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Instruction of Distinct CD4 T Helper Cell Fates by Different Notch Ligands on Antigen-Presenting Cells

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Summary

Antigen-presenting cells (APC) tailor immune responses to microbial encounters by stimulating differentiation of CD4 T cells into the Th1 and Th2 lineages. We demonstrate that APC use the Notch pathway to instruct T cell differentiation. Strikingly, of the two Notch ligand families, Delta induces Th1, while Jagged induces the alternate Th2 fate. Expression of these different Notch ligands on APC is induced by Th1- or Th2-promoting stimuli. Th2 differentiation has been considered a default process as APC-derived instructive signals are unknown. We demonstrate that Jagged constitutes an instructive signal for Th2 differentiation, which is independent of IL4/STAT6. Th2 differentiation induced by APC is abrogated in T cells lacking the Notch effector RBPJκ. Notch directs Th2 differentiation by inducing GATA3 and by directly regulating *il4* gene transcription through RBPJκ sites in a 3′ enhancer.

Introduction

Naive T cells differentiate into various effector lineages to orchestrate effective immune responses. The best characterized lineages are T helper1 (Th1) cells, producing γIFN, and T helper 2 (Th2) cells, producing IL4, IL5, and IL13 (Mosmann and Coffman, 1989). Th1 cells are essential for cellular immunity against intracellular pathogens. Th2 cells are crucial in humoral immunity and in defense against nematode parasitic infections. Aberrant responses result in autoimmune disorders such as EAE, arthritis, and diabetes (Th1) or in asthma and allergies (Th2) (Abbas et al., 1996; Murphy, 1998; O'Garra, 1998).

CD4 T cell differentiation is controlled by APC, which translate information about the microbial threat to the T cells. Dendritic cells (DC) recognizing DNA, RNA, or bacterial structures such as LPS promote Th1 differentiation (Kapsenberg, 2003). Conversely, recognition of parasitic nematode or fungal products, or cholera toxin, enables DC to induce strong Th2 responses (Kapsenberg, 2003).

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Signals from the surrounding tissue are also interpreted by APC to promote different types of T cell responses (Kalinski et al., 1999). DC from, e.g., the intestinal and bronchial mucosa skew responses toward Th2, whereas those isolated from spleen promote Th1 responses (Iwasaki and Kelsall, 1999; Stumbles et al., 1998). One such tissue signal is PGE2, a proinflammatory mediator that instructs DC to promote responses in Th2 and is produced constitutively in the intestine (Kalinski et al., 1999; Newberry et al., 2001).

The major APC factor driving Th1 differentiation is IL12 (Moser and Murphy, 2000), but Th1 responses still occur in IL12$^{-/-}$ mice (Jankovic et al., 2002). The APC-derived factors responsible for that residual Th1 activity are not clear. Little is known about APC-derived signals that induce Th2 responses nor whether such signals exist. It has been argued that Th2 differentiation is a default fate, which occurs in the absence of Th1-inducing stimuli (Moser and Murphy, 2000). However, IL12 deficiency does not necessarily yield Th2 responses, suggesting that Th2 instructive signals must exist (Jankovic et al., 2002).

IL4 is an important differentiation factor for Th2 cells (Murphy and Reiner, 2002) as shown for instance by the reduced Th2 responses in mice deficient for STAT6$^{-/-}$, a factor required for IL4 receptor signaling (Murphy and Reiner, 2002). This raises the paradox that IL4 is required for the generation of the cell type that is its major producer. As Th2 differentiation does not depend on IL4 produced by non-T cells (Schmitz et al., 1994), signals other than IL4 might initiate Th2 differentiation. IL4 produced by the developing Th2 cells may then serve to further promote differentiation and/or expansion of these cells. The existence of non-IL4 signals inducing Th2 differentiation is suggested by the finding that strong Th2 responses occur in STAT6$^{-/-}$ mice infected with parasites (Finkelman et al., 2000; Jankovic et al., 2000; Noben-Trauth et al., 1997). Similarly, DC incubated with parasite products, cholera toxin, or PGE2 induce IL4-independent Th2 responses (de Jong et al., 2002; d'Ostiani et al., 2000; Gagliardi et al., 2000; Jankovic et al., 2000). The nature of these Th2-promoting signals is not known.

Several transcription factors regulate Th2 differentiation, including STAT6, NFAT family members, c-Maf, and GATA3 (Murphy and Reiner, 2002). Expression of the latter two is Th2 specific and responsive to IL4 receptor signaling, which explains at least in part the requirement for IL4 Th2 generation. Among these, GATA3 is key, as its expression is sufficient for Th2 commitment (Zheng and Flavell, 1997).

Here we show that the Notch pathway regulates T helper differentiation. Notch, an evolutionarily conserved receptor involved in cell fate decisions (Artavanis-Tsakonas et al., 1999; Bray, 1998), is a heterodimer with an extracellular domain associated with a polypeptide containing a transmembrane region and a long intracellular tail. Upon binding ligand, the intracellular domain (ICD) is released from the membrane through proteolytic cleavages enabling cytoplasmic and nuclear

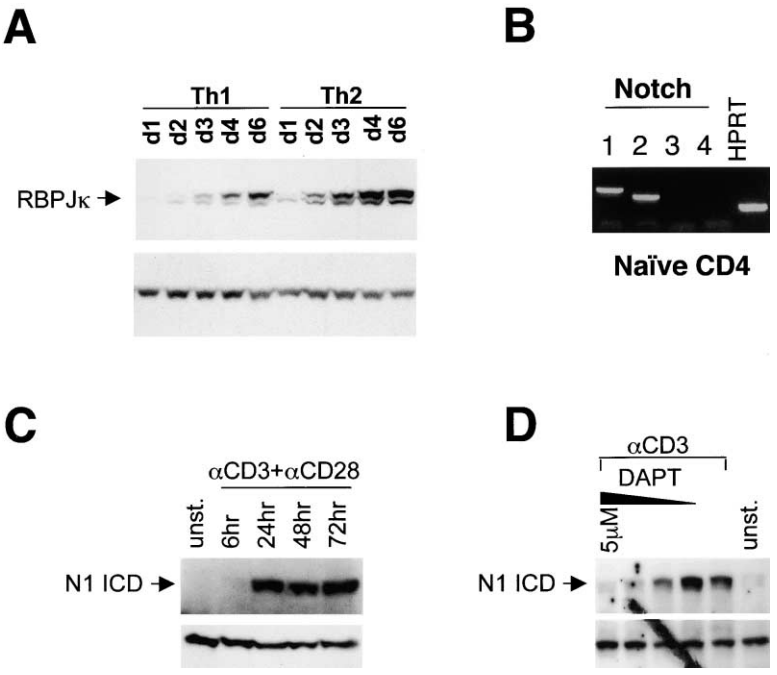

Figure 1. Expression of Molecules in the Notch Pathway and Activation of Notch in T Cells

(A) (top) Western blot for RBPJκ using 50 μg protein per lane of lysates from $CD4^+$ T cells stimulated in vitro for 1, 2, 3, 4, or 6 days with plate bound anti-CD3 and -CD28 under Th1- or Th2-skewing conditions. The blot reprobed for β-actin (bottom). Results are representative of three independent experiments.

