AGA ACC TGC TGT GTA CCA GTT AA; TCR Cα reverse CAT GAG CAG GTT AAA TCC GGC T. PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. All PCR products shown correspond to the expected molecular size.

Results

β selection proceeds normally on OP9-DL1 stroma

We have recently demonstrated that ectopic expression of a Notch ligand, DL1, on the bone marrow stromal cell line OP9-DL1 permits the efficient differentiation of fetal liver-derived hemopoietic progenitor cells into CD4$^+$CD8$^+$ DP T cells (12). To address the suitability of OP9-DL1 cells for the study of the β selection checkpoint, hemopoietic progenitor cells from wild-type and RAG-2$^{-/-}$ fetal livers were cultured on OP9-DL1 monolayers. In both cultures, progenitors underwent phenotypic progression through the various DN stages as defined by CD44 and CD25 expression. However, by day 8, coculture-derived RAG-2$^{-/-}$ cells displayed the expected developmental arrest at the DN3 stage (CD4$^-$CD8$^-$CD44$^-$CD25$^+$) due to a defect in TCRβ gene rearrangement (Fig. 1*A*), recapitulating the in vivo phenotype (30). Moreover, RAG-2$^{-/-}$ cells remained CD4$^-$CD8$^-$ DN at day 12 of culture, and up to day 24 (Fig. 1*A*; data not shown). In contrast, cultures with wild-type progenitors gave rise to 48% DP cells by day 8. The striking 10-fold difference in cellularity between wild-type and RAG-2$^{-/-}$ cultures by day 12 reflects the proliferative expansion resulting from successful pre-TCR formation (Fig. 1*B*). Thus, these observations reveal that the OP9-DL1 coculture system is appropriate for the study of β selection events. In particular, the Notch receptor-DL1 interactions experienced by developing RAG-2$^{-/-}$ progenitors on OP9-DL1 monolayers are sufficient to support differentiation to the DN3 stage, but insufficient to overcome the requirement for pre-TCR expression for proliferation and differentiation to the DP stage.

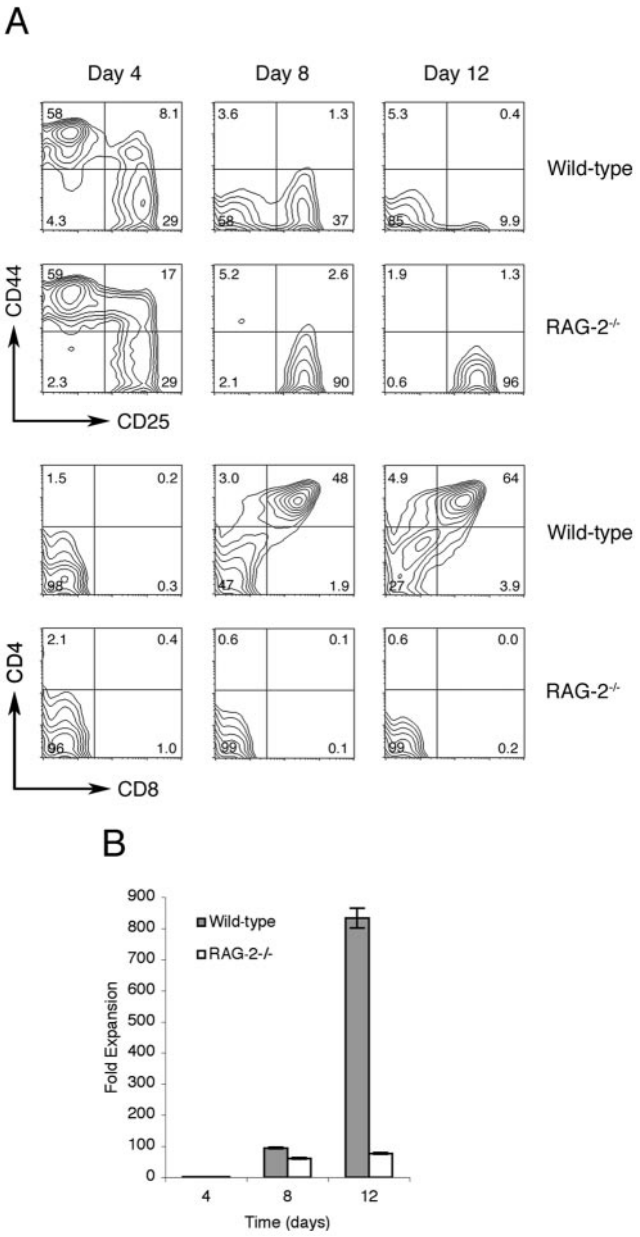

FIGURE 1. Developmental arrest of RAG-2$^{-/-}$ hemopoietic progenitor cells cultured on OP9-DL1 cells. Hemopoietic progenitor cells obtained from wild-type and RAG-2$^{-/-}$ day 14/15 FL were cocultured with OP9-DL1 stroma cells. *A*, Developmental progression of T lineage cells was examined on days 4, 8, and 12 by flow cytometric analysis of CD25, CD44, CD4, and CD8 cell surface expression. *B*, Increase in cellularity (fold expansion) from the initial number of wild-type and RAG-2$^{-/-}$ FL-derived progenitors cultured with OP9-DL1 cells for various time points. Data represent mean ± SEM of three independent cultures.

