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Article   Immunology

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Notch ligation by Delta1 inhibits peripheral immune responses to transplantation antigens by a CD8+ cell–dependent mechanism

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Notch signaling plays a fundamental role in determining the outcome of differentiation processes in many tissues. Notch signaling has been implicated in T versus B cell lineage commitment, thymic differentiation, and bone marrow hematopoietic precursor renewal and differentiation. Notch receptors and their ligands are also expressed on the surface of mature lymphocytes and APCs, but the effects of Notch signaling in the peripheral immune system remain poorly defined. The aim of the studies reported here was to investigate the effects of signaling through the Notch receptor using a ligand of the Delta-like family. We show that Notch ligation in the mature immune system markedly decreases responses to transplantation antigens. Constitutive expression of Delta-like 1 on alloantigen-bearing cells renders them nonimmunogenic and able to induce specific unresponsiveness to a challenge with the same alloantigen, even in the form of a cardiac allograft. These effects could be reversed by depletion of CD8+ cells at the time of transplantation. Ligation of Notch on splenic CD8+ cells results in a dramatic decrease in IFN-γ with a concomitant enhancement of IL-10 production, suggesting that Notch signaling can alter the differentiation potential of CD8+ cells. These data implicate Notch signaling in regulation of peripheral immunity and suggest a novel approach for manipulating deleterious immune responses.

Introduction

The Notch-signaling pathway plays an integral role in cell fate decisions in all organisms, mutations in the Notch receptor, or its ligands, resulting in developmental abnormalities and embryonic lethality (1). Signaling through the cell surface receptor, Notch is activated by ligation to DSL (from: Delta, Serrate, and Lag proteins). In vertebrates there are four identified Notch receptors (Notch1–4) and five ligands of the Delta-like families (Delta-like 1 [Dl1], Dl3, Dl4) and Jagged families (Jagged1, Jagged2). The precise functions of these alternate families of ligands, and in particular whether they have redundant activities, is not completely understood. Recent studies, however, have indicated that during lymphoid differentiation, signaling through Delta-like proteins inhibits B cell differentiation (2), while that through Jagged proteins allows it. Furthermore, while the activity of Delta is potentiated by the Notch modifier, Fringe, the activity of Jagged is inhibited (3). As well as lateral specification and inductive signaling, Notch can affect cell fate through influencing apoptosis or cell cycle arrest (4–6). Taken together, these findings suggest that Notch signaling may act as a "gatekeeper" in controlling cell fate choices by influencing proliferation, differentiation, and death.

A number of studies have implicated Notch signaling in hematopoiesis, T-B lineage commitment, and thymic T cell development (4, 7–13; for review see refs. 14–16). There are also a number of studies that suggest a role for Notch signaling in differentiation and regulation of the mature peripheral immune system. First, APCs retrovirally transduced to constitutively express

the Notch ligand, Jagged-1, can induce peripheral tolerance to the house dust mite antigen Der p 1 (17). Second, the Notch ligand Dl1 can inhibit the differentiation of monocytes into macrophages, but allows their differentiation into DCs (18). Finally, in elegant studies involving conditional inactivation of the Notch pathway transcription factor, RBP-j, Tanigaki et al. (19) demonstrated that Notch signaling regulates the lineage commitment of mature peripheral B cells. As yet, however, there are no data addressing the activities of ligands of the Delta-like family on T cells nor the effects of Notch signaling on CD8^{+} cells. The aim of the studies reported here was to investigate the effects and mechanisms of action of Delta-mediated ligation of Notch on peripheral T cell–dependent immunity to transplantation antigens.

Methods

Production and characterization of Notch ligand transfectants. L cells express endogenous MHC class I antigens of the H-2[k] haplotype, but no endogenous MHC class II antigens. K[b] and A[b] (20) are L cells transfected with MHC class I (*h-2K[b]*) or MHC class II (*h-2A[b]*), respectively, of the H-2[b] haplotype (kindly provided by K. Wood, University of Oxford, Oxford, United Kingdom). Mouse Dl1 cDNA was subcloned into the mammalian expression vector, pIRES2/enhanced GFP (EGFP) (BD Biosciences Clontech UK, Cowley, United Kingdom) for transfection into L cells using Effectene (Qiagen Ltd., Crawley, United Kingdom). Stable transfectants were selected in G418 (Life Technologies Ltd., Paisley, United Kingdom) and twice sorted using flow cytometry to produce a population of EGFP-expressing cells named K[b]/Dl1 and A[b]/Dl1. Control K[b] and A[b] transfected with empty pIRES/EGFP were similarly selected and shown to behave in the same way as the K[b] and A[b] cells in popliteal lymph node (PLN) assays.

Ab's. Biotinylated AF6-88 (anti–H-2K[b]) and 1D9 (anti–H-2A[b]) were provided by A. Livingstone (University of Rochester Medical Center, Rochester, New York, USA). Anti-CD3 (FITC conjugated), streptavidin-phycoerythrin, and annexin V staining kits were from PharMingen (Cowley, United Kingdom). Depleting CD8 (YTS169) and CD4 (YTS191) Ab's were a kind gift of S. Cobbold (University of Oxford, Oxford, United Kingdom) (21). Rat Ig was from Sigma-Aldrich Co. Ltd. (Gillingham, United Kingdom).

Flow cytometry. Viable cells (10[5]) were aliquoted into wells of a 96-well plate (Bibby Sterilin Ltd., Stone, United Kingdom) and incubated with titrated amounts of primary Ab's in 50 μl PBS/FCS. After washing, cells were resuspended with secondary Ab. Stained cells were analyzed using a FACscan II flow cytometer with Cellquest software (Becton-Dickinson UK Ltd., Oxford, United Kingdom).