(B) RT-PCR analysis of *notch1, 2, 3*, and *4* genes in cDNA from naïve CD4 T cells. Results are representative of two experiments.

(C) Western blot using an antibody specific for the activated intracellular tail of Notch1. Twenty microgram protein per lane was used of nuclear extracts from fresh CD4 T cells stimulated with plate bound antibodies to CD3 and CD28 for various times. The blot was reprobed for β-actin. Results are representative of four independent experiments.

(D) Western blot as in (C) using nuclear extracts from Th2 cells stimulated with plate bound anti-CD3 and -CD28 for 6 hr in the presence of 10-fold increasing amounts of the γ-secretase inhibitor DAPT (starting at 5 μM). DMSO used as vehicle for DAPT was present in all groups at a final concentration of 1%. A background band is shown as loading control (bottom). Results are representative of four independent experiments.

functions. Indeed, this ICD is constitutively active, and ICD expression phenocopies Notch signaling (Artavanis-Tsakonas et al., 1999).

A target of the ICD is RBPJκ (the mammalian homolog of Su(H), also known as CBF1 or CSL) (Jarriault et al., 1995; Tamura et al., 1995). ICD binding converts RBPJκ from a transcriptional repressor to an activator (Maillard et al., 2003). Although RBPJκ-independent activities of Notch are known, RBPJκ is a major effector of all four Notch receptors (Artavanis-Tsakonas et al., 1999; Kato et al., 1996).

Mammals express four Notch genes. Notch1 and 2 are closely related, while Notch3 and 4 are more divergent. In addition, five genes encode ligands for Notch from two conserved families, Jagged (Jagged1 and Jagged2) and Delta (Dlk1, Dlk3, Dlk4) (Maillard et al., 2003). Both types of ligands appear to induce the same type of signaling through Notch receptors (Shimizu et al., 2000a). Nonetheless, conservation of these families during invertebrate and vertebrate evolution suggests that they may perform distinct functions. The different ligands have specific and often nonoverlapping expression patterns (Lindsell et al., 1996) and are not always interchangeable (Huppert et al., 1997; Jaleco et al., 2001). The functional importance of Fringe, an enzyme that glycosylates Notch and thereby inhibits its binding to Jagged but not Delta, also suggests unique functions of these molecules (Justice and Jan, 2002).

Specific functions of the ligands may reflect different responses by different cell types selectively responding to one ligand but not the other. In contrast, we here show that one and the same cell type can generate distinct responses to ligands of the two families: Both ligands promote differentiation of naïve CD4 T cells into effector cells, but while Delta promotes Th1 responses, Jagged instructs naïve CD4 T cells to differentiate into the Th2 lineage.

Results

Expression of Molecules of the Notch Pathway

A comparison of the gene expression profiles of Th1 and Th2 cells revealed the DNA binding factor RBPJκ to be preferentially expressed in Th2 cells (Lu et al., 2004). Western blotting confirmed this preferential expression in Th2 effector cells, although some RBPJκ protein is present in Th1 effector cells (Figure 1A). RBPJκ is expressed at a low level in naïve CD4 T cells and its level increases rapidly upon activation of T cells (Lu et al., 2004; D.A. and R.A.F., unpublished data).

We hypothesized that Notch might regulate differentiation of effector T cells. Consistent with this, both *notch1* and *notch2* are expressed in naïve CD4 T cells (Figure 1B). Furthermore, Notch1 is activated in naïve $CD4^+$ T cells and in effector cells after T cell receptor crosslinking (Figures 1C and 1D) in a γ-secretase-dependent manner since the inhibitor DAPT (Dovey et al., 2001) inhibited Notch activation (Figure 1D).

Different Ligands for Notch Are Expressed on APC under Conditions that Promote Th1 or Th2 Differentiation

We considered it likely that Notch ligands might be expressed on APC. Splenic $CD11c^+$ cells were found previously to express Delta4 and low levels of Jagged2 and Delta1 (Tanigaki et al., 2002; Yamaguchi et al., 2002). Furthermore, Jagged1 is expressed on activated B cells (data not shown).

We found low levels of Jagged1 and Jagged2 mRNA on immature BMDC prior to stimulation (Figures 2A and