β selection requires Notch receptor-ligand interaction

To characterize the requirement for Notch receptor-ligand interactions during β selection, we assessed the ability of TCRβ expression to rescue the development of RAG-deficient DN cells in the presence or absence of the Notch ligand, DL1. Transgenic expression of a productively rearranged TCR β-chain has been previously demonstrated to rescue the developmental arrest observed in RAG-2$^{-/-}$ mice (41). Genetic reconstitution of RAG-deficient cells permitted temporal control over pre-TCR formation and, moreover, allowed us to address the requirement for Notch signals during β selection independently of the role of Notch1 in TCRβ gene recombination (26). Coculture-derived RAG-2$^{-/-}$ DN cells were retrovirally transduced with either a TCRβ-encoding or an empty GFP-only vector, and FACS-purified GFP$^+$ DN3 cells were further cultured on OP9-DL1 or OP9-control cells. RAG-2$^{-/-}$ progenitors transduced with the empty retroviral vector (GFP) remained DN when cultured on OP9-control cells, and consistent with our results from Fig. 1*A*, we observed the expected developmental block of RAG-2$^{-/-}$ GFP$^+$ progenitors cultured on OP9-DL1 cells (Fig. 2*A*). RAG-2$^{-/-}$ DN3 cells transduced to express TCRβ failed to induce β selection when cultured on OP9-control cells, retaining the DN phenotype (Fig. 2*A*). In striking contrast, when cultured on OP9-DL1 cells, RAG-2$^{-/-}$ DN3 cells expressing TCRβ effectively underwent β selection, resulting in 80% DP cells by day 6 of culture (Fig. 2*A*). Moreover, we observed a concomitant 300-fold increase in the number of TCRβ-transduced cells (Fig. 2*B*). The paucity of thymocytes recovered from OP9-control cocultures likely reflects the combined failure to induce proliferation and to rescue apoptosis of pre-TCR-expressing cells in the absence of Notch ligand interaction (Fig. 2*B*). Therefore,

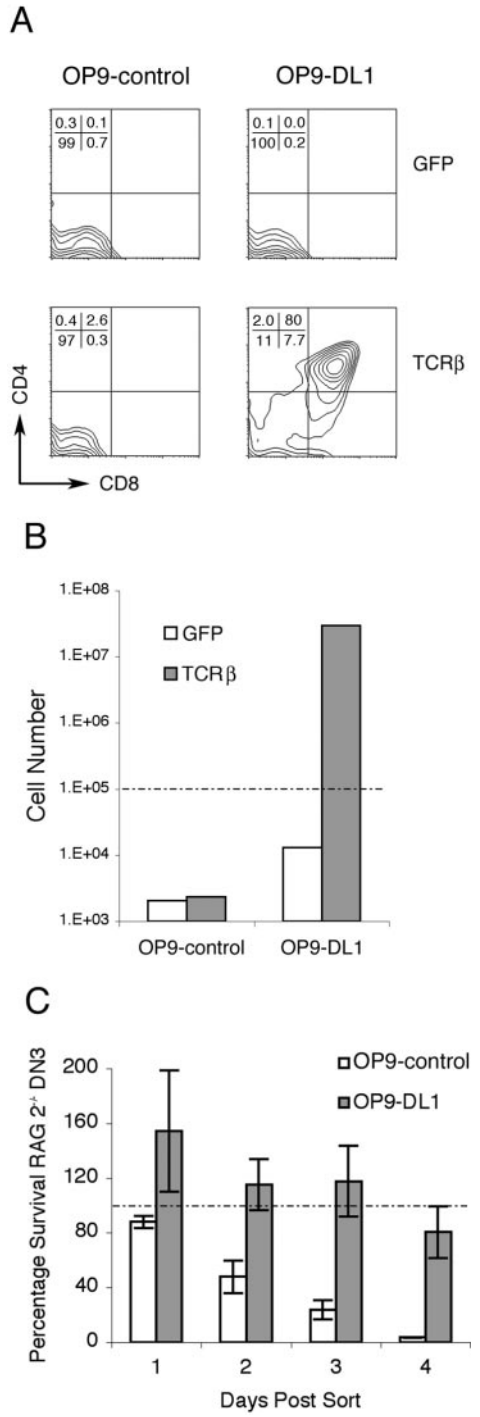

FIGURE 2. Requirement for Notch receptor and DL1 ligand interaction for TCR β selection. *A*, OP9-DL1 coculture-derived RAG-$2^{-/-}$ DN cells were retrovirally transduced with either TCRβ-encoding (TCRβ) or empty GFP-only control vector (GFP). After 16 h, GFP-expressing DN3 ($CD44^{-}CD25^{+}$) cells were sorted and subsequently cultured with OP9-control or OP9-DL1 cells for 6 days. Flow cytometric analysis of CD4 and CD8 cell surface expression is shown for cells gated for GFP and CD45 expression. *B*, Corresponding cellularity for the day 6 cocultures shown in *A*. –•–, Initial number of sorted cells placed in culture. Data are representative of three independent experiments. *C*, Percent survival of sorted OP9-DL1 coculture-derived RAG-$2^{-/-}$ DN3 cells plated on OP9-control vs OP9-DL1 monolayers. –•–, 100% survival. Data represent mean ± SEM of three independent experiments.

these results reveal that Notch and pre-TCR signals are required concurrently for the functional outcomes of β selection including survival, proliferation, and differentiation.

Several studies have suggested a role for Notch1 activation in promoting survival of T lineage cells (42, 43). Thus, we wanted to address the possibility that the lack of differentiation of TCRβ-transduced RAG-$2^{-/-}$ DN3 cells cultured on the OP9-control cells could reflect an underlying requirement for Notch-mediated signals for pre-T cell survival. To this end, sorted DN3 cells from RAG-$2^{-/-}$/OP9-DL1 cocultures were plated onto OP9-DL1 and OP9-control cells and assessed for survival by cell counts over 4 days. In the absence of pre-TCR signals, RAG-deficient thymocytes undergo apoptosis (44); however, we observed that the kinetics of cell death was much faster on OP9-control vs OP9-DL1 stroma, with 3 and 80% of RAG-$2^{-/-}$ DN3 cells remaining, respectively, by day 4 (Fig. 2*C*). Thus, it appears that signals through Notch receptors impact on the survival of pre-T cells, independently of pre-TCR-mediated signaling. However, it is important to note that ~90 and 50% of cells were maintained on the OP9-control cells over the first 24 and 48 h, respectively (Fig. 2*C*). Thus, a sufficient number of live cells were available at the onset of TCRβ expression and pre-TCR signaling following transduction to assess the necessity for Notch signals at the β selection checkpoint.