Quantitative RT-PCR. Total RNA was extracted using StrataPrep (Stratagene Europe, Amsterdam, The Netherlands) or Qiagen RNeasy kits (Qiagen Ltd.). Total RNA (400–500 ng) was used for cDNA synthesis using a multi-cDNA kit and random oligonucleotide

primers (according to the manufacturer's instructions; ABI, Warrington, United Kingdom) or Superscript II (Invitrogen, Paisley, United Kingdom) and oligo-dT[+] random decamers (Ambion Europe Ltd., Huntingdon, United Kingdom). Samples were resuspended in 100 μl final volme with distilled water, and 2.5 μl of this was used per well in a final volume of 25 μl for PCR. Primer and probe sequences were as follows: *Mouse notch1*: forward, TCCAGAGTGCCACCGATGT; reverse, TCCACCG-GCTCACTCTTCAC; probe, CTGCCTTCCTAGGTGCTC-TTGCGTCA; *Mouse notch2*: forward, ACCCTCCGCCGA-GACTCT; reverse, TCCCAGAACCAATCAGGTTAGC; probe, CCTGTCCCACAGGTTCACGGCG; *Mouse notch4*: forward, TGTCTCCCCCATAGAGTATGCA; reverse, CTCGAAAT-CAACTTTGTCCTCTTG; probe, CCGGACATCCTAAACC-CTCCCATTG; *Mouse dl3*: forward, GGCGGTGAAGATC-CTGACTCT; reverse, CGGTCCACCCTCTTCTCACA; probe, ATTGCCCACCTGGTTTCCAAGGCTC; *Mouse dl1*: forward, TCCTTTCGCGTATGCCTCAA; reverse, CATCAGGCAG-GCTGAAGGA; probe, ACTACCAGGCCAGCGTGTCACCG; *Mouse jagged1*: forward, CCCGCACCCAGGAGTTGT; reverse, CACCCCAGTTGGTCTCACAGA; probe, CACCTG-CAATGAACCCTGGCAGTG; *Mouse jagged2*: forward, CAGCTGGACGCCAATGAGT; reverse, GCCAATCAGGTT-TTTGCAAGA; probe, AGCATTAAGGCACGGCTTCCCT-TCA; *Mouse Hes1*: forward, GGTGCTGATAACAGCGGAAT; reverse, CCATCCAATCGGTAGTAGCG; *18s*: forward, GTAACCCGTTGAACCCCATT; reverse, CCATCCAATCG-GTAGTAGCG; or off the shelfprimers from ABI. All primers were purchased from ABI.

All probes were labeled with 5′-6-carboxyfluoroscein and 3′-6-carboxytetramethylrhodamin. The Hes1 amplicon was detected with SYBR green.

Samples were run on an ABI 7700 sequence detection system or a Lightcycler (Roche Diagnostics Ltd., Lewes, United Kingdom). Relative quantification of mRNA levels was performed using the comparative cycle threshold (C_T) method, following the manufacturer's instructions (ABI).

Notch-signaling reporter assay. CHO cells were stably transfected with a full-length cDNA encoding human Notch2, which was isolated from a commercially available cDNA bank (OriGen Technologies Inc., Rockville, Maryland, USA). These cells were further transfected with a construct containing a multimerized (10 times) CBF1 response element upstream of a luciferase cDNA. Stable transfectants (CHO/N2-luc, 2×10^4 cells/well) were placed into white 96-well OptiPlates (Canberra Packard Ltd., Pangbourne, United Kingdom) and allowed to adhere for 1 hour at 37°C before adding the L cell transfectants or control untransfected CHO cells at 2×10^5/well in medium containing 10 mM LiCl. L cells and control untransfected CHO cells were mitomycin C treated (100 μg/ml, 70 minutes, 37°C) before they were added to the assay. Cultures were incubated at 37°C for 24 hours. Supernatants were removed from wells, and cells were washed and lysed by the addition of 100 μl of Glo lysis buffer (Promega UK Ltd., Southampton, United Kingdom). After 5 minutes at

room temperature, 100 μl of SteadyGlo luciferase assay reagent (Promega UK Ltd.) was added, and the luminescence was read in a TopCount (Canberra Packard Ltd.).

Mice. C57BL/10 (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) mice between 8 and 12 weeks old were obtained from Harlan Olac Ltd. (Bicester, United Kingdom) and kept in the animal facilities in Central Biomedical Services at Imperial College in accordance with Home Office guidelines [Animals (Scientific Procedures) Act 1986]. The mice were used in both PLN assays and heart transplantation experiments. C57BL/10 mice were used as allogeneic donors, BALB/c mice were used as third-party donors, and C3H/HeJ mice were used as recipients.

Heterotopic heart transplantation. Heterotopic heart transplants were performed as described by Corry (22). Animals were injected with cells 14 days before transplantation, as indicated. Doses of cells were based on those used previously (20). Injections were as follows: K^b, 2.5 × 10^6 or 5 × 10^6; A^b, 1 × 10^6; K^b/Dl1, 2.5 × 10^6 or 5 × 10^6; A^b/Dl1, 1 × 10^6; K^b, 5 × 10^6, + A^b, 1 × 10^6; and K^b/Dl1, 5 × 10^6, plus A^b/Dl1, 1 × 10^6. Doses of 2.5 × 10^6 or 5 × 10^6 K^b/Dl1 were compared with 2.5 × 10^6 or 5 × 10^6 K^b due to the sharp dose-response curve reported previously regarding the effect of K^b in transplantation experiments (20). Both doses of both cells had the same effect on graft survival, and, consequently, doses of 5 × 10^6 K^b or K^b/Dl1 cells were given in all mixing experiments. Where shown, animals were treated with 1 mg YTS169, YTS191, or rat IgG intraperitoneally on day 1 and day 0.

PLN assay. Cells (10^6) in 15 μl of PBS were injected subcutaneously into the left foot pad (LFP). The ratio of K^b-expressing to A^b-expressing cells used in a mixed population was 5:1, as above. Allogeneic lymph node cells (LNCs) were irradiated with 30 Gy before use. Challenges were with 1 × 10^6 cells or 0.6 × 10^6 DCs. PLNs were harvested, dissected free of fat and surrounding tissue, and weighed (at 7 days), or made into a single cell suspension (at times shown). Results are expressed as mean weight or cell number ratio of left PLN to right PLN (LPLN/RPLN) ± SEM. A LPLN/RPLN ratio of 1 equates to no response.

Bone marrow–derived DCs. Bone marrow cells were harvested, red cells lysed, and the remaining cells were cultured at a concentration of 2 × 10^6 cells/ml in RPMI-1640 plus 10% FCS plus antibiotics (GibcoBRL; Life Technologies Ltd.) with GM-CSF and IL-4 (10 ng/ml each; PeproTech EC Ltd., Northampton, United Kingdom). Two milliliters of the medium was replaced on days 2 and 4. DCs were used on day 7.

Indirect allopresentation. Seven day–cultured DCs were treated with mitomycin C (Sigma-Aldrich Co. Ltd.) at 50 μg/ml for 1 hour at 37°C. Cells were washed three times and incubated overnight in serum-free RPMI-1640 to induce apoptosis. These were fed to growing DCs on days 3 to 5 of their culture. On day 7, DCs that had been fed with apoptotic DCs were used in PLN assays.