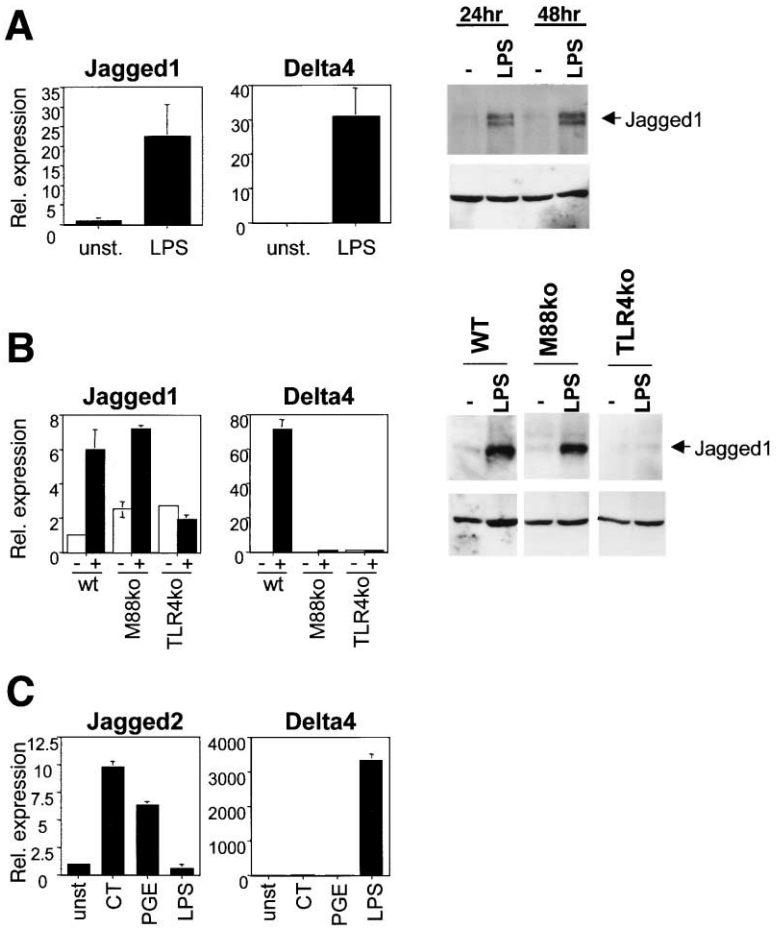

Figure 2. Expression of Notch Ligands on APCs

(A) Real-time quantitative RT PCR for Jagged1 (left) and Delta4 (middle) using RNA from day 5 BMDC, unstimulated (unst.) or treated for 6 hr with LPS. cDNA contents were normalized on basis of predetermined levels of β-actin. (Right) Western blot for Jagged1 using 50 μg protein per lane of lysates from BMDCs unstimulated (−) or stimulated for 24 and 48 hr with LPS. The blot was reprobed for β-actin. Results are representative of at least five independent experiments.

(B) Real-time quantitative RT PCR for Jagged1 (left) and Delta4 (middle) using RNA from wild-type, Myd88-deficient, and TLR4-deficient BMDCs without stimulation (−) or after 6 hr stimulation with LPS (50 ng/ml) (+). cDNA contents were normalized as in (A). Results are representative of two independent experiments. (Right) Western blot for Jagged1 using 50 μg protein per lane of lysates from wild-type, Myd88-deficient, or TLR4-deficient BMDCs without (−) or with LPS stimulation for 24 hr. The blot was reprobed for β-actin. Results are representative of five independent experiments.

(C) Real-time quantitative RT PCR for Jagged2 (left) and Delta4 (right) using RNA obtained from day 5 BMDCs, unstimulated (unst.) or treated for 6 hr with cholera toxin, PGE2, or LPS. cDNA contents were normalized as in (A). Results are representative of four independent experiments.

2C). Expression of Delta1, 3, and 4 was undetectable, although high levels were detected in brain cDNA (not shown). Interestingly, LPS stimulation rapidly induced Jagged1 and Delta4 mRNA (Figure 2A). LPS is a potent maturation stimulus for DC, allowing these cells to induce differentiation of CD4 T cells into effector cells. Although LPS strongly promotes Th1 responses, it also promotes Th2 responses (Eisenbarth et al., 2002). Th1 responses induced by LPS depend on the TLR adaptor Myd88, whereas a Myd88-independent pathway promotes Th2 differentiation (Kaisho et al., 2002; Schnare et al., 2001). Thus, LPS stimulation of DC from Myd88$^{-/-}$ mice enables these cells to induce Th2 but not Th1 responses (Kaisho et al., 2002). Interestingly, treatment of DC from Myd88$^{-/-}$ mice with LPS leads to normal Jagged1 expression (Figure 2B). Conversely, Delta4 expression is dependent on Myd88 (Figure 2B). Thus, expression of Jagged1 correlates with the ability of LPS to promote Th2 responses, while Delta4 expression correlates with the ability of LPS to promote Th1 responses.

PGE2 and cholera toxin cause DC to become Th2 inducers (Kapsenberg, 2003). Interestingly, Jagged2 is strongly responsive to PGE2 and cholera toxin (Figure 2C), while Jagged1, Delta1, Delta3, and Delta4 expression are barely affected (Figure 2C; not shown). Expression of the Jagged family is therefore associated with conditions that stimulate Th2 responses, while expression of a Delta member on DC was associated with Th1-promoting conditions.

We therefore hypothesized that the two families of Notch ligands might serve different roles in T helper differentiation. To test this, we used IE^{k}-expressing L cell lines expressing these ligands as APC to differentiate naïve AND TCR transgenic CD4 T cells. Surface Delta expression on APC strongly promoted generation of Th1 cells and reduced Th2 responses. In contrast, Jagged expressing L cell APC stimulated Th2 effector generation (Figure 3). Thus, while Jagged and Delta both effect T helper differentiation, the outcomes of their ligation on naïve CD4 T cells are diametrically opposite.

Induction of Th2 Differentiation by APCs Requires an Intact Notch Pathway

The major common pathway downstream of all Notch receptors involves RBPJκ. To delete RBPJκ expression in CD4 T cells, we crossed mice carrying floxed *rbpj*κ alleles (Tanigaki et al., 2002) with CD4-Cre transgenic mice (Wolfer et al., 2001). Thymic and peripheral T cells were normal as judged by expression of numerous surface markers (not shown).

We examined Th2 differentiation in RBPJκ-deficient T cells using LPS-treated (Jagged-expressing), Myd88-deficient BMDC as APC. As expected (Kaisho et al., 2002), these DC strongly stimulated the differentiation of IL4-producing effector CD4 T cells derived from wild-type (+/+) or mice heterozygous for the floxed *rbpj*κ allele (Figure 4B). In sharp contrast, very few IL4-produc-

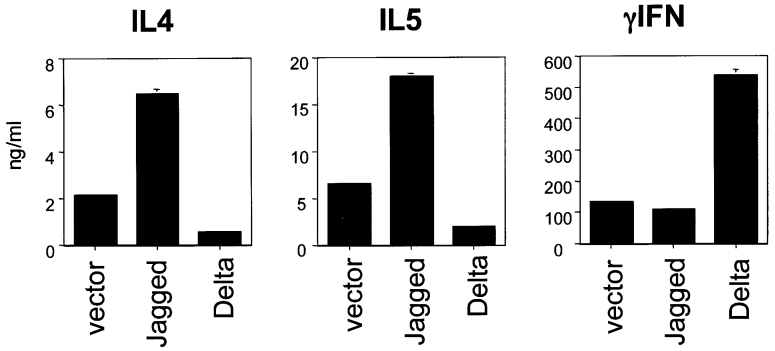

Figure 3. Different Notch Ligands Induce Opposite T Cell Fates

(A) Naïve CD4 T cells were isolated from AND TCR transgenic mice and cultured in vitro with IE^k+ control (vector), Jagged1-, or Delta1-expressing APC (DCEK hi7) pulsed with 0.1 μg/ml mcc peptide. After 5 days, viable T cells were restimulated with plate bound anti-CD3. Supernatants were taken after 48 hr and cytokine concentrations determined by ELISA. The results are representative of three independent experiments.

ing T cells emerged in cultures of RBPJκ-deficient cells (flox/−), and the intensity of the IL4 signal in the few positive cells was lower than in control cells (Figures 4B and 4C). Differentiation of IL4-producing cells was partially restored by addition of exogenous IL4, suggesting that IL4 may be downstream of RBPJκ. Thus, an intact Notch pathway in CD4 T cells is required for differentiation into the Th2 lineage in response to signals from DC.