Anti-CD3-induced differentiation and proliferation requires Notch signals

The developmental progression of DN thymocytes to the DP stage can be mimicked by injection of anti-CD3ϵ mAb into RAG-deficient mice, or by treatment of RAG$^{-/-}$ fetal thymic organ cultures (FTOC) with anti-CD3ϵ in vitro (45–47). However, attempts to recapitulate anti-CD3-induced β selection events by thymocytes in suspension cultures have failed (40, 48), suggesting that additional thymus-derived signals are required for this differentiation step. In light of our results (Figs. 1 and 2), we hypothesized that the missing signals may in part reflect a requirement for Notch receptor-ligand interactions. To directly address this possibility, day 15 RAG-$2^{-/-}$ fetal thymocytes (of which ~85% are DN3 cells) were cultured on OP9-control or OP9-DL1 cells and treated with either anti-CD3ϵ mAb or hamster IgG isotype control Ab. Flow cytometric analysis on day 4 revealed that the developmental progression to the DP stage had been effectively induced in anti-CD3 treated RAG-$2^{-/-}$ thymocytes cultured on OP9-DL1 cells, giving rise to 40% $CD4^{+}CD8^{+}$ cells (Fig. 3*A*). In marked contrast, thymocytes engaged with anti-CD3 but cultured on OP9-control cells failed to differentiate, resembling cells treated with isotype control Ab (Fig. 3*A*). These observations are in agreement with our above finding that Notch receptor-ligand interactions are required for β-selected thymocytes to transit from the DN to DP stage. Furthermore, an important implication of these data is that Notch- and CD3-mediated signals are required concurrently, such that previous Notch activation obtained in the thymus is insufficient to support subsequent development to the DP stage in vitro.

Because Notch activation ultimately results in transcriptional modulation, the failure of RAG-$2^{-/-}$ fetal thymocytes to differentiate in response to anti-CD3 on OP9-control cells could reflect a reduction in CD3 complex expression in the absence of Notch-DL1 interaction. To address this possibility, we performed semi-quantitative RT-PCR analysis of cDNA prepared from thymocytes stimulated on OP9-control and OP9-DL1 cells during a period of 48 h (Fig. 3*B*). CD3ϵ expression was only slightly reduced in OP9-control-cultured cells from the levels detected in ex vivo RAG-$2^{-/-}$ fetal thymocytes before culture. Nevertheless, a sufficient density of signaling-competent CD3 complexes were available on thymocytes as induction of germline TCR-Cα transcripts, indicative of pre-TCR signaling (6), were detected in cells following anti-CD3 treatment on both stroma (Fig. 3*B*). Thus, the facilitation

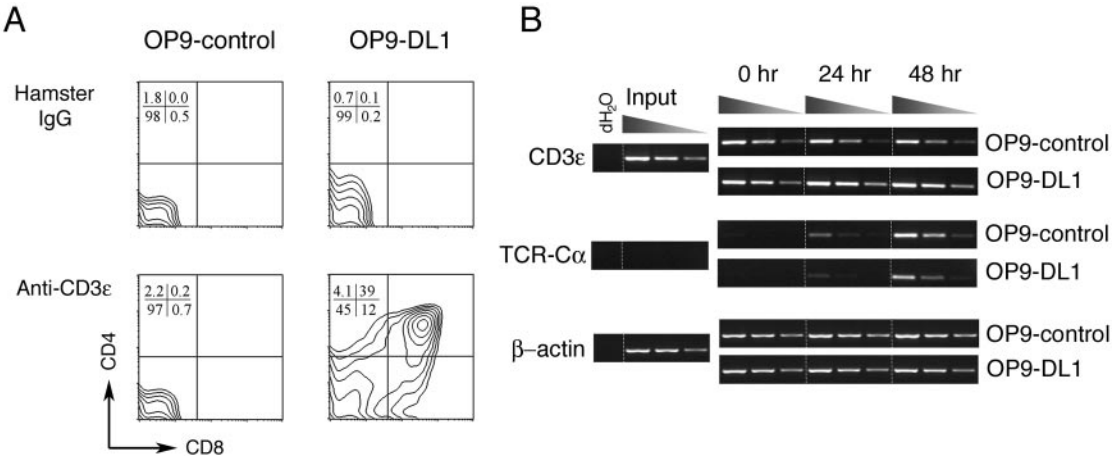

FIGURE 3. Notch receptor-ligand interactions are necessary for anti-CD3-induced DN to DP transition. *A*, Flow cytometric analysis of CD4 and CD8 surface expression of ex vivo RAG-$2^{-/-}$ fetal thymocytes stimulated with 10 μg/ml anti-CD3ϵ mAb or 10 μg/ml hamster IgG isotype control Ab on OP9-control or OP9-DL1 cell monolayers. Day 15 RAG-$2^{-/-}$ fetal thymocytes were incubated on the corresponding stromal cells for 12 h before Ab addition and analyzed after 4 days of stimulation. Similar results were obtained when Ab treatment was initiated immediately (data not shown). Data are representative of at least five independent experiments. *B*, Day 15 RAG-$2^{-/-}$ fetal thymocytes (input) were stimulated with 10 μg/ml anti-CD3 mAb for 0, 24, and 48 h on either OP9-control or OP9-DL1 cells. The 0-h time point reflects thymocytes preincubated on the stromal cells for 12 h before Ab addition. Expression of CD3ϵ and germline TCR-Cα mRNA was analyzed by RT-PCR. Data represent 3-fold serial dilutions of template cDNA normalized to a β-actin-specific signal. Control PCRs using OP9-control and OP9-DL1 cDNAs were negative for CD3ϵ- and TCR-Cα-specific transcripts (data not shown). A water (dH_2O) control is included adjacent to input samples. Data are representative of two independent experiments.

of β selection outcomes by Notch signaling does not reflect a requirement for Notch activation during the initial signaling complex formation or function.