Dl1-Fc cultures. Ninety-six– or 24-well plates were coated with anti-hamster IgG1 and anti-human IgG4 cap-ture Ab's at 1 μg/ml in PBS. Plates were incubated overnight at 4°C and washed three times with PBS before addition of hamster anti-mouse CD3 (clone 145.2C11, concentrations as shown) and Dl1-Fc (various concentrations as shown). Dl1-Fc protein was purified from stably transfected CHO cell supernatants and was shown to be active in a Notch-signaling assay (23). Plates were then incubated for 2 hours at 37°C and washed three times with PBS. CD4$^+$ or CD8$^+$ cells were positively selected from red cell lysed spleen cell suspensions using Miltenyti microbeads according to the manufacturer's instructions. For cytokine analysis in 96-well plates, 2 × 10^5 to 3 × 10^5 cells/well were added to treated plates with soluble anti-CD28 at 2 μg/ml. For RT-PCR in 24-well plates, 2.5 × 10^6 cells/well were added. Coating and activating Ab's were from PharMingen. Cells were incubated for 5 days before removal of supernatants for cytokine analysis by ELISA using Ab pairs from R&D Systems Europe Ltd. (Oxon, United Kingdom) or for 4 hours before collection for RNA preparation.

Statistical analysis. Statistics were performed using Mann-Whitney test (PLN assay) or Wilcoxon's log-rank test (transplants) with Bonferroni correction as appropriate.

Results

Characterization of L cells. L cells express endogenous MHC class I antigens of the H-2^k haplotype, but no endogenous MHC class II antigens. K^b and A^b are L cells transfected with allogeneic MHC class I or MHC class II, respectively, of the H-2^b haplotype (20). K^b and A^b cells were further transfected with a mouse Dl1/EGFP bicistronic cDNA. We chose to use a Notch ligand of the Delta-like family because the effects of this ligand family have not been studied previously in the context of T cell–dependent peripheral immunity. Following selection, the Notch ligand transfectants were sorted into EGFP-expressing populations (MHC class I–expressing Dl1 transfectants were termed K^b/Dl1, and MHC class II–expressing Dl1 transfectants were termed A^b/Dl1) and analyzed by flow cytometry (Figure 1a). The initial flow-cytometric analysis of EGFP expression indicated that higher expression of Dl1 would be found in K^b/Dl1 than in A^b/Dl1 cells, and this was confirmed at the transcript level using quantitative (Taqman) RT-PCR (Figure 1b). Furthermore, Dl1 transcripts were undetectable in the parental K^b and A^b cells. By flow cytometry, Dl1 transfectants were shown to express the same levels of H-2^b MHC proteins as their parental counterparts (Figure 1c).

Notch signaling can be induced by Dl1-mediated ligation in both reporter assays and in T cells. A reporter assay system was used to determine the ability of Dl1 transfectants and their parental counterparts to actively signal through the Notch receptor. Notch2-expressing CHO cells were transfected with a plasmid construct that reports Notch pathway activation by expression of luciferase. Such cells were then cocultured with the various L cell transfectants and assayed for luciferase activity. The Dl1 protein, introduced by transfection into
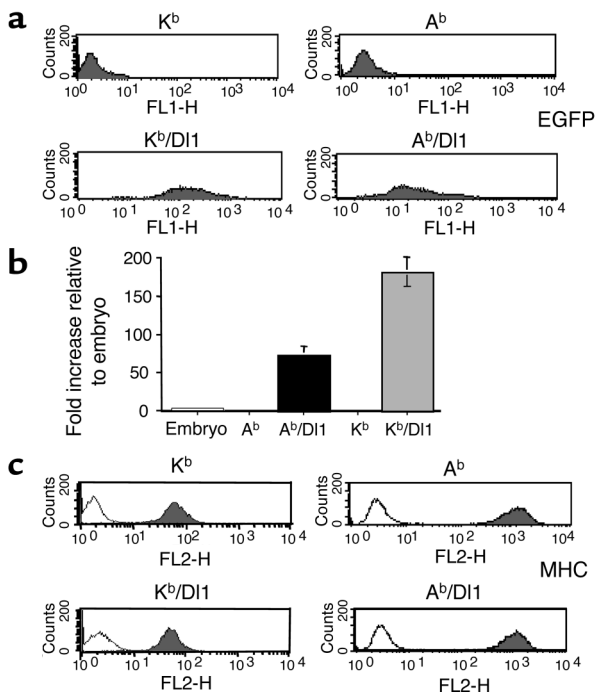
a

K^b — A^b — EGFP

$K^b/Dl1$ — $A^b/Dl1$

b

Embryo A^b $A^b/Dl1$ K^b $K^b/Dl1$

c

K^b — A^b — MHC

$K^b/Dl1$ — $A^b/Dl1$

Figure 1

Characterization of L cell transfectants. (**a**) MHC class I and II (K^b and A^b, respectively) and MHC Dl1-transfected ($K^b/Dl1$ and $A^b/Dl1$) cells were analyzed using flow cytometry. EGFP expression is shown on FL1-H and indicates the level of expression of the transfected Dl1:EGFP bicistronic construct. EGFP expression reflects Dl1 expression. FL1-H, level of green fluorescence. (**b**) Quantitative RT-PCR for Dl1 transcripts in parental K^b and A^b cells and their Dl1-transfected counterparts. All samples were normalized by assessment of 18s RNA. Mouse embryonic RNA (day 11) was used as a positive control in the experiment, and transcript levels in other samples are expressed relative to this. (**c**) Flow-cytometric analysis of MHC class I (H-2K^b, left panels) and MHC class II (H-2A^b, right panels) expression on all cell lines using biotinylated AF6-88 (MHC class I) and 1D9 (MHC class II) mAb's (filled traces) or no first-stage Ab (open traces). All data are representative of at least three repeat experiments. FL2-H, level of red fluorescence.
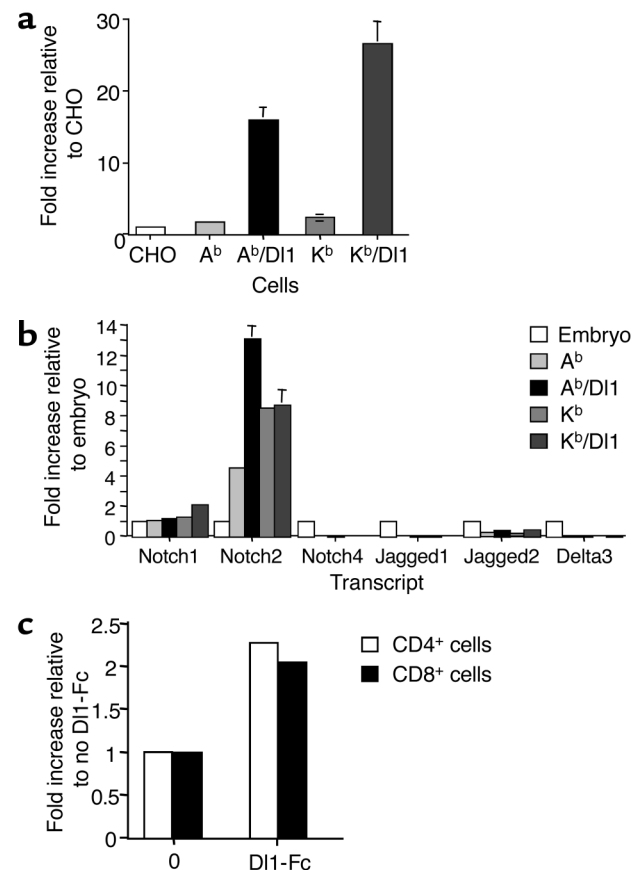
Notch receptor by ligand-expressing cells can affect the ability of that cell to signal or to express other ligands. In our experiments, however, the Dl1–transfected cells signaled well and did not have upregulated expression of alternative ligands. Taken together, these findings indicate that the effects observed in functional assays were due solely to the expression of Dl1 introduced into these cells by transfection.

