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Notch Signaling Augments T Cell Responsiveness by Enhancing CD25 Expression

Scott H. Adler,* Elise Chiffoleau,* Lanwei Xu,†‡ Nicole M. Dalton,* Jennifer M. Burg,* Andrew D. Wells,** Michael S. Wolfe,† Laurence A. Turka,†* and Warren S. Pear†‡§

Notch receptors signal through a highly conserved pathway to influence cell fate decisions. Notch1 is required for T lineage commitment; however, a role for Notch signaling has not been clearly defined for the peripheral T cell response. Notch gene expression is induced, and Notch1 is activated in primary CD4\(^+\) T cells following specific peptide-Ag stimulation. Notch activity contributes to the peripheral T cell response, as inhibition of endogenous Notch activation decreases the proliferation of activated T cells in a manner associated with the diminished production of IL-2 and the expression of the high affinity IL-2R (CD25).

Conversely, forced expression of a constitutively active Notch1 in primary T cells results in increased surface expression of CD25, and renders these cells more sensitive to both cognate Ag and IL-2, as measured by cell division. These data suggest an important role for Notch signaling during CD4\(^+\) T cell responses, which operates through augmenting a positive feedback loop involving IL-2 and its high affinity receptor. The Journal of Immunology, 2003, 171: 2896–2903.

T he mammalian Notch family of transmembrane receptors (Notch1–4) signal through a highly conserved pathway known to participate in cell fate decisions (1). Signaling through the Notch receptor is initiated by interaction with one of its ligands (Delta or Serrate/Jagged) (2, 3) located on an adjacent cell, which results in a series of proteolytic cleavages. This leads to the release and nuclear translocation of the Notch intracellular domain (Notch IC) (4), which converts the transcription factor CBF1, Su(H), and Lag-1 from a repressor to an activator (5).

Notch signaling has important consequences for T lymphocytes in vivo (5). Dysregulated Notch1 signaling leads to T cell leukemia in humans, cats, and mice (6). The ability of Notch to cause T cell leukemia most likely results from a perturbation of its normal role in thymocyte development, where Notch receptor expression and signaling occur at distinct developmental stages. There is compelling evidence for Notch activity at the earliest stage of lymphoid development: committing progenitors to the T cell lineage (7–11). Notch may also influence other stages of T cell development, including the CD4 vs CD8 and the TCR \(\alpha\beta\) vs \(\gamma\delta\) lineage choices (12–15).

In contrast to thymic development, there is a paucity of information regarding a role for Notch in peripheral T cells. Here we address the function of Notch signaling in CD4\(^+\) T lymphocyte responses. We demonstrate that Notch (1–4) mRNA is induced, and Notch1 is activated following CD4\(^+\) T cell activation. When endogenous Notch activation is blocked pharmacologically, T cell proliferation is inhibited in vitro. This inhibition in T cell division is associated with a decrease in IL-2 production and CD25 expression. Conversely, when Notch1-IC is expressed in primary CD4\(^+\) T cells using retroviral transduction, enhanced proliferative sensitivity is observed. This reduced threshold for responsiveness is due to increased IL-2 sensitivity, which is mediated by enhanced surface expression of CD25. These data, obtained by loss- and gain-of-function experimental approaches, suggest that Notch signaling contributes to regulation of the CD4\(^+\) T cell immune response by modulating CD25 expression.

Materials and Methods

Mice

DO11.10 TCR transgenic mice were bred and maintained at the university laboratory animal research facility, University of Pennsylvania. Six-week-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cell culture

Lymphoid tissue was harvested from mice according to approved standardized protocols, and single-cell suspensions of mononuclear cells were prepared. Cells were stained with CFSE (Molecular Probes, Eugene, OR) as previously described (16) and were cultured in the presence of OVA peptide (residues 323–339) with or without the potent \(\gamma\)-secretase inhibitor (GSI), \(^3\) compound E (0.5 \(\mu\)M) (17, 18). Recombinant mouse IL-2 (R&D Systems, Minneapolis, MN) was added. IL-2 levels were measured in the culture supernatants using a standard ELISA protocol.