In contrast, Th1 differentiation was not decreased in RBPJk-deficient T cells cultured either with LPS-treated Myd88$^{-/-}$ or (Delta-expressing) wild-type DC (Figures 4A and 4C) or T cell-depleted splenic APC (data not shown).

Notch-Induced Th2 Differentiation Is Independent of STAT6 and Leads to Expression of IL4 and GATA3

STAT6 is required for IL4-mediated Th2 differentiation. When T cells derived from STAT6-deficient mice were

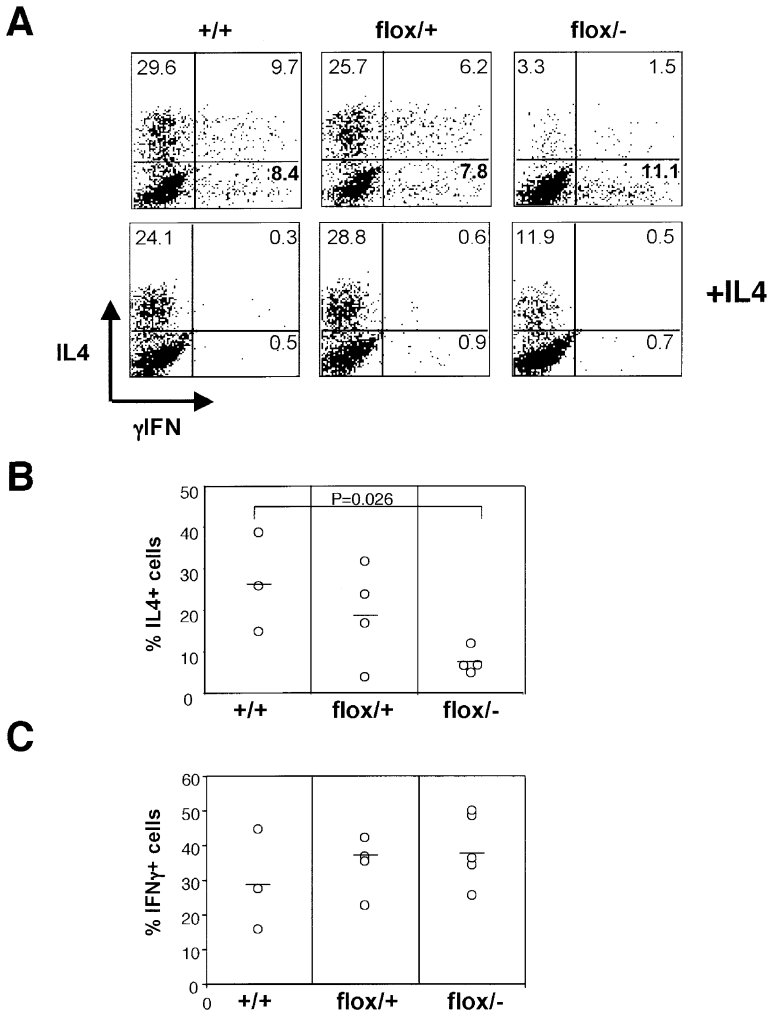

Figure 4. RBPJκ Is Necessary for Induction of Th2 Differentiation by DC

(A) CD4 T cells from RBPJκ$^{+/+}$, RBPJκ flox/+, and RBPJκ flox/− littermate mice (all Cre$^+$) were cultured with LPS pretreated BMDCs from Myd88$^{-/-}$ mice and 0.1 μg/ml anti-CD3. After 5 days, viable cells were restimulated with PMA and ionomycin and intracellular levels of IL4 and γIFN determined by flow cytometry. Similar numbers of viable cells were recovered from the different genotypes after 5 day differentiation. The results are representative of four independent experiments.

(B) The percentage IL4$^+$ cells from individual RBPJκ$^{+/+}$, RBPJκ flox/+, and RBPJκ flox/− mice (all Cre$^+$) after differentiation as in (A). Each mouse is represented by a single dot. The average value for each group is represented by a horizontal line. The difference between the RBPJκ$^{+/+}$ and the RBPJκ flox/− group was statistically significant ($p = 0.026$) as determined by Student's t test.

(C) Naive CD4 T cells as in (B) were cultured with wild-type BMDC stimulated with LPS in the presence of 0.1 μg/ml anti-CD3 and the percentage of γIFN producing effector cells determined by flow cytometry. The differences between the groups are not statistically significant.