Cellular proliferation is one of the hallmarks of β selection. Because we found that TCR β-chain expression in RAG-$2^{-/-}$ DN3 T cells failed to induce proliferation on OP9-control cells (Fig. 2*B*), we sought to further examine the requirement for Notch signals for cellular expansion at this checkpoint. To this end, OP9-DL1 culture-derived RAG-$2^{-/-}$ DN cells (Fig. 1*A*, day 8) were treated with increasing concentrations of anti-CD3 mAb in the context of either OP9-DL1 or OP9-control monolayers. Analysis after 6 days of coculture revealed a dose-dependent increase in cellularity for DN thymocytes receiving Notch signals through DL1, reflecting a 5-fold expansion in cell number over nonstimulated controls (Fig. 4*A*). This degree of proliferation is consistent with similar treatments performed in RAG$^{-/-}$ FTOC (47). In marked contrast, cellularity remained unaffected by anti-CD3 addition to OP9-control cocultures, even at the highest concentrations of Ab (30 μg/ml) (Fig. 4*A*). This finding supports the conclusion that Notch signals enable proliferation events during the differentiation of DN cells to the DP stage.

OP9-DL1 coculture-derived RAG-$2^{-/-}$ DN cells display the same dependence on Notch ligand interaction for anti-CD3-mediated differentiation as demonstrated for ex vivo fetal thymocytes (Fig. 2A; data not shown). To characterize the population of cells undergoing proliferation in anti-CD3-stimulated cultures, coculture-derived RAG-$2^{-/-}$ DN cells were labeled with CFSE before stimulation with anti-CD3 on OP9-DL1 cells. After 4 days, analysis of cell division by flow cytometry revealed that both cultures were proliferating (see Fig. 4*B*; compare day 1 time point with day 4 time point). However, the cells induced to differentiate by anti-CD3 stimulation, which had up-regulated CD4 and CD8 surface expression while down-regulating CD25 expression, had undergone additional rounds of cell division over that observed in nonstimulated cultures (Fig. 4*B*). Increased proliferation of cells from stimulated as compared with control cultures was noted after 2 days, at which time surface expression of CD8 and CD4 were first detected in only a small fraction of the cells (data not shown). Indeed, proliferation and differentiation events appear to occur sequentially after anti-CD3 stimulation.

Signaling requirements for anti-CD3-mediated differentiation

Formation of the pre-TCR results in the activation of various signaling cascades (5, 6). To establish the suitability of OP9-DL1 coculture-derived RAG-$2^{-/-}$ DN cells for the study of β selection-associated signaling, we performed a kinetic analysis of protein phosphorylation events in response to anti-CD3 engagement in vitro (Fig. 5*A*). Immunoblot analysis of cellular lysates revealed the induction of protein tyrosine phosphorylation within 30 s of stimulation, with increased phosphorylation of proteins corresponding in molecular mass to the Src tyrosine kinase Lck, and adaptor molecule LAT (linker for activation of T cells; Fig. 5*A*, asterisks). In addition, ERK phosphorylation was induced after 30 s and sustained for at least 10 min after CD3 aggregation. These results indicate that proximal and downstream phosphorylation events of the pre-TCR pathway are functionally intact in coculture-derived RAG-$2^{-/-}$ DN cells.

To characterize the signaling pathways required for the β selection-mediated DN to DP transition, coculture-derived RAG-$2^{-/-}$ DN cells were treated with pharmacological inhibitors of specific signaling mediators during CD3 engagement in OP9-DL1 coculture. Previous studies have implicated members of the Ras/Raf/mitogen-activated protein/extracellular signal-related kinase kinase (MEK) MAPK signaling cascade, in promoting the differentiation of DN cells (35, 37, 49–51); however, a requirement for this pathway during β selection has not been demonstrated. Fig. 5*B* shows that inhibition of MEK1 with PD98059 effectively attenuated anti-CD3-mediated differentiation and proliferation in a dose-dependent manner, such that at the highest inhibitor concentration the number of DP cells was reduced by 97%, and the total cellularity resembled that of nonstimulated cultures. Inhibition of protein kinase C (PKC) function with BIM produced a similar effect, reducing DP cellularity by 92% at the highest dose (Fig. 5*B*). This observation supports our previous findings that PKC is required for the development of immature DN cells to the DP stage (35). Importantly, nonstimulated cultures maintained a constant cellularity