Quantitative RT-PCR

CD4\(^+\) T cells were enriched to >95% purity by negative selection using MACS LS separation columns (Miltenyi Biotec, Auburn, CA). CD4\(^+\) T cells (2 \times 10\(^5\)) were stimulated with anti-CD3 (2.5 \(\mu\)g/ml; 145-2C11; BD PharMingen, San Diego, CA) and anti-CD28 (2 \(\mu\)g/ml; hybridoma 37-51 obtained from J. Bluestone, University of California, San Francisco, CA)-coated latex beads (5 \(\mu\)m; Interfacial Dynamics, Portland, OR) in a 1/1 ratio for 16 h. Total RNA was extracted using TRIzol reagent (Invitrogen,

Abbreviations used in this paper: GSI, \(\gamma\)-secretase inhibitor; NGFR, nerve growth factor receptor; sNGFR, truncated form of NGFR.
Carlsbad, CA), and genomic DNA was digested with amplification grade DNase I (Roche, Indianapolis, IN) before RT with the Superscript First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed with PRISM 7700 (PE Applied Biosystems, Foster City, CA). Specific Notch primers were designed using Primer Express software (PE Applied Biosystems): Notch1: forward, 5'-AGCGGTACGTCACCAGACAGAC-3'; reverse, 5'-ACGGAGTACGGCCATGGTT-3'; Notch2: forward, 5'-ACAAATACTGTCAGACACCACCTCTCA-3'; reverse, 5'-AGCCACGATGATCAGGGT-3'; Notch3: forward, 5'-CGAGAACAC-3'; reverse, 5'-GCCATGTCCTCCATCCC-3'; and Notch4: forward, 5'-GGCCGATTTTGCTGAAA-3'; reverse, 5'-TCTGAGTGTCACCAACAGCCCG-3'. Standards were prepared by PCR amplification of each target sequence and diluted to 10^2–10^5 copies/well. A constant amount of cDNA corresponding to the RT of 100 ng of total RNA, or each dilution of the standard, was amplified using the SYBR Green PCR kit (PE Applied Biosystems) containing the gene-specific primers. 18S rRNA was used as an internal positive control according to the manufacturer's instructions (PE Applied Biosystems). The PCR efficiencies of all the standards were >99%, and the correlation index between the input copy numbers and the fluorescence was always >0.95. The copy number for the specific gene product was normalized to the copy number for 18S rRNA.

Western blot analysis
Lysates from 3 x 10^6 CD4^+ T cells were run on a 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-cleaved Notch1 (Val1744) Ab (Cell Signaling Technology, Beverly, MA; 1:1000), an anti-Notch Ab (8), or an anti-actin Ab. The primary Ab was detected with a horseradish peroxidase-conjugated Ab (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL Plus (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Retroviral transduction
Retroviral vectors and production of high titer retroviral supernatants in Bosc23 cells were previously described (20). Primary CD4^+ T cells were transduced following a 16-h stimulation with PMA (0.1 μg/ml) and ionomycin (1 μg/ml). Cells were washed, spin-infected with retroviral supernatants containing 2 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) at 2500 rpm for 90 min at 25°C, and cultured for 3 days. The cells were washed and recultured for an additional 3 days in the absence of stimulation.

Flow cytometry
Flow cytometry was performed on a FACS Calibur (BD Biosciences, Franklin Lakes, NJ) maintained at the flow cytometry facility, University of Pennsylvania. Directly conjugated mAbs used were KJ1-26-PE (clone 3, 21) and CD28-coated latex beads. Treatment with GSI diminished the proliferation induced by anti-CD3 stimulation alone, but had little or no effect in the presence of CD28 costimulation (Fig. 2B and data not shown). These data suggest that Notch signaling supports CD4^+ T cell proliferation under conditions of low antigenic stimulation, and this effect is most prominent in the absence of CD28 signaling.