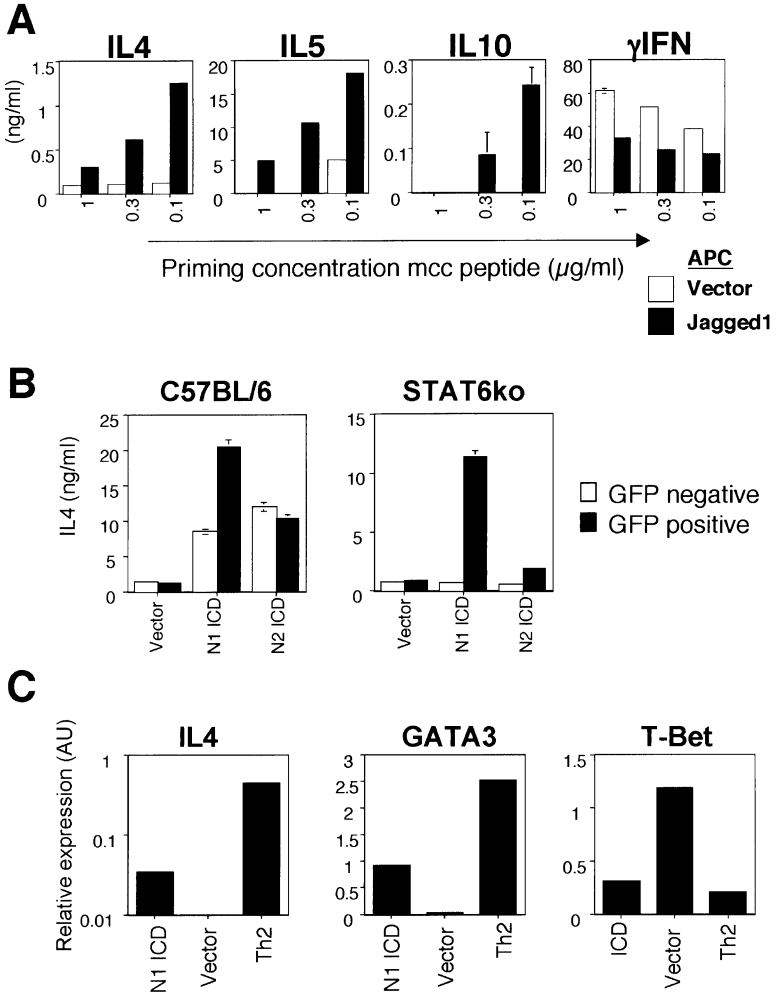

Figure 5. Notch Signaling Induces STAT6-Independent Production of IL4 and Expression of GATA3

(A) Naïve CD4 T cells from STAT6$^{-/-}$ AND TCR transgenic mice were cultured with IE^{k}+ control (vector) or Jagged1-expressing APC (DCEK hi7) pulsed with different concentrations of mcc peptide. After 5 days, viable T cells were harvested and equal numbers of cells per group restimulated with plate bound anti-CD3. Supernatants were taken after 48 hr and cytokine concentrations determined by ELISA. Results are representative of three independent experiments.

(B) Naïve CD4 T cells from C57BL/6 mice (left) or STAT6$^{-/-}$ mice (right) were activated and transduced with retrovirus encoding Notch1 or Notch2 ICD linked to GFP by an IRES or with control GFP virus. Seventy-two hours after transduction, viable GFP-positive and GFP-negative cells were separated and equal numbers of cells restimulated with plate bound anti-CD3. IL4 concentrations were determined by ELISA in 48 hr supernatants. Similar results were obtained using the MSCV Thy1.1 retroviral vector. Results are representative of over five experiments.

(C) CD4 T cells from STAT6$^{-/-}$ mice were activated and transduced with Notch1 ICD. Thirty-six hours after transduction, viable Thy1.1^{+} (over 90% pure) cells were isolated by MACS sorting and RNA was prepared. Also, RNA was prepared from day 5 Th2 effector cells. Relative expression of *il4* (left), *gata3*, and *t-bet* was determined by quantitative real-time RT PCR and expressed in arbitrary units (AU). cDNA contents were normalized on basis of predetermined levels of β-actin. Results are representative of three independent transduction experiments.

used, Jagged-expressing L cell APC still supported the development of Th2 effector cells, characterized by IL4, IL5, and IL10 production. (Figure 5A). Similarly, the induction of Th2 differentiation by Jagged-expressing APC was unaffected by the addition of anti-IL4 (not shown). Therefore, Th2 differentiation induced by Jagged is independent of IL4/STAT6.

To study the mechanism of Notch-mediated Th2 differentiation, we introduced an activated Notch1 (N1 ICD) allele into CD4 T cells, using retrovirus. This activated Notch allele promoted IL4 production in effector cells independently of STAT6 (Figure 5B) and IL4 (using anti-IL4—not shown). Expression of activated Notch resulted in reduced production of γIFN wild-type, but not in STAT6$^{-/-}$ T cells (not shown).

CD4 T cells lacking expression of only Notch1 are not defective at Th2 differentiation (D.A. and R.A.F., unpublished data). We reasoned that other Notch family members might perform the same function as Notch1, as redundancy was found in other differentiation processes (Krebs et al., 2003). Expression of Notch2 ICD also led to STAT6-independent generation of IL4-producing cells (Figure 5B). (Notch2 ICD expression, as measured by the intensity of the GFP marker, was 10-fold lower than that of Notch1, potentially explaining the lower efficacy at promoting IL4 production by Notch2 ICD—data not shown). We conclude that signaling through both Notch1 and Notch2 directly activates a Th2 differentiation program that bypasses the need for IL4 receptor signaling.

T helper differentiation depends on the relative expression of the transcriptional regulators GATA3 and T-Bet (Murphy and Reiner, 2002). We transduced STAT6$^{-/-}$ CD4 T cells with a retrovirus encoding Notch1 ICD and examined whether this would affect expression of these key regulatory genes. To minimize indirect effects, RNA was isolated from the cells within 36 hr after transduction and no effector restimulation was provided. Expression of GATA3 was greatly elevated in response to Notch1 ICD, whereas expression of T-Bet was reduced (Figure 5C). Ectopic expression of GATA3 is sufficient to induce Th2 differentiation in STAT6-deficient CD4^{+} T cells (Ouyang et al., 2000). Therefore, induction of GATA3 expression is one mechanism through which Notch instructs Th2 differentiation.

Notch Directly Regulates Transcription of the il4 Locus

Expression of the *il4* gene itself was elevated in T cells expressing Notch1 ICD without the need for restimula-