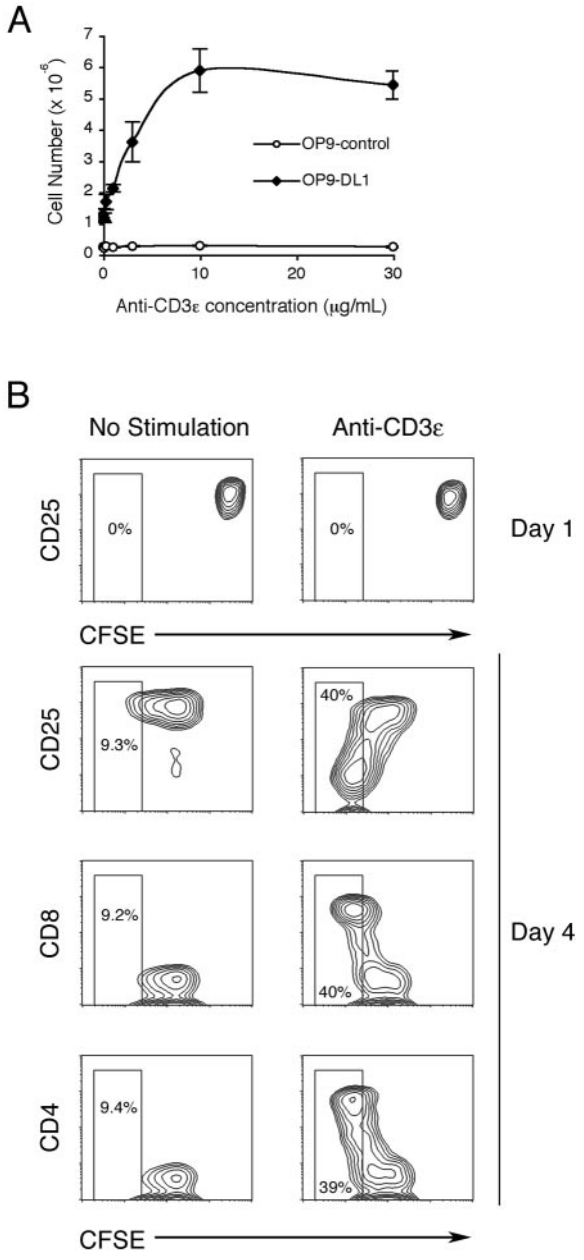

FIGURE 4. Analysis of proliferation following anti-CD3 induction of RAG-2$^{-/-}$ thymocytes in vitro. *A,* Anti-CD3-mediated proliferation requires Notch receptor-ligand interactions. Total cell counts for coculture-derived RAG-2$^{-/-}$ DN cells stimulated with increasing concentrations of anti-CD3 mAb on OP9-control vs OP9-DL1 monolayers are shown. Cells were plated at an initial density of 2.5×10^5/ml and analyzed after 6 days of Ab treatment. Data represent mean ± SEM of independent culture conditions performed in triplicate. *B,* Analysis of cell division with CFSE labeling. Coculture-derived RAG-2$^{-/-}$ DN cells were labeled with CFSE, were plated onto OP9-DL1 monolayers, and received either 10 μg/ml anti-CD3 or no stimulation for 4 days. Cultures were then stained for CD4, CD8, or CD25 and analyzed by flow cytometry. Values are percent of cells in the gated region. Data are representative of four independent experiments.

with increasing concentrations of both inhibitors, indicating a lack of general toxicity within the concentration range used. Conversely, treatment of RAG-2$^{-/-}$ cocultures with the highly specific p38 MAPK inhibitor, SB203580, enhanced proliferation of nonstimulated and anti-CD3-induced cultures equally, reinforcing the suggested role of p38 as a negative regulator of cell cycle in pre-T cells (52).

To directly address the requirement of Notch signaling during the DN to DP transition, we preformed anti-CD3 induction of culture-derived RAG-2$^{-/-}$ DN cells on OP9-DL1 monolayers in the presence or absence of a presenilin-1/2 inhibitor. A presenilin-dependent γ-secretase activity has been shown to mediate the cleavage of the intracellular *trans*-activating domain of each of the four mammalian Notch receptors in response to ligand engagement (53). Addition of increasing concentrations of presenilin inhibitor resulted in an abrogation of anti-CD3-mediated differentiation and proliferation, similar in effect to MEK1 and PKC inhibition (Fig. 5*B*). This finding further establishes the concomitant requirement of Notch signaling for the β selection-mediated DN to DP transition. Notably, doses of inhibitor that effectively blocked Notch-supported proliferation to anti-CD3 also resulted in a decrease in the total cell number of nonstimulated cultures, which were down to 46% of those treated with DMSO alone (Fig. 5*B*). These data complement our previous conclusion that Notch receptor-ligand interactions provide essential survival signals to DN3 thymocytes (Fig. 2*C*).

Importantly, the trends observed in this experiment are not due to inhibitory effects on the stromal cells, as pretreatment of OP9-DL1 cells for 4 days with the highest concentrations of inhibitor used did not affect the ability of the OP9-DL1 cells to support subsequent anti-CD3-mediated differentiation (data not shown). Furthermore, treatment of wild-type FL-derived hemopoietic progenitors cultured on OP9-DL1 cells with the presenilin inhibitor (0.3 μM) permitted B lymphopoiesis (data not shown), which is otherwise not observed (12), indicating that the inhibitor does not affect the ability of the OP9-DL1 cells to support lymphocyte development. Collectively, the data presented here reveal that the combination of pre-TCR- and Notch-mediated signals are indispensable for the developmental transition of DN cells to the DP stage.

Insufficiency of pre-TCR signals in the absence of Notch signals

The β selection checkpoint can be experimentally bypassed in RAG-deficient thymocytes via the expression of active versions of signaling molecules that act downstream of the pre-TCR (5). This approach has revealed the sufficiency of the Src tyrosine kinase Lck (34), the GTPase Ras (37, 50), and the serine-threonine kinase PKC (35) in promoting all or a subset of the β selection-associated outcomes. However, each of these studies was conducted in the context of a Notch signaling-competent thymic environment.

With this in mind, we wanted to assess whether constitutive activation of pre-TCR-mediated signaling pathways could overcome the requirement for Notch receptor-ligand interactions during the DN to DP transition. To this end, TCRβ, active signaling mutants (LckF505, FynF528, RasV12, and PKCαCAT) and empty control GFP-only vector were introduced into coculture-derived RAG-2$^{-/-}$ DN cells via retroviral transduction, and the development of FACS-purified GFP$^+$ DN3 cells was assessed on OP9 monolayers in the presence or absence of ectopic DL1 expression (Fig. 6). After 6 days, varying degrees of differentiation to the CD4$^+$ or CD8$^+$ immature single-positive and/or DP stages, and proliferation (indicated as fold increase in cellularity) were observed in the OP9-DL1 cocultures, with the exception of the vector GFP-only negative control cells (Fig. 6). These data reflect the hierarchical positioning of each active molecule in the pre-TCR cascade; TCRβ, the membrane proximal LckF505, and RasV12 induced both proliferation and differentiation events, whereas active Fyn and PKC displayed a more restricted potential toward differentiation in the absence of significant cellular expansion (Fig. 6). In striking contrast, little if any differentiation was observed in transduced RAG-2$^{-/-}$ DN cells cultured on the OP9-control cells.