We also wished to know whether Notch signaling could be directly activated in T cells. To explore this, T cells stimulated in the presence or absence of Notch ligand were assessed for increased transcript levels of Hes1.

both $A^b/Dl1$ and $K^b/Dl1$, was shown to be functionally active in this reporter assay for Notch ligand activity, but as predicted by RT-PCR, the level of reporter activation by the $A^b/Dl1$ transfectants was found to be lower than that by the $K^b/Dl1$ cells (Figure 2a). Importantly, no signaling was observed when K^b or A^b cells were introduced into the assay, suggesting that there is no, or insignificant, endogenous Notch ligand expressed by these cells. The expression of endogenous ligands was further explored by using quantitative RT-PCR. Of the Notch ligands, only low levels of Jagged2 were detected in all L cells at similar levels, although all cells did express significant levels of both Notch1 and Notch2 (Figure 2b). It is known that transendocytosis of the

Figure 2

Dl1 is able to activate Notch signaling in reporter cell assays and in T cells. (**a**) CHO/N2-luc cells, which contain a construct that reports Notch pathway activation by expression of luciferase, were cultured with mitomycin C–treated K^b, $K^b/Dl1$, A^b, or $A^b/Dl1$. Untransfected CHO cells were used to control cell numbers in the assay and do not activate the Notch pathway in CHO/N2-luc cells. Cell lysates were assayed for luciferase activity after 24 hours. (**b**) Quantitative RT-PCR was used to assess the expression of Notch receptors and Notch ligands by L cells. All samples were normalized by assessment amplification of 18s RNA. Mouse embryonic RNA (day 11) was used as a positive control in the experiment, and transcript levels in other samples are expressed relative to this. (**c**) Positively selected CD4^{+} or CD8^{+} cells were cultured in the presence of immobilized CD3 Ab (0.12 μg/ml), CD28 Ab (2 μg/ml), and immobilized Dl1-Fc fusion protein (40 μg/ml). After 4 hours, cells were harvested and RNA extracted. Samples were analyzed for Hes1 transcripts and normalized using 18s amplification by quantitative RT-PCR. Data are expressed as fold induction compared with cells incubated with CD3 and CD28 Ab's in the absence of Dl1-Fc fusion protein. All data are representative of at least three repeat experiments.
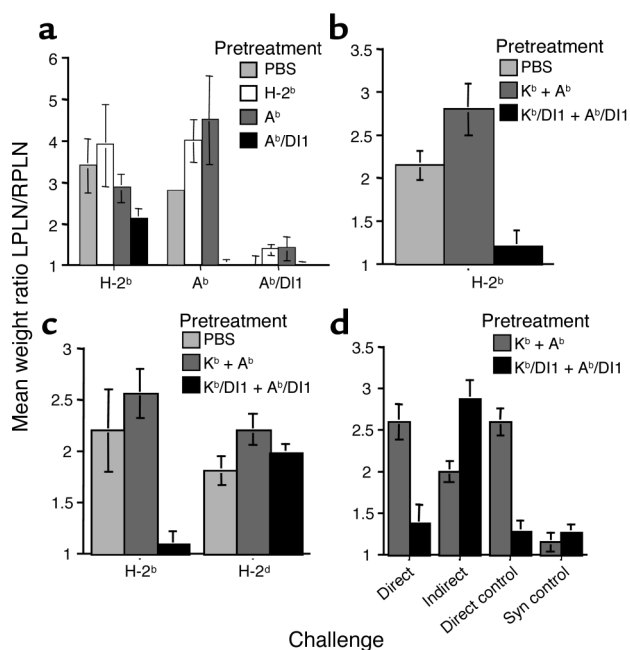
a

CHO A^b $A^b/Dl1$ K^b $K^b/Dl1$
Cells

b

Notch1 Notch2 Notch4 Jagged1 Jagged2 Delta3
Transcript

□ Embryo
░ A^b
■ $A^b/Dl1$
▨ K^b
▩ $K^b/Dl1$

c

0 Dl1-Fc

□ CD4^{+} cells
■ CD8^{+} cells

Figure 3

Notch ligand transfectants are not immunogenic and can inhibit the response to a challenge with antigen in a PLN assay. (**a**) PBS, H-2^b LNCs, A^b, or A^b/Dl1 cells were injected into the LFP of C3H/HeJ mice. After 14 days, animals were challenged in the LFP with irradiated allogeneic LNCs (H-2^b, from C57BL/10), A^b, or A^b/Dl1 cells, and after an additional 7 days, PLNs from both sides were harvested, weighed, and the results expressed as a ratio of weight (LPLN/RPLN). (**b**) A mixed population of either K^b + A^b or K^b/Dl1 + A^b/Dl1 was used as the first injection, and a second challenge with H-2^b LNCs was given as above. PLNs were harvested and data expressed as indicated above. (**c**) Mixed populations of cells or PBS were given as a first injection as shown. Animals were challenged with H-2^b or H-2^d (third party–derived) LNCs. PLNs were harvested and data expressed as indicated above. (**d**) Either K^b + A^b or K^b/Dl1 + A^b/Dl1 were given as a first injection. DCs were used to test responsiveness to directly (direct, C57BL/10 DCs) or indirectly (indirect, C3H/HeJ DCs fed with C57BL/10 DCs) presented antigen. To check that the feeding protocol did not alter the function of DCs, control injections were with C57BL/10 DCs fed with C57BL/10 DCs (direct control) and C3H/HeJ DCs fed with C3H/HeJ DCs (syn control). Syn, syngeneic. n = 3–4 in all groups; experiments are representative of at least three repeats.