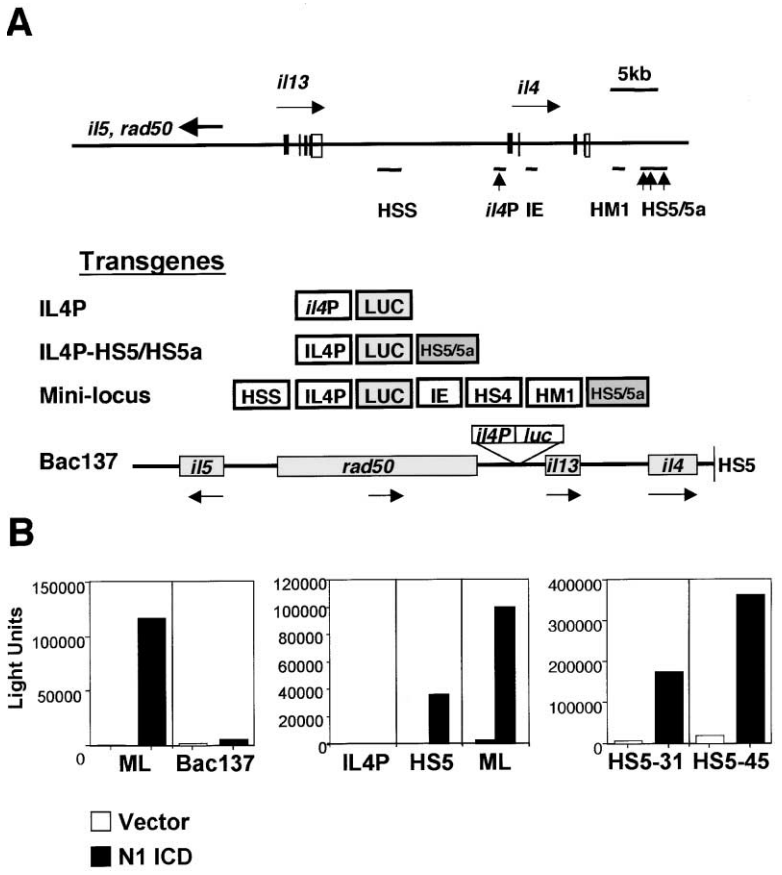

Figure 6. The *il4* Locus Is Notch Responsive

(A) Schematic of the *il4* locus (top) ranging from the *il13* gene to the *il4* gene. Positions of promoter (*il4*P) and enhancer elements are indicated underneath the locus. Potential RBPJκ sites are indicated by vertical arrows. (Bottom) Transgenic constructs used. The core of all transgenes consists of the *il4* promoter driving expression of luciferase. To this core was added the region encompassing HS5 and HS5a. A minilocus (ML) was made containing this core together with all proximal regulatory regions. A 120 kB Bac transgene (Bac137) was used in which *il4* promoter-luciferase was integrated into the *il4* locus. This Bac is truncated just downstream of HM1 on the 3′ end (thus lacking HS5).

(B) Transgenic mice for these constructs were tested for Notch responsiveness. CD4 T cells were isolated from Minilocus and Bac transgenic mice (left) and from *il4* promoter (*il4*P), *il4* promoter-HS5/5a, and minilocus (ML) (middle) transgenic mice. Cells were transduced with Notch1 ICD IRES GFP (N1 ICD) or control GFP virus (Vector). Neutralizing anti-IL4 was added to prevent autocrine effects from Notch ICD-stimulated IL4 production. Forty-eight hours after transduction, viable GFP-positive cells were isolated, lysed without restimulation, and luciferase measured. Results are representative of at least three independent experiments.

The same experiment was performed with two additional il4 promoter-HS5/5a founder lines to exclude integration effects. (Right) Transgenic founders were selected for responsiveness to TCR stimulation.

tion through the TCR (Figure 5C). To examine whether the *il4* gene is a direct target of Notch signaling, we searched for RBPJκ elements in the *il4* locus. We focused our search on the regions that were previously shown to regulate *il4* transcription (Lee et al., 2001; Mohrs et al., 2001). Three RBPJκ sites were found in HS5 (three elements), an enhancer region downstream of the *il4* coding region (vertical arrows—Figure 6A) (Agarwal et al., 2000), and one site in the *il4* promoter. The most 3′ RBPJκ element in HS5 lies within a highly conserved region between mouse and human and constitutes a conserved high-affinity RBPJκ element (Bailey and Posakony, 1995; Tun et al., 1994). The other two elements, while not conserved in that location, are an almost exact match to the tandem RBPJκ sites found in the *hes1* promoter (Jarriault et al., 1995).

To examine the function of these sites, we used transgenic mice in which a luciferase reporter is controlled by different regulatory elements of the *il4* gene (Figure 6A). Initially, we tested Notch responsiveness in T cells from mice, which carry a "minilocus" transgene, consisting of the luciferase reporter combined with all the proximal regulatory elements of the *il4* gene, including HS5. This minilocus has previously been shown to drive high level Th2-specific expression of luciferase (Lee et al., 2001). Retroviral expression of ICD in cells from these mice resulted in robust luciferase induction (Figure 6B). This response was observed in the absence of TCR restimulation, suggesting that Notch directly activated transcription of this transgene.

Because HS5 has RBPJκ sites, we tested Notch responsiveness of a 120 kB Bac reporter transgene, which encompasses the entire 5′ region of the *il4* locus but is truncated just upstream of (and therefore lacks) the HS5 enhancer element (Figure 6A). An integrated *il4* promoter-luciferase reporter allows quantitative measurement of the Th2-specific transcriptional activity of this Bac transgene. Expression of this Bac transgene lacking HS5 was not promoted by expression of Notch1 ICD (Figure 5B). Transcriptional activity of this transgene is induced by TCR crosslinking (Lee et al., 2003), demonstrating that the lack of Notch responsiveness of this Bac is not a consequence of position effect silencing. These results suggested that Notch responsiveness of the *il4* locus resides in HS5.

To test directly whether the HS5 region is Notch responsive, we introduced Notch1 ICD into CD4 T cells from transgenic mice carrying either the *il4* promoter-luciferase reporter (IL4P) or a transgene in which this core reporter has been combined with HS5 (IL4P-HS5/5a—Figure 6A). Reporter transgenes containing HS5 responded vigorously to Notch1 ICD in multiple transgenic founder lines, while the *il4* promoter alone did not respond (Figure 6B).

To determine whether this Notch responsiveness depends on the presence of the RBPJκ binding sites in HS5, we mutated each of the sites to perturb RBPJκ binding and made transgenic mice. None of the RBPJκ sites in HS5 overlap with NFκB sites as found elsewhere (Palmieri et al., 1999), such that the introduced muta-