Inhibition of Notch activity diminishes T cell proliferation
To examine the role Notch signaling plays in CD4^+ T cell proliferation, we cultured primary T lymphocytes from DO11.10 TCR transgenic mice in the presence of OVA peptide while pharmacologically inhibiting Notch activity. We observed that treatment with GSI significantly reduced clonotypic T cell proliferation in response to low peptide concentrations, but not to higher peptide concentrations, after 72 h in culture, as determined by analysis of CFSE proliferation profiles (Fig. 2A). The apparent role of Notch signaling in supporting the response of CD4^+ T cells to a low Ag concentration is reminiscent of the effect of CD28 costimulation, which increases T cell sensitivity to low Ag concentrations (25) and promotes proliferation in part through induction of IL-2 (26). To separately examine the role of CD28 costimulation in the requirement for Notch signaling, purified BALB/c CD4^+ T cells were stimulated with either anti-CD3- or anti-CD3- and anti-CD28-coated latex beads. Treatment with GSI diminished the proliferation induced by anti-CD3 stimulation alone, but had little or no effect in the presence of CD28 costimulation (Fig. 2B and data not shown). These data suggest that Notch signaling supports CD4^+ T cell proliferation under conditions of low antigenic stimulation, and this effect is most prominent in the absence of CD28 signaling.

To better understand the mechanism of Notch signaling on T cell proliferation, we next measured IL-2 levels in supernatants derived from cultured DO11.10 mononuclear cells using a concentration of OVA peptide (0.1 μg/ml) at which pharmacologic inhibition of Notch inhibited T cell proliferation (see Fig. 2A). At this concentration of OVA peptide, IL-2 concentrations were decreased in tissue culture supernatants treated with GSI compared with those in supernatants from control cultures (Fig. 2C). At peptide concentrations at which no proliferative differences were observed (i.e., 1 μg/ml), IL-2 production was also impaired in cultures containing GSI (data not shown), indicating that IL-2 is not limiting under these conditions. In addition to its effects on IL-2 production, we explored the effect of GSI on CD25 expression. In a dose-dependent manner, GSI diminished CD25 expression on activated CD4^+ T cells, as shown by CD25 vs CFSE dot plots (Fig. 2D and data not shown). Importantly, treatment with GSI did not affect CD4^+ T cell activation, as determined by CD69 and CD44 expression at 24 h (Fig. 2E). As IL-2 itself regulates CD25

stimulation with OVA peptide, the cleaved form of Notch1 was detected within 12 h (Fig. 1B, left panel). These data demonstrate that Notch gene (Notch1–4) expression is induced, Notch1 protein expression is induced, and Notch1 protein is activated in CD4^+ T cells during cognate interaction with APCs.

Notch cleavage and subsequent activation can be inhibited pharmacologically with GSIs (17, 24). When DO11.10 TCR transgenic T cells were cultured in the presence of OVA peptide for 12 h, the cleaved form of Notch1 was not detected when the potent GSI, compound E (17, 18), was added to the culture (Fig. 1B, right panel). Treatment with GSI, however, had no effect on the expression of Notch1 at this time point (Fig. 1B, right panel). When DO11.10 mononuclear cells were cultured with OVA peptide in the presence of GSI, cleaved Notch1 was not detected in MACPSpurified CD4^+ T cells for up to 72 h (Fig. 1C). In contrast to the 12-h culture, Notch1 levels were diminished in the GSI-treated cultures at 24 and 48 h. This result is consistent with the positive feedback loop previously described for Notch-induced Notch expression (1). Taken together, these data demonstrate that Notch1 activity can be inhibited pharmacologically in a peptide-specific CD4^+ T cell immune response.