Hes1 is a downstream target of Notch signaling, and an increase in transcripts of this gene indicates active Notch signaling in cells, including those of the hematopoietic system (2, 24, 25). Preliminary experiments using L cell stimulators indicated that, indeed, the Dl1 transfectants, but not their parental counterparts, could deliver a signal through Notch as assessed by increased expression of Hes1 (data not shown). We were concerned, however, that because the Dl1 transfectants expressed high levels of Hes1 (data not shown), small contamination with these cells could bias our results. We therefore used a Dl1-Fc fusion protein to stimulate Notch signaling in T cells stimulated with CD3 and CD28 Ab's. These experiments showed that Dl1-mediated ligation of Notch is able to induce upregulated transcript levels of Hes1 in both CD4^+ and CD8^+

cells, thereby indicating that Notch signaling can be directly activated in T cells (Figure 2c).

Dl1-transfected L cells are not immunogenic and can induce antigen-specific unresponsiveness. The immunogenicity and ability to regulate immune responses of the L cells was first assessed using a system of local antigen challenge, the PLN assay. In this assay animals are challenged in the footpad with antigen, and responses are measured in the draining PLN. In PLN assays, neither A^b/Dl1 nor K^b/Dl1 were immunogenic. Moreover, these Notch ligand transfectants were capable respectively of inducing unresponsiveness to a subsequent challenge with A^b (Figure 3a) or K^b (data not shown) cells not expressing the Notch ligand. By contrast, A^b and K^b cells were immunogenic and could prime the animals to a subsequent challenge with A^b (Figure 3a) or K^b (data not shown), respectively. Neither A^b/Dl1 nor K^b/Dl1 alone could induce unresponsiveness to a subsequent challenge with H-2^b LNCs that express the entire H-2^b haplotype (H-2^b; Figure 3a), but mixtures of A^b/Dl1 and K^b/Dl1 could do so (Figure 3b). The unresponsiveness induced by pretreatment with A^b/Dl1 and K^b/Dl1 was antigen specific in that third-party responses to LNCs of H-2^d origin were unaltered (Figure 3c).

Unresponsiveness is not induced to MHC antigens presented through the indirect pathway. Transplantation antigens can be presented either directly, that is on cells expressing the alloantigen, or indirectly, that is as processed peptides on self APCs. Both pathways have been shown to be important in evoking acute graft rejection (26–28). The experiments described above indicated that T cells recognizing alloantigen directly on the expressing cell were inhibited by the pretreatment protocol, and this was confirmed in PLN assays where the APCs used in challenge were DCs rather than LNCs (direct; Figure 3d). To test responses of T cells recognizing the alloantigen indirectly, we took DCs of H-2^k origin and fed them with mitomycin C–treated DCs of H-2^b origin. The fed DCs are able to present peptides of H-2^b origin in the context of H-2^k MHC, that is indirectly, and are able to stimulate a good PLN response in H-2^k mice whether animals were pretreated with K^b plus A^b or K^b/Dl1 plus A^b/Dl1 (indirect; Figure 3d). These data show that while pretreatment with K^b/Dl1 plus A^b/Dl1 inhibits responses stimulated by directly presented alloantigen, it does not affect responses to indirectly presented antigen. Pretreatment with K^b plus A^b cells is unable to inhibit responses to alloantigen presented either directly or indirectly.

Kinetics and apoptosis in PLN responses. To follow more closely events in K^b/Dl1 plus A^b/Dl1–pretreated animals following antigen challenge, PLNs were removed at various times and total or T cell numbers counted and expressed as a ratio of injected to noninjected sides (total cells, Figure 4a; T cells, Figure 4b). The response in animals pretreated with K^b plus A^b was clearly primed, with cell numbers rising rapidly in the first 18–48 hours and remaining elevated during the entire time course. Animals pretreated with K^b/Dl1 plus
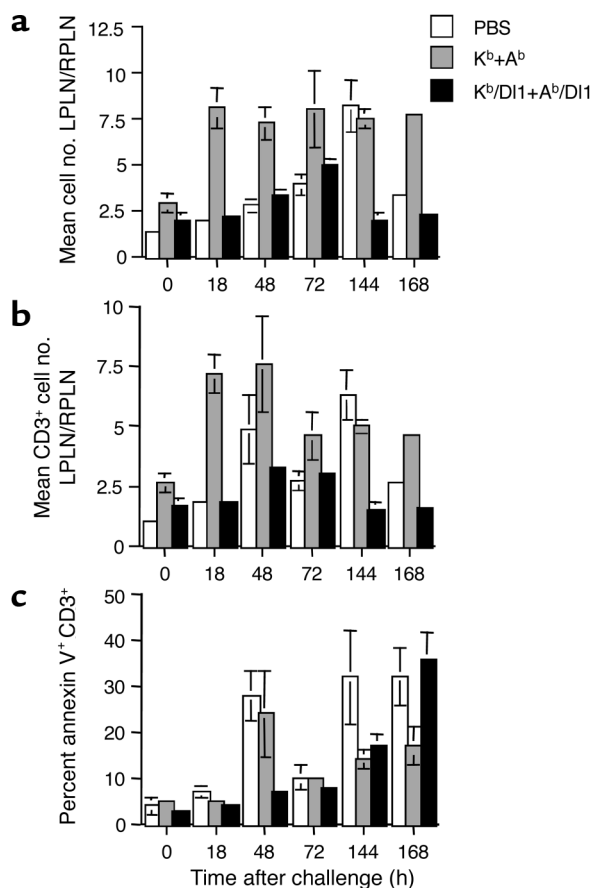
Figure 4
Kinetics of cell accumulation and apoptosis in PLN assay. C3H/HeJ mice were first injected with PBS, mixtures of $K^b + A^b$, or mixtures of $K^b/Dl1 + A^b/Dl1$ in the LFPs, as in Figure 1. After 14 days, they were challenged with irradiated allogeneic LNCs of H-2^b origin. At various time points after the second injection, PLNs were harvested for analysis of cell numbers (**a**) expressed as the ratio of mean cell numbers (LPLN/RPLN ± SEM) (**b**) expressed as ratio of mean T cell numbers (LPLN/RPLN ± SEM), and (**c**) annexin V staining. Data are expressed as the percentage of CD3$^+$ cells coexpressing annexin V. Results of two separate experiments are pooled such that n = 3–6 animals per time point.