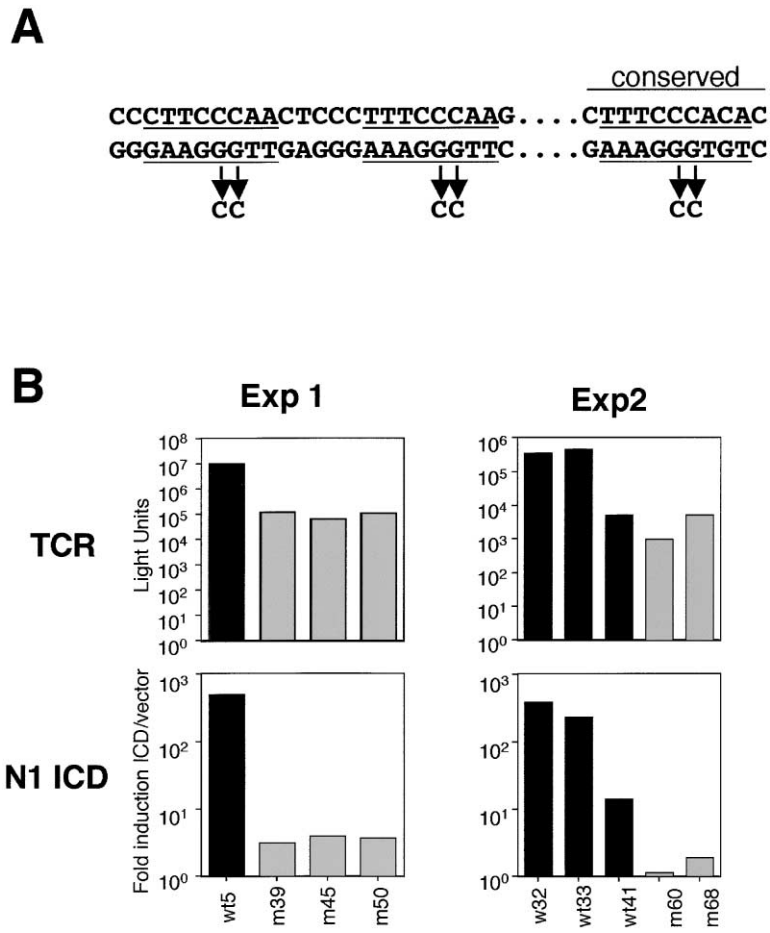

Figure 7. Notch Responsiveness of the *il4* Locus Depends on RBPJκ Elements Located in HS5

(A) Partial sequence of the HS5 regions containing RBPJκ binding sites (underlined). Indicated are the G residues essential for RBPJκ binding, which were mutated to C to disrupt binding.

(B) Transgenic mice were made with these mutated constructs and several individual founder lines for unmutated minilocus transgene (wt) or the mutated minilocus transgene (m) were tested for responsiveness to TCR stimulation. Effector CD4 T cells were stimulated with plate bound anti-CD3 for 16 hr (exp1) or 6 hr (exp2) (top). Also, cells from these mice were tested for responsiveness to Notch1 ICD (as in Figure 6) (bottom). Results are depicted as the ratio between the luciferase counts in the N1 ICD sample divided by the counts in the corresponding vector control sample. Two independent experiments (out of four) using different founder lines are shown. Transgenic founders were selected for responsiveness to TCR stimulation.

tions presumably affect RBPJκ binding only. Mutant minilocus constructs almost completely lost responsiveness to Notch in all five transgenic lines (Figure 7B). Although TCR responsiveness from the founders carrying mutated transgenes was consistently lower than that of founders carrying wild-type minilocus trangenes, a strong TCR response was measurable nonetheless (Figure 7B), excluding position effect silencing as a cause of Notch unresponsiveness. We conclude that the *il4* gene is a direct target of Notch signaling through RBPJκ sites in HS5.

Discussion

Different classes of microorganisms require different defense mechanisms. (Abbas et al., 1996; Murphy, 1998; O'Garra, 1998). Also, different tissues require distinct T cell responses (Kalinski et al., 1999). APC translate recognition of different microbial products or inflammatory mediators into specific signals instructing T cells to activate appropriate differentiation programs (Kapsenberg, 2003). Here we show that Notch ligands act as signals mediating this translation. Remarkably, while both classes of Notch ligands function as instructive differentiation signals, their effects on CD4 T cells are opposite.

Jagged Is an APC-Derived Signal for IL4-Independent Th2 Differentiation

Although Th2 differentiation has been regarded by some as a default pathway, several observations have suggested the existence of an IL4-independent instructive mechanism (see Introduction). The identity of the IL4 receptor-independent signals driving Th2 differentiation has been a mystery. Factors, such as OX40 ligand, ICOS ligand, and IL6 promote Th2 responses. However, these factors either promote Th1 responses as well (OX40, ICOS) or were shown to depend on IL4 for Th2 promotion (OX40, IL6)(Lane, 2000; Rincon et al., 1997; Sharpe and Freeman, 2002).

Jagged, on the other hand, seems to fit the criteria for a specific Th2 instructing signal on APC. Instruction of Th2 differentiation by Notch is independent of IL4 receptor signaling (Figures 5A and 5B), and expression of Jagged is induced on APC by environmental and microbial signals that predispose to Th2 responses, such as PGE2 and cholera toxin (Figure 2).

Perhaps counterintuitively, Jagged1 expression occurs in response to LPS, which is generally considered a Th1 inducer. Nonetheless, this finding is not inconsistent with a role for Jagged as a specific Th2 signal, as LPS in fact also promotes Th2 responses. The clearest evidence for this is the finding that LPS treatment of Myd88$^{-/-}$ DC enables these cells to induce Th2 differen-

tiation (Kaisho et al., 2002; D.A. and R.A.F., unpublished results), a process which we show to be Notch dependent (Figure 4). The function of Jagged induction by LPS in wild-type DC, which produce Th2-antagonizing IL12, is not clear. Indeed, even Th2 differentiation resulting from overexpression of N1 ICD is suppressed significantly by the addition of IL12 (D.A. and R.A.F., unpublished results). One possibility is that Jagged expression functions at later time points after LPS stimulation when DC no longer produce IL12 but still express Jagged (Figure 2A) and, indeed, promote Th2 differentiation (Langenkamp et al., 2000).

Delta Is an APC-Derived Th1 Differentiation Signal

We found that, in contrast to Jagged, Delta on APC stimulates Th1 responses (Figure 3). Expression of Delta4 correlated with the ability of DC to induce Th1 responses since LPS but not Th2 inducers stimulated expression of this ligand in wild-type but not MyD88-deficient DC (Figure 2). We cannot yet draw conclusions about the importance of Delta-mediated Th1 differentiation since RBPJκ deficiency did not result in reduced Th1 responses (Figure 4). However, a recent report using Delta Fc fusion proteins indicated an important role for Delta in Th1 induction under similar conditions (Maekawa et al., 2003). Although caution is warranted with the interpretation of experiments using soluble Notch ligands (Hicks et al., 2002), the combination of their and our data suggests that Delta uses an RBPJκ-independent pathway for promoting Th1 differentiation.

How Do Different Notch Ligands Evoke Distinct Responses?

How can Delta and Jagged evoke such drastically different responses in T cells? One possibility is that these different ligands preferentially activate different Notch receptors. Consistent with this, activated Notch1 and Notch2 alleles activate the Th2 differentiation program, while an activated Notch3 allele was reported to promote production of γIFN (Maekawa et al., 2003). Certainly, different Notch family members have distinct preferences for transcriptional regulatory elements (Beatus et al., 1999; Shimizu et al., 2002), which could lead to distinct effects on differentiation. Furthermore, Notch3 may antagonize Notch1-mediated transactivation (Beatus et al., 1999, 2001), but this is controversial (Shimizu et al., 2002). While Notch3 is not expressed in naïve T cells (Figure 1), expression occurs within 24 hr after T cell activation (Adler et al., 2003), consistent with a possible role in T cell differentiation.