Results

Induction of Notch signaling in activated T cells
Real-time PCR was used to analyze Notch gene expression in unstimulated and stimulated CD4^+ T cells. Following T cell activation with anti-CD3- and anti-CD28-coated beads, the expression of all four Notch genes (Notch1–4) was markedly increased within 16 h (Fig. 1A). We next determined the kinetic expression of Notch1 protein. Mononuclear cells from DO11.10 TCR transgenic animals were cultured with OVA peptide for different times. CD4^+ T cells were MACS-purified from these cultures, and cell lysates were analyzed by Western blotting. Notch1 expression was induced within the first 3–6 h and peaked 12–24 h after stimulation in CD4^+ T cells (Fig. 1B, left panel, and data not shown). After expression, the engagement of Notch by one of its ligands results in proteolytic cleavage at an intracellular site between glycine 1743 and valine 1744. This cleavage event is dependent upon the enzymatic activity of the γ-secretase complex that contains presenilins and nicastrin (3, 21–23). Cell lysates were also analyzed for this cleaved form of Notch1 using a cleavage-specific Ab. During
expression through a positive autocrine feedback loop (27), exogenous IL-2 was added to cultures treated with GSI in an attempt to rescue CD25 expression. While untreated cells that received exogenous IL-2 showed a significant increase in CD25 expression, cells that were treated with GSI did not (Fig. 2F). These data demonstrate that in the absence of Notch signaling, T cell responses are inhibited through effects on CD25 expression, and this is independent of a decrease in IL-2 production.

Retroviral expression of intracellular Notch1 enhances T cell proliferation

The above data, observed in primary T cells using a loss-of-function approach, suggest that Notch signaling can influence CD4+ T cell proliferation at low antigenic concentrations through effects on CD25 expression. To further understand the role of Notch1 in CD4+ T cells, we used a complimentary gain-of-function approach. Previously described murine stem cell virus-based retroviral vectors (8, 19) were used to express a bicistronic mRNA encoding a test protein and an inactive human tNGFR as a marker of transduction. Vectors expressing the constitutively active intracellular domain of Notch1, Notch1-IC (ICN1 NGFR), a Notch1-IC loss of function mutant lacking the ankyrin repeat domain (∆ANK NGFR), or the surrogate tNGFR marker alone (MIG NGFR) were used to transduce CD4+ T cells from DO11.10 mice. We have shown that ∆ANK NGFR is a loss-of-function mutant allele and used it as an additional negative control (8).

Efficient retroviral transduction of primary CD4+ T lymphocytes requires cycling cells. This was achieved by activating MACS-purified CD4+ T cells with PMA and ionomycin before retroviral transduction and then reculturing the cells in medium alone for a 3-day rest. Following the rest period, the cells were labeled with CFSE and recultured with fresh OVA peptide-loaded APCs (BALB/c splenocytes). Clonotypic CD4+ T cells transduced with either the control retroviral vector (MIG NGFR) or the inactive Notch1-IC mutant (∆ANK NGFR) proliferated to the same extent as nontransduced (MIG NGFR) cells cultured in the same wells at all peptide concentrations (Fig. 3, A and C). In contrast, clonotypic CD4+ T cells transduced with the Notch1-IC-expressing retroviral vector (ICN1 NGFR) proliferated significantly more than nontransduced (ICN1 NGFR) cocultured cells at the lower peptide concentrations (Fig. 3B). Importantly, proliferation was largely Ag dependent, as minimal proliferation was observed without the addition of OVA peptide (Fig. 3, A and B).
insets, and data not shown). Thus, the net effect of Notch1 activity is to lower the threshold required for Ag-specific CD4+ T cell proliferation. In experiments designed to inhibit the proliferation of Notch1-IC-transduced cells, treatments that blocked IL-2 signaling (e.g., blocking mAbs against IL2 and CD25, rapamycin, or CTLA-4 Ig) were capable of inhibiting Ag-specific T cell proliferation (data not shown). These data suggest that Notch-enhanced T cell proliferation is mediated via IL-2R signaling.
**FIGURE 3.** Notch1-IC expression enhances T cell proliferation. DO11.10 CD4+ T cells were transduced with control (MIG NGFR), Notch1-IC (ICN1 NGFR), or Notch mutant (Δ ANK NGFR) retroviruses. Rested, CFSE-labeled CD4+ T cells (1 x 10^6) were cocultured with 2 x 10^6 fresh syngeneic splenocytes with OVA peptide for 3 days. The CFSE proliferation profiles for live (FSC vs SSC), clonotypic (KJ1-26+), CD4+ T cells are shown. A. Control transduced (MIG NGFR+) and nontransduced (MIG NGFR−) cells. B, Notch1-IC-transduced (ICN1 NGFR+) and nontransduced (ICN1 NGFR−) cells. C, Notch1-IC mutant (ΔANK NGFR+) and nontransduced (ΔANK NGFR−). Insets, Cells cultured without OVA peptide. These data are representative of three independent experiments.