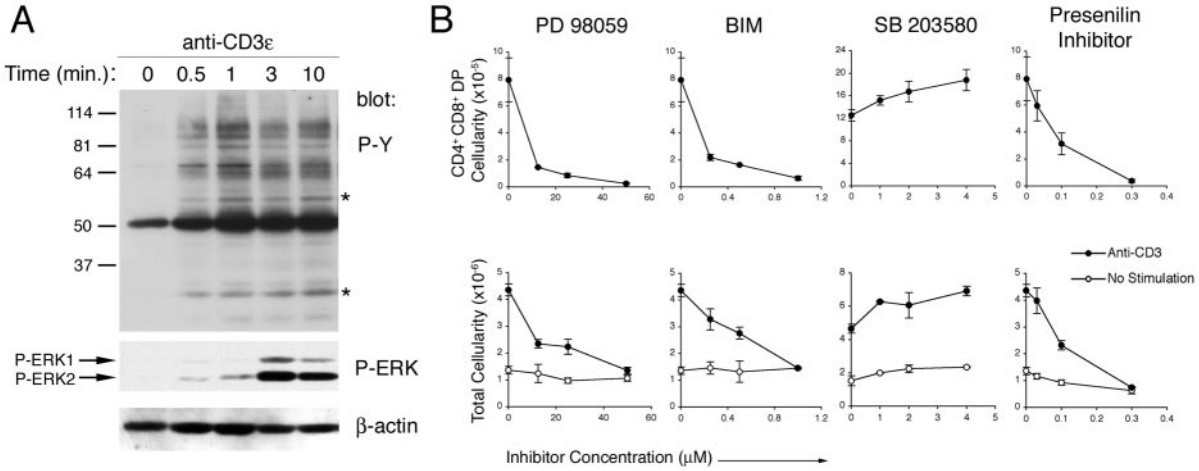

FIGURE 5. Signaling requirements for anti-CD3-induced DN to DP transition. *A*, Detection of protein phosphorylation after anti-CD3 induction of RAG-2$^{-/-}$ DN cells in vitro. Culture-derived RAG-2$^{-/-}$ DN cells were harvested, treated with biotin-conjugated anti-CD3 mAb, and stimulated with avidin at 37°C for the indicated time in vitro. Cell lysates were subjected to SDS-PAGE and immunoblot analysis with Abs to total phosphotyrosine, and phospho-ERK 1/2. The same blots were stripped and reprobed with an anti-β-actin Ab to determine protein loading. ∗, Proteins with molecular masses consistent with Lck (56 kDa) and LAT (35 kDa). Molecular mass standards (kilodaltons) are indicated on the left ordinate. *B*, Anti-CD3-induced differentiation and proliferation requires MEK1-, PKC- and Notch-mediated signals. Culture-derived RAG-2$^{-/-}$ DN cells were seeded onto OP9-DL1 cells and pretreated with DMSO or increasing concentrations of specific inhibitors of MEK1 (PD 98059), PKC (BIM), p38 MAPK (SB 203580), and presenilin 1/2 (γ-secretase inhibitor X). After 2 h, cultures received either 14 μg/ml anti-CD3ε or no stimulation. Developmental progression was assessed on day 4 by flow cytometric analysis of cell surface CD4 and CD8 expression. CD4 CD8 DP cellularity was obtained by multiplying the total cellularity with the percent of DP cells present in the cultures. Total cellularity is plotted as a function of inhibitor concentration. Error bars reflect the SD of the mean from culture conditions conducted in duplicate. Data are representative of four independent experiments.

In fact, in most cases, cellularity suffered a substantial decline. Thus, in the absence of Notch receptor-ligand interactions, even constitutive pre-TCR signals are insufficient to mediate the transition of DN3 thymocytes to the DP stage of T cell development. Taken together, these data indicate that Notch receptor-DL1 ligand interactions provide a necessary signal to enable the proliferation and differentiation of DN thymocytes, which is dependent on specific signals downstream of the pre-TCR complex.

Discussion

We demonstrate a novel requirement for Notch receptor-ligand interactions for productive β selection outcomes, namely rescue from apoptosis, proliferation, and differentiation to the DP stage of T cell development. Use of RAG-2$^{-/-}$ thymocytes in coculture with OP9-control/OP9-DL1 monolayers in vitro allowed temporal control over the initiation of pre-TCR-mediated signals in the presence or absence of DL1-triggered Notch signals. Here we show that Notch- and pre-TCR-mediated signals are required concurrently during β selection as transit of RAG-2$^{-/-}$ DN cells to the DP stage after transduction with a TCR β-chain or treatment with anti-CD3 mAb was dependent on DL1 expression by OP9 cells. In support of this notion, anti-CD3-induced differentiation was effectively abrogated on pharmacological inhibition of either pathway. Importantly, our findings do not reflect a requirement for Notch signals for surface expression of pre-TCR or CD3 complexes, given that bypass of receptor formation via the expression of active downstream signaling mutants was equally dependent on OP9 cell expression of DL1 for the developmental progression of RAG-2$^{-/-}$ DN cells.

Our findings provide a functional basis for the observed pattern of Notch receptor expression and activation in developing thymocytes. Several reports showing that levels of Notch1 and Notch3 expression, and activity (as revealed by target Hes1 and Deltex1 gene expression) are significantly higher in DN vs DP thymocytes correlate well with the necessity for Notch signaling for differentiation past the β selection checkpoint (14, 15, 17, 18, 27). Moreover, our data extend the findings of a recent study by Wolfer et al. (26), in which conditional disruption of *Notch1* at the DN2/3 boundary revealed a requirement for Notch1 during Vβ-DJβ rearrangement. In addition, Notch signals have been demonstrated to regulate the expression of pre-Tα (54), another component of the pre-TCR complex. Taken together with our findings, it is clear that Notch signals are absolutely critical for continued differentiation of T lineage-committed progenitors past the first checkpoint in αβ T cell development, not only by facilitating TCRβ rearrangement and pre-TCR expression but also by potentiating the signaling outcomes of β selection.