$A^b/Dl1$ did not show this early accumulation of cells (e.g., $P < 0.002$ K^b plus A^b–pretreated versus $K^b/Dl1$ plus $A^b/Dl1$–pretreated animals at 18 hours), although there was some elevation in cell numbers between 48 and 72 hours. This elevation rapidly declined thereafter and remained low until the end of the experiment. Annexin V staining indicated that apoptosis in T cells could not account for the early difference in cell numbers observed between K^b plus A^b–pretreated and $K^b/Dl1$ plus $A^b/Dl1$–pretreated animals (Figure 4c), and indeed there was a clearly reduced level of apoptosis in the T cells of the latter animals at the 48-hour time point ($P < 0.05$ K^b plus A^b versus $K^b/Dl1$ plus $A^b/Dl1$). This is consistent with previous reports indicating that Notch can protect T cells from apoptosis (5). It therefore appears that preconditioning with Dl1 transfectants affects early accumulation, proliferation, and/or retention, but not death of cells at the site of

antigen challenge. At later time points in the assay, however, apoptosis increased in T cells from animals with all pretreatment regimens, but particularly in those pretreated with either PBS or $K^b/Dl1$ plus $A^b/Dl1$. The reasons for this were not investigated, but because it was observed in both PBS-pretreated and $K^b/Dl1$ plus $A^b/Dl1$–pretreated animals we concluded that this was not of direct importance to the regulatory effects induced by the $K^b/Dl1$ plus $A^b/Dl1$ cells.

Pretreatment with Dl1 transfectants can inhibit graft rejection in an antigen-specific fashion. We next tested the ability of the Notch ligand transfectants to inhibit graft rejection using the mouse, fully vascularized, heterotopic heart transplant model (C57BL/10,H-2^b>C3H/HeJ,H-2^k). In this strain combination, H-2K^b– and H-2A^b–expressing L cells delivered intravenously before transplantation have been shown previously to modestly enhance graft survival (20). As with PLN experiments, pretreatment of recipients with either $K^b/Dl1$ or $A^b/Dl1$ alone failed to effectively inhibit the response to a challenge with H-2^b, in this case in the form of a heart allograft (Figure 5a). When we compared animals pretreated with mixtures of K^b plus A^b or $K^b/Dl1$ plus $A^b/Dl1$, however, expression of the Notch ligand on donor MHC-expressing cells considerably enhanced their ability to prolong graft survival (Figure 5a, $P < 0.05$). This effect was antigen specific because third-party (H-2^d) grafts were rejected with normal kinetics (Figure 5a).

Inhibition of allogeneic responses by delivery of alloantigen in the context of Dl1 was seen in both the PLN and the transplant experiments, despite a difference in the route of administration (subcutaneous versus intravenous), yet the inhibitory effect of the K^b and A^b cells was observed only after intravenous delivery. To further investigate if ligation of Notch could provide an overriding inhibitory signal even when antigen is delivered by a normally sensitizing route, recipient mice were pretreated with mixtures of either K^b plus A^b or $K^b/Dl1$ plus $A^b/Dl1$ cells intraperitoneally before receiving an allograft. This route of pretreatment completely negated the modest beneficial effect seen with K^b plus A^b pretreatment, while the prolongation of allograft survival remained intact in animals that received $K^b/Dl1$ plus $A^b/Dl1$ cells (Figure 5b).

Inhibition of rejection is dependent on a CD8$^+$ cell population. We next tested the possibility that an inhibitory population of cells was involved in the Notch ligand–induced effects by depleting CD4$^+$ or CD8$^+$ cells at the time of transplantation. Consistent with previous findings (29), depletion of CD4$^+$ cells further enhanced graft survival in animals pretreated with either K^b plus A^b or $K^b/Dl1$ plus $A^b/Dl1$ cells (Figure 6a). Because CD4$^+$ T cells are known to be critical in the rejection of fully MHC-incompatible mouse heart allografts, this was perhaps not surprising (29). Depletion of CD8$^+$ cells completely abrogated the beneficial effects of pretreatment with the $K^b/Dl1$ plus $A^b/Dl1$ cells, however, even though CD8$^+$ cells can be involved as effector cells in the rejection of heart allografts (29)

a

% Grafts surviving vs Days after transplantation

····□···· L cells
····◇···· L-A^b/Dl1
····○···· L-K^b/Dl1
──▲── L-K^b + L-A^b
──■── L-K^b/Dl1 + L-A^b/Dl1
──◆── L-K^b/Dl1 + L-A^b/Dl1
 (third party graft)

b

% Grafts surviving vs Days after transplantation

····△···· L-K^b + L-A^b, i.v.
····□···· L-K^b/Dl1 + L-A^b/Dl1, i.v.
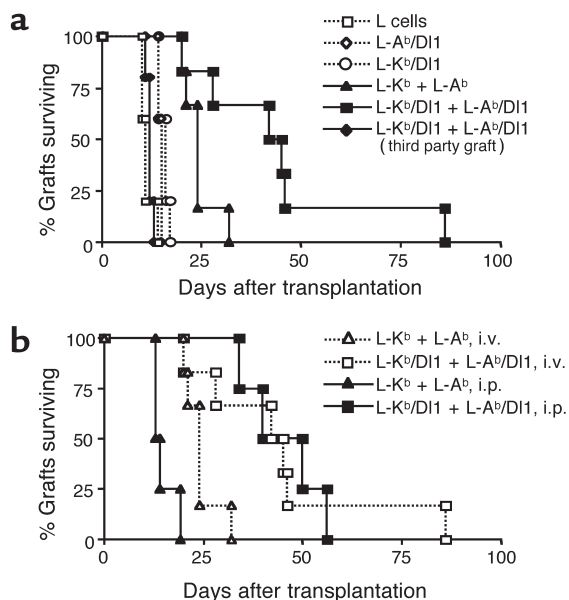──▲── L-K^b + L-A^b, i.p.
──■── L-K^b/Dl1 + L-A^b/Dl1, i.p.