**OVA peptide concentration**

- 0.003 μg/mL
- 0.01 μg/mL
- 0.03 μg/mL

CD25 expression is independent of cell division

CD25 expression on the surface of CD4+ T cells has been linked with cell division in vitro (16) and in vivo (28). Therefore, it was important to determine whether the expression of CD25 in Notch1-IC-transduced cells was a direct result of Notch signaling or merely a consequence of the ability of Notch1-IC to augment proliferation through some other mechanism. To differentiate between these two possibilities, we analyzed CD25 expression as a function of cell division. In these experiments purified CD4+ T cells were labeled with CFSE, stimulated and transduced, and then rested for 3 days (in contrast to the experiments in Fig. 3 in which cells were CFSE-labeled after the rest period). The proliferation that occurred during the rest period for both the control and Notch1-IC transduced T cells was similar, with all cells undergoing at least one round of proliferation, and some dividing as many as seven times (Fig. 5A). Under these conditions (PMA and ionomycin stimulation), persistent Notch activity does not enhance T cell proliferation, as the cells are optimally stimulated. Similar results were seen in other experiments (e.g., Fig. 3) in which cells were optimally stimulated, and proliferation was maximal. When gating on individual CFSE division peaks, CD25 expression in the control-transduced cells was progressively down-regulated as a function of the number of mitoses the cells had undergone (Fig. 5B). In contrast, CD25 expression was maintained on Notch1-IC-transduced cells independent of the number of cell divisions (Fig. 5B). These data support a role for Notch1 activity in maintenance of the high affinity IL-2R complex on the cell surface of CD4+ T cells independent of cell proliferation. Although we did not observe any significant differences in the CFSE proliferation profiles during the rest period (Fig. 5A), the Notch1-transduced cells were 9 ± 4.5% larger by forward scatter compared with the control-transduced cells (data not shown). In addition, a small proportion (<5%) of the Notch1-IC-transduced cells remained in cell cycle, as determined by BrdU incorporation, after a 6-h pulse (data not shown). These effects were not due to IL-2, as ELISAs performed on supernatants from control and Notch1-transduced cells did not reveal any detectable IL-2. These results demonstrate that Notch1-transduced cells display some features of an activated phenotype and are primed for activation.

**Notch1-IC enhances the response to IL-2**

As Notch1 activity leads to enhanced expression of the high affinity IL-2R, we hypothesized that Notch1-IC-expressing T cells were more sensitive to IL-2. To address this, we reanalyzed the CFSE profiles (i.e., the proliferative response) of transduced cells (cells from Fig. 5A) after an additional 72 h of culture in limiting concentrations of exogenous IL-2. In the absence of IL-2, the Notch1-IC-transduced cells exhibited a slightly increased tendency to proliferate compared with control-transduced cells (Fig. 5C, left panel). This slight difference in proliferative capacity is greatly enhanced in the presence of subthreshold amounts of IL-2 (Fig. 5, C and D). The enhanced response to IL-2 by the Notch1-IC-transduced cells was greatest at the lowest IL-2 concentration (0.01 U/ml), a dose at which both the nontransduced NGFR−) and control-transduced (MIG NGFR−) cells failed to respond (Fig. 5D). These data suggest that the enhanced proliferative response of Notch1-IC-expressing CD4+ T cells is in part dependent upon IL-2 sensitivity and is mediated by the high affinity IL-2R.

**Discussion**

In this report we identify a role for Notch signaling in peripheral T cell activation. We find that Notch gene expression is rapidly
induced following T cell activation for multiple Notch receptors and that the Notch1 signaling pathway is activated during peptide Ag stimulation. Pharmacologic inhibition of Notch activity attenuates T cell responses through CD25 expression, and gates were determined for negative, low, intermediate, and high expression. The percentages of CD4+ T cells in the NGFR gates were comparable between the control and Notch1-IC-containing retroviral vectors, respectively (negative, 53 vs 53%; low, 4 vs 3%; intermediate, 7 vs 4%; high, 2 vs 5%). Bottom, Analysis of CD25 expression on CD4+ T cells expressing different levels of tNGFR. Histograms of CD25 expression for NGFR−, NGFR low, NGFR intermediate, and NGFR high are shown. These data are representative of three independent experiments.