The data presented here demonstrate that Notch and pre-TCR signals are required concurrently for the developmental transition of DN cells to the DP stage. Interestingly, pre-DP (DN4) cells purified from wild-type mice are capable of differentiating to the DP stage within 48 h in suspension culture (11), and similarly when cultured on OP9-control cells (data not shown). Taken together with the present findings, these observations suggest that Notch signals are necessary at the onset of pre-TCR signaling but appear to be dispensable for the late phase of DN to DP transition (18).

Survival is one of the hallmarks of β selection. It has been proposed that pre-TCR signaling promotes survival by blocking TNFR-mediated death signals that eliminate thymocytes that fail β selection (55). Here we demonstrate that rescue of RAG-2$^{-/-}$ DN cells from apoptosis after pre-TCR formation and signaling was critically dependent on Notch-DL1 interactions in vitro. In contrast, a recent study reported a role for Notch1 in eliminating pre-TCR-defective DN3 thymocytes, based on the observation that conditional deletion of Notch1 at the DN2 stage resulted in the appearance of an aberrant population of intracellular TCRβ^{-} intracellular CD3ε^{+} DN4 thymocytes (26). We found no evidence of increased survival of pre-TCR-deficient RAG-2$^{-/-}$ DN3 cells cultured in the absence vs the presence of Notch-DL1 interaction.

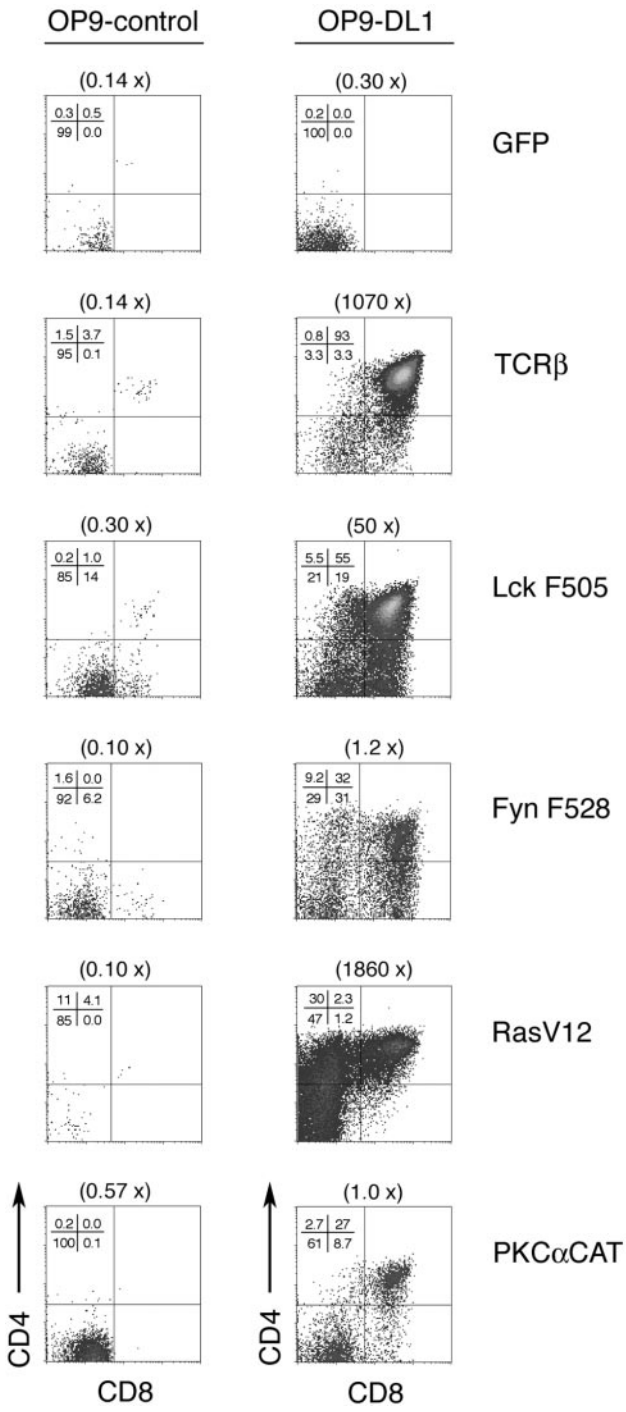

FIGURE 6. Constitutively active pre-TCR signals are insufficient to rescue the developmental arrest of RAG-2$^{-/-}$ DN cells in the absence of Notch receptor-ligand interaction. Flow cytometric analysis for surface expression of CD4 and CD8 from coculture-derived RAG-2$^{-/-}$ DN cells retrovirally transduced with TCRβ, active signaling mutants (LckF505, FynF528, RasV12, and PKCαCAT), or empty control vector (GFP), initially sorted as GFP^{+} DN CD44^{-} CD25^{+}, and cultured on OP9-control or OP9-DL1 for 6 days is shown. CD4 and CD8 profiles were gated for GFP and CD45 expression to identify construct-expressing cells. Increase in cellularity (fold expansion) over the input number of cells is provided in parentheses. FynF528 and RasV12 constructs were assayed in separate experiments. Results are representative of a least three independent experiments.