Figure 5

Notch ligand transfectants induce prolonged graft survival. (**a**) C3H/HeJ recipients (H-2^k) were injected intravenously with either untransfected L cells (H-2^k), K^b, A^b, K^b/Dl1, A^b/Dl1, a mixed population of K^b + A^b, or a mixed population of K^b/Dl1 + A^b/Dl1. The dose of K^b-expressing cells was 5×10^6, and the dose of A^b-expressing cells was 10^6. These animals received heart allografts of H-2^b origin 14 days later. To test antigen specificity of graft-survival prolongation, one group that had been pretreated with K^b/Dl1 and A^b/Dl1 received third-party grafts of BALB/c origin (H-2^d). (**b**) C3H/He mice were pretreated as above intraperitoneally (i.p.) or intravenously. Data for intravenous injections are from the same animals in both **a** and **b** and shown in both for comparative purposes. Heart grafts were assessed daily by palpation for rejection. $n = 4$–6 in all groups.

enhancement of IL-10 production following TCR-mediated activation (23). As demonstrated (Figure 6b) using splenic CD8+ cells, ligation of Notch in the presence of T cell receptor and costimulatory signals also has profound effects on the type of cytokine produced, dramatically decreasing the level of IFN-γ while increasing over threefold the amount of IL-10 produced. Levels of IL-2 were also decreased and those of IL-13 were unaffected, suggesting that Notch signaling inhibits differentiation down a T1 pathway in these experiments.

Discussion

We have shown, we believe for the first time, that pretreatment with cells expressing a ligand of the Delta-like family together with alloantigen is able to inhibit the response to subsequent exposure to the same antigen in an antigen-specific fashion. In the context of organ transplantation this resulted in the prolongation

(Figure 6a). These findings implicate a CD8+ cell in the beneficial Notch ligand–induced effects observed.

Notch ligation of CD8+ cells alters their cytokine expression potential. We were interested in the possibility that ligation of Notch on T cells might alter their cytokine-expression pattern. In preliminary experiments we observed that the production of IFN-γ by T cells was reduced significantly upon incubation with K^b/Dl1 plus A^b/Dl1 cells in comparison to incubation with K^b plus A^b cells (data not shown). Under these culture conditions, however, the secretion of other cytokines was very low. Use of the Dl1-Fc fusion protein described above allowed us to further explore the effects of Notch ligation on isolated populations of cells in culture under conditions where many different cytokines could be produced. Indeed, we have shown recently that Notch ligation in CD4+ cells by Dl1-Fc induces a selective

a

Percent grafts surviving vs Days after transplantation

	Cells	Antibody
──◆──	-	
····○····	K^b+A^b	Anti-CD8
──●──	K^b/Dl1+A^b/Dl1	
····◇···	-	
····△····	K^b+A^b	Anti-CD4
──▲──	K^b/Dl1+A^b/Dl1	
····□···	K^b+A^b	IgG
──■──	K^b/Dl1+A^b-Dl1	

Figure 6

CD8+ cell depletion reverses Dl1 enhancement of graft survival, and purified CD8+ splenic cells make altered cytokine responses upon ligation with Dl1. (**a**) Animals were pretreated with K^b + A^b or K^b/Dl1 + A^b/Dl1 as in Figure 5. All cell injections were given intraperitoneally. One day before and on the day of transplantation animals received 1 mg CD8-depleting (YTS169) or CD4-depleting (YTS 191) Ab intraperitoneally. Graft survival was monitored by palpation, $n = 4$–6/group. (**b**) Purified splenic CD8+ cells were cocultured with immobilized anti-CD3 (0.04 µg/ml), soluble anti-CD28, and immobilized Dl1-Fc or IgG-4 control protein at the concentrations shown. Supernatants were harvested after 5 days and tested in ELISA for cytokines as shown. One representative experiment of three is shown.

b

Cytokine concentration (ng/ml) vs Dl1-Fc concentration (µg/ml)

IFN-γ, IL-10, IL-2, IL-13
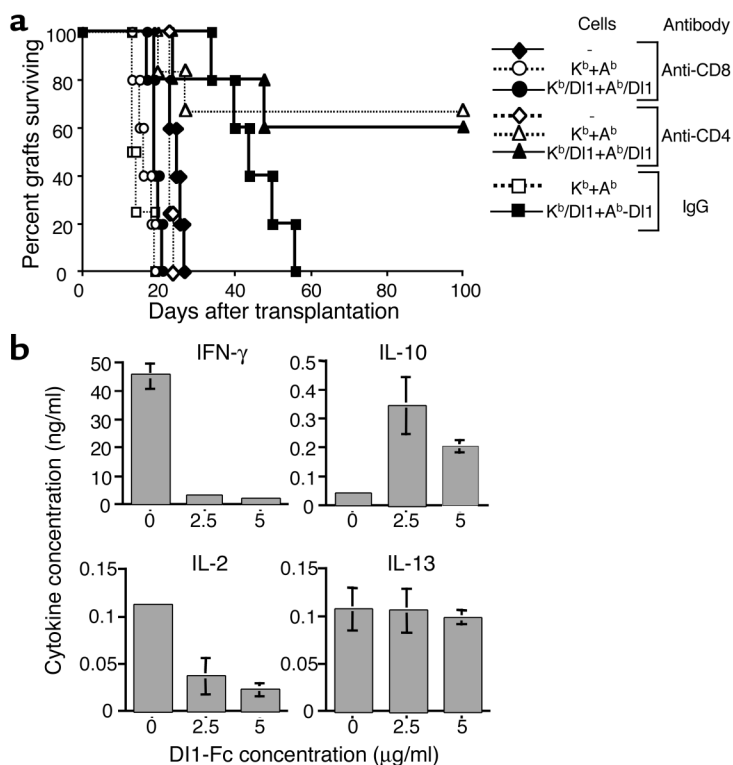
of graft survival under conditions in which pretreatment with alloantigen alone had no beneficial effect. Mechanistically, this inhibition was shown to be dependent upon the presence of CD8+ cells and did not associate with increased apoptosis in responding cells. Although engraftment was not permanent, we provided evidence that this was due to the fact that our pretreatment regimen did not target the indirect pathway of presentation. Cell culture experiments indicated that ligation of Notch on CD8+ responder cells was able to alter their cytokine expression potential, diverting them away from a T1-type response while enhancing their ability to express IL-10.