The CD25 promoter has been well characterized, and many factors regulating its transcription have been described (30, 31). Several recent studies of T cell development suggest that CD25 may be downstream of Notch signaling (32-34). In our own work we found that Notch1-IC induced ectopic immature T cell development to the DN2 (CD3−CD25−CD44+) and DN3 (CD3−CD25+CD44+) stages in recombinase-activating gene−/− mice, both of which are associated with CD25 expression (9). It is not clear whether CD25 is a direct or an indirect transcriptional target of Notch signaling. Analysis of the CD25 promoter failed to identify canonical CBF1, Su(H), and Lag-1 binding sites. Nevertheless, CD25 is positively regulated by NF-κB activity, which is downstream of Notch in some circumstances (33). An alternative possibility suggested by our results is that Notch signaling prevents CD25 degradation or stabilizes its surface expression.

Although the mechanism by which Notch enhances CD25 cell surface expression remains to be elucidated, this example of Notch activity provides a clue to the variety of ways that Notch has been suggested to function. Up-regulation of IL-2R per se does not specify a particular cellular response. Depending on the cellular milieu, signaling through IL-2R may be associated with proliferation, survival,
or activation-induced death. In our assays the system is skewed toward IL-2R-induced T cell proliferation. In other contexts, however, Notch induced IL-2R up-regulation may result in a different IL-2R-associated phenotype. In addition to enhancing the proliferative response of T cells to IL-2, Notch activity has been reported to induce cyclin D1 and promote entry into S phase in transformed cells (35). In our system we cannot rule out the possibility that Notch may affect entry into the cell cycle at other points downstream of the IL-2R.

There are multiple ways in which Notch might facilitate cell fate determination during the evolution of an immune response, including Th1 vs Th2 differentiation, survival or apoptosis, and proliferation or anergy in response to Ag. In addition, Notch signaling has been linked to protection from apoptosis during thymocyte development (32, 36). We have not detected any survival differences when comparing cells in which endogenous Notch activity was inhibited or in Notch1-IC-transduced cells when cultured in the absence of antigenic stimulation or exogenous IL-2 (data not shown). It is conceivable that Notch may function to both expand undifferentiated cells and regulate differentiation in response to other external signals. The circumstances in which Notch signaling acts in vivo and how this type of signaling fits with our current understanding of costimulation remain to be determined.

In one published study Ag-specific regulatory T cells, capable of transferring tolerance to naive mice, developed in mice that were injected with APCs engineered to overexpress the Notch ligand, Serrate1 (Jagged1) (37). Our results appear to be distinct from these findings and are probably related to differences between the two experimental systems used. The precise cell context is likely to be an important component of how cells respond to Notch signals, and it would not be surprising if in some circumstances Notch signaling was associated with anergy, and in other situations it primed the T cell response. Future studies characterizing the specific interactions between Notch receptors and ligands on T cells and APCs will aid in understanding this signaling pathway.

FIGURE 5. Notch1-IC-expressing cells have enhanced sensitivity to IL-2. A, CFSE profiles for MIG NGFR+ and ICN1 NGFR+ are shown for CD4+ T cells following a 3-day rest. Gating by the CFSE division peaks is shown (zero to seven cell divisions). B, CD25 histograms are based on division peak gates described in A and are shown for control-transduced (MIG NGFR+) and Notch1-IC-transduced (ICN1 NGFR+) cells. C, Resting, transduced (MIG NGFR+ or ICN1 NGFR+), CFSE-labeled total lymph node cells derived from BALB/c were recultured in limiting concentrations of IL-2 for 3 days. CFSE profiles are shown for live CD4+ NGFR+ T cells. D, Percentages of transduced and nontransduced cells undergoing more than five cell divisions in response to IL-2. Numbers in bold are represented in the histograms of C.
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