In fact, we observed the opposite trend for both purified ex vivo RAG-2$^{-/-}$ DN3 thymocytes (data not shown) and OP9-DL1 coculture-derived RAG-2$^{-/-}$ DN3 cells. This discrepancy may reflect differences between the analysis of T cell differentiation in the steady state thymus, where aberrant cell populations may accumulate, and our approach which permits a particular cell subset to be examined in a controlled setting over time. Moreover, support for an antiapoptotic role of Notch signals has been provided by gain-of-function experiments: expression of active Notch1-IC promotes resistance of DP cells to glucocorticoid-induced apoptosis (42) and of T cell lines to Nur77-dependent cell death (43). In addition, Notch3-IC-transgenic thymocytes display constitutive NFκB activation and increased expression of the downstream antiapoptotic A1/Bfl-1 protein (28). It remains to be determined whether these molecules represent direct physiological targets of Notch signaling in DN thymocytes.

The requirement for pre-TCR formation can be experimentally bypassed via the introduction of active mutants of downstream signaling mediators. Indeed, expression of constitutively active forms of Lck, Ras, and PKC can rescue the developmental block of RAG$^{-/-}$ thymocytes in vivo, or in FTOC, and additionally, in coculture with OP9-DL1 cells (this study). Strikingly, here we report that in the absence of Notch signaling triggered by DL1, such active pre-TCR-associated signals are rendered insufficient to mediate β selection outcomes. Our findings are also in line with published reports in which transgenic expression of active Notch1-IC in RAG$^{-/-}$ thymocytes failed to rescue differentiation of DP cells (18, 21). In a related study, ectopic development of DP cells in the bone marrow from Notch1-IC-transduced progenitors was abrogated when pre-TCR signaling-incompetent RAG$^{-/-}$ and SLP-76$^{-/-}$ progenitors were used (56). Taken together, constitutive pre-TCR- or Notch-mediated signals in isolation are insufficient to drive the development of DN thymocytes to the DP stage, because one pathway does not sufficiently overcome the requirement for the other. Therefore, the available evidence supports a model in which Notch and pre-TCR signals cooperate in a nonredundant manner to facilitate β selection outcomes.

Here we demonstrate that as with differentiation, mitogenesis following pre-TCR signaling requires simultaneous Notch activation, which is induced by DL1 expressed on the OP9-DL1 cells. This is highlighted by the observation that anti-CD3-induced proliferation of RAG-2$^{-/-}$ DN cells is effectively abrogated by pharmacological inhibition of either Notch- or pre-TCR-associated MEK and PKC signaling pathways. We were surprised to find that active signaling mutants LckF505 and RasV12, proven to induce proliferation of developing T cells in vivo (34, 37, 50), failed to mediate cellular expansion of RAG-2$^{-/-}$ thymocytes in the absence of DL1-mediated Notch signaling in vitro. Collectively, these findings suggest that Notch and pre-TCR signals coordinate the regulation of proliferation events during β selection. Given the requirement for Notch signals for proliferation, the reduction of Notch receptor expression in DP thymocytes may provide a means by which to extinguish cellular expansion after β selection.

How do Notch and pre-TCR signals cooperate to mediate β selection outcomes? Our findings clearly demonstrate that Notch signals facilitate various cellular processes during β selection (differentiation, proliferation, and rescue from apoptosis), thus supporting a model in which Notch signals integrate multiple key cellular pathways. In line with this, Notch signaling has been associated with the transcriptional up-regulation of a diverse sets of genes in DN thymocytes, including *pre-Tα*, *Hes-1*, *Deltex*, *Meltrin β*, and *Ifi-204*, each possessing the potential to regulate additional signaling pathways (27). Our finding that concurrent Notch and pre-TCR signaling is required for functional β selection outcomes, and that neither is sufficient for promoting this transition, suggests that these pathways converge, contributing to the regulation or assembly of transcriptional and/or cytosolic complexes. In support

of this theory, active Notch signals have been reported to suppress E2A activity (20, 57, 58), perhaps through the induction of negative regulators of basic helix-loop-helix transcription factors, such as the Id proteins, and lead to NFκB activation (28), which are known outcomes of pre-TCR-mediated signals (5). The OP9-DL1 in vitro system should prove useful for the identification of further targets of Notch signaling in the context of physiological levels of activation. Our finding that Notch signals are required for β selection, coupled with the relatively high level of Notch activity detected in DN subsets (17, 18, 27), raises the interesting possibility that Notch signals set the low threshold of cellular activation of pre-T cells (10), thus accommodating the low surface density and ligand-independent signaling of the pre-TCR. Alternatively, our findings are also consistent with a model in which the inductive signals for differentiation and proliferation are provided from Notch ligands in the surrounding microenvironment, and pre-TCR signals serve a classical checkpoint function (59).

The dependence of the β selection checkpoint on Notch signaling allows us to exploit the OP9-DL1 system to examine questions relevant to pre-TCR signal transduction. The paucity of DN thymocytes present in wild-type and $RAG^{-/-}$ mice has posed a constant challenge to research in this area. The OP9-DL1 stromal cells represent a powerful tool, with which large pools of DN T lineage cells may be generated for developmental and biochemical assays. Thus far, the introduction of active signaling mutants into RAG-$2^{-/-}$ DN cells in OP9-DL1 coculture has recapitulated the results observed using transgenic mouse and FTOC systems (5). Further to this, our in vitro system is versatile and permits the direct comparison of several individual signaling molecules. For example, our demonstration that expression of active mutants of Lck, Fyn, Ras, and PKC in RAG-$2^{-/-}$ cells can promote differentiation from the DN3 stage to the DP stage on supporting OP9-DL1 monolayers, but only Lck and Ras activation results in significant cellular expansion provide important insights into the hierarchical structure of pre-TCR-proximal signaling mediators.

The molecular interactions provided by the thymic microenvironment that support T cell developmental transitions are largely unknown. Here, we identify a requirement for Notch receptor-ligand interactions for productive β selection outcomes. An important implication of our findings is that Notch signals mediated by DL1 are sufficient for the transition of β-selected thymocytes to the DP stage. The OP9-DL1 in vitro culture system will prove instrumental in future studies aimed at characterizing the molecular mechanisms that underlie the requirement for Notch activation during the β selection checkpoint.

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