Whether the different mammalian ligand families, Jagged and Delta-like, signal for the same outcome in mature lymphocytes is not known. There is evidence from the developing hematopoietic system that these ligands do not have completely redundant activities, with Dl1 being able to completely inhibit B cell differentiation whereas Jagged1 has no effect. Furthermore, there is evidence that their signaling potential is modulated differentially by proteins such as fringe (2, 30). Previously, we performed experiments in which DCs expressing human Jagged1 and pulsed with peptide were able to inhibit responses to the soluble protein Der p 1 (17) and showed that the inhibition was mediated by a CD4+ population of cells. In those studies CD8+ cells capable of inhibiting responses in adoptive transfer experiments were not generated, but this may well be a function of the antigen used, rather than an inherent difference in the ability of the two different Notch ligands used to affect different populations of cells. Experiments are in progress to further compare the effects of signaling through the two families of ligands on the peripheral immune system.

The lack of an effect of the Dl1-expressing L cells on T cell responses to indirectly presented antigen could be due to the fact that Notch ligand on APCs is *cis*- and not *trans*-acting, or it may be that in the PLN, T cells with specificity for indirectly presented alloantigen would not come sufficiently close to the Dl1-transfected L cells to be affected by the presence of Notch ligand. Alternatively, it could be that inherent differences in the requirements for stimulation by direct and indirect pathways as suggested by others (31) affects the unresponsiveness induced by Notch ligation. These issues, and in particular whether Notch ligand on APCs is either *cis*- or *trans*-acting in this system are currently under investigation.

Despite the significant prolongation observed in transplants of all animals pretreated with K^b/Dl1 plus A^b/Dl1 cells, all animals eventually rejected their grafts. This was perhaps not surprising in view of the fact that our pretreatment regimen targeted only the direct pathway of antigen presentation, and it is clear from the work of others that indirect presentation can result in rapid graft rejection (26–28). We presented evidence that an inhibitory CD8+ cell is responsible for the prolonged graft survival observed since depletion of this population

at the time of grafting, a strategy used previously by others to demonstrate a similar phenomenon (32), completely abrogated the beneficial effects of the pretreatment protocol. There are many reports of CD8+ suppressor/inhibitory cells that operate through a variety of cell contact–dependent and independent mechanisms (33–39). In the work of others, CD8+ regulatory cells have been documented recently both in patients with antigen-specific unresponsiveness to influenza matrix peptide (40) and after culture of human T cells with CD40 ligand–activated plasmacytoid DCs (41). In the latter study the regulatory cells were shown to produce elevated levels of IL-10, but reduced levels of all other cytokines tested compared with CD8+ effector cells activated by monocyte-derived DCs. Our data provide further evidence implicating the activity of CD8+ cells in antigen-specific unresponsiveness in vivo, although our data do not define the cell lineage involved.

Our data do not exclude the possibility that a CD4+ cell is also involved in the beneficial effects of Notch ligand–transfected cells. Indeed, the requirement for the inclusion of MHC class II–expressing transfectants in the pretreatment protocol, taken together with our previous data (17), argues strongly in favor of the involvement of CD4+, MHC class II–reactive cells in collaboration with the CD8+ cells. There are many reports of CD4+ regulatory cells with a variety of phenotypic and functional characteristics (42–48), and it will be of interest to examine whether any of these cells are induced or expanded in the present system. Such cells, which include the naturally occurring CD4+CD25+ regulatory cells (44, 45) and those induced or expanded by experimental manipulation (42, 48–52), have been reported variably to depend upon cytokines, including IL-10, for their induction and/or function. The relationship between naturally occurring regulatory cells and those induced or expanded by experimental manipulation is poorly defined, but it is apparent for at least one cell type (i.e., IL-10–producing Tr1 cells) that they have different origins (53). The eventual loss of grafts in our experiments could be explained by the loss of the CD8+ (and putative CD4+) inhibitory populations since the activating signal for them (i.e., APCs of graft origin) is lost rapidly from the graft (54), and it is clear in other models of transplantation that inhibition or tolerance can be maintained only in the continued presence of antigen (55).

Epitope spreading, in which tolerance induced to one peptide of an antigen extends to the entire protein, is a common feature of many experimental models of tolerance. Similarly, in other experimental transplantation models, long-term engraftment or tolerance may be achieved with preoperative treatment regimens that include only single-donor alloantigens, suggesting that in these experiments spreading of tolerance can not only account for unresponsiveness to haplotypes recognized directly, but also to alloantigen recognized indirectly (56). Indeed, it is becoming clear that in long-term surviving recipients of allogeneic grafts, it is the

CD4^{+} T cells that recognize antigen indirectly that maintain the tolerant state (57, 58). These and other transplantation tolerance studies have in common a phase of nonspecific immunosuppression during the induction of unresponsiveness that will target all T cells whether they recognize alloantigen directly or indirectly. This is not the case in our experiments, and it is likely that nonspecific immunosuppression will result in the ablation and/or inactivation of T cells recognizing alloantigen directly and indirectly, allowing the emergence of a dominant population of regulatory cells. Since in long-term surviving grafts the primary stimulus will be to regulatory T cells recognizing alloantigen indirectly, it is not surprising that this population is maintained in such experiments. It has been difficult to raise inhibitory cells that regulate responses to directly presented alloantigen (31), but our data establish that this may indeed be possible.

Our understanding, as derived from developmental systems, indicates that the Notch-signaling pathway controls cell fate choices through influencing proliferation, differentiation, and death (1). While Notch may itself direct cell fate, more usually it acts by inhibiting differentiation along one pathway, thereby allowing the cell to be influenced by other cell extrinsic or intrinsic cues to differentiate down an alternate pathway. The data presented in this paper is consistent with the hypothesis that Notch signaling in mature CD8^{+} lymphocytes inhibits differentiation along a T1 program, thereby allowing them to mature into a T2/inhibitory population. The integration of Notch signaling with T cell receptor and costimulatory and cytokine-mediated signaling is obviously critical to the outcome of this process and the subject of further investigation in our laboratories.

We have demonstrated that delivery of cells coexpressing antigen and a Notch ligand of the Delta-like family can subvert, in an antigen-specific fashion, the normally powerful immune response to alloantigen, even when that antigen is delivered by a route that would otherwise be sensitizing. This inhibition is dependent on the presence of CD8^{+} cells at the time of antigen challenge. We have shown that animals pretreated with Dl1-expressing cells showed a very early inhibition of cell accumulation at the site of antigen challenge and that direct ligation of the Notch receptor on splenic CD8^{+} cells enhances the production of IL-10 while dramatically reducing the production of IFN-γ. These latter data suggest that Notch signaling can inhibit differentiation of mature CD8^{+} cells down a T1 pathway.

The ability to induce antigen-specific unresponsiveness without concomitant nonspecific immunosuppression is highly desirable and should not only be beneficial in the context of bone marrow and organ transplantation, but also in the context of diseases of autoimmune or allergic pathology.

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