Despite the conceptual appeal of differential Notch usage by Jagged or Delta, no clear preferences have been observed in experiments designed to test this (Shimizu et al., 1999, 2000b), and Notch3 ICD expression in transgenic mice was found to increase IL4 instead of γIFN production (Anastasi et al., 2003). Decisive experiments on this issue will require knock out studies.

An alternative possibility is differential signaling through the same Notch receptors. In fact, as in *Drosophila* only a single Notch gene is expressed, different responses to the ligands may involve signaling differences (Fleming et al., 1997; Justice and Jan, 2002; Panin et al., 1997). Currently available data show all ligands activate the same core Notch signaling pathway through multiple Notch members (Jarriault et al., 1998; Shimizu et al., 2000a). Differences may therefore lie in other pathways activated by Notch, perhaps not involving RBPJκ.

Molecular Mechanisms Used by Notch to Induce Th2 Differentiation

Notch promotes expression of GATA3; GATA3 is sufficient to induce Th2 differentiation even in STAT6-deficient cells by virtue of its ability to reorganize the *il4* locus and antagonize Th1-promoting pathways (Murphy and Reiner, 2002). Initial induction of GATA3 expression by activated Notch would probably be stabilized in wild-type T cells by an IL4/STAT6-responsive mechanism as well as by GATA3-mediated autoactivation (Ouyang et al., 2000). It has been speculated that GATA3 expression initially occurs stochastically, as a small proportion of $STAT6^{-/-}$ cells expresses this factor in differentiation cultures (Ouyang et al., 2000). It is conceivable, however, that such "spontaneous" expression results from Notch activation in only these cells, which happen to have interacted with Jagged-expressing APC present in the splenocyte populations used in such experiments.

How does Notch regulate expression of GATA3? One possibility is that Notch indirectly regulates expression of the *gata3* gene through NFκB. The p50 NFκB subunit is reportedly required for Th2 differentiation and for expression of GATA3 (Das et al., 2001), and expression of several NFκB members is reduced in transgenic mice expressing an antisense Notch (Cheng et al., 2001; Palaga et al., 2003). There is also potential for direct regulation of GATA3 expression by Notch since we have found a conserved RBPJκ site in the Th2-specific *gata3* promoter that was described recently (Asnagli et al., 2002).

In addition to increasing GATA3, Notch promotes Th2 differentiation by directly regulating transcription of the *il4* gene. Three RBPJκ sites (Bailey and Posakony, 1995; Tun et al., 1994) are present in the 3′ enhancer located in HS5. The enhancer located in HS5 is a strong activator of IL4 transcription, deletion of which leads to a pronounced reduction in IL4 expression (Solymar et al., 2002). We find that mutation of the RBPJκ sites in HS5 abrogates Notch responsiveness of a minilocus transgene, which contains all the proximal *il4* regulatory elements (Figure 7).

Our data do not allow us to conclude the stage at which Notch regulates IL4 transcription or its mechanism. Notch activation in naïve T cells may promote initial transcription of the *il4* gene. While high level expression of the *il4* gene develops only several days after the initial activation of naïve T cells, IL4 transcription does take place within just hours (Nakamura et al., 1997). This early IL4 production, promoted by Notch, could subsequently serve to augment the Th2 response in an auto/paracrine fashion. Notch ICD association with chromatin-modifying molecules such as p300 and PCAF (Maillard et al., 2003) may also help condition the *il4* locus for high level expression. Although the characteristic DNase 1 hypersensitive sites in the il4 locus are not altered in HS5-deficient mice (Solymar et al., 2002), the addition of exogenous IL4 in that study may have bypassed the requirement for this region under physiological conditions.

Finally, since TCR-mediated expression of the mutated minilocus is reduced in effector cells (Figure7B), Notch may also promote IL4 expression at later stages, a notion consistent with our demonstration that Notch is rapidly activated in effector cells (Figure 1D).

Alternative Differentiation Programs Activated by Jagged in T Cells?

Overexpression of Jagged was found to induce T cell tolerance in one study, although it is not clear that the observed effects were direct (Hoyne et al., 2000). In a more direct study, alloantigen presentation by Jagged-expressing B cell lymphoma cells led to the generation of effector T cells producing reduced IL4 and IL5 (Vigouroux et al., 2003). Instead, these cells produced TGFβ and inhibited activation of naïve T cells, suggesting a role for Jagged in generation of regulatory T cells. These results contrast with ours. In our hands, Notch activation by Jagged or by expression of an activated allele consistently resulted in enhanced production of IL4, the signature Th2 cytokine. We saw no elevated TGFβ production. Although Notch activation leads to enhanced production of IL10, a cytokine implicated in regulatory T cell function, this cytokine is also made by Th2 cells. Finally, we found that cells expressing Notch1 ICD did not express elevated levels of Foxp3, a master regulator of regulatory T cell differentiation (Ramsdell, 2003) (data not shown). While we have no definitive explanation for the discrepancies between that study and ours, it is possible that the effects of Jagged-mediated Notch engagement are dependent on surrounding signals.

Conclusion

Collectively, our data make a compelling case for the instruction of Th2 differentiation by Jagged-mediated Notch activation. We reach this conclusion on the basis of the expression characteristics of Jagged molecules and RBPJκ, gain- and loss-of-function experiments, and identification of Notch responsive molecular mechanisms driving Th2 differentiation.

On the other hand, our data reveal that Delta induces the diametrically opposite Th1 fate, showing that different Notch ligands can evoke different responses in a homogeneous group of precursor cells. The molecular mechanisms involved in Delta-mediated Th1 induction are not yet understood.

Experimental Procedures

Reagents and Antibodies

DAPT (Calbiochem), LPS from *Salmonella abortus equi*, cholera toxin, PGE2 (all from Sigma), anti-rat Jagged1 (AF599, R&D Systems), anti-Notch1 ICD (Cat# 2421, Cell Signaling Technology), anti-RBPJκ(T6709), anti-β-actin (sc-1616, Santa Cruz Biotechnology), anti-CD3 (145-2C11), anti-CD28 (37.1), anti-IL4 (11B11), anti-γIFN (XMG1.1), anti-Th1 (Y19) (all American Type Tissue Culture Collection, Manassas, Virginia), anti-Thy1.1 FITC, anti-CD44 Cychrome, anti-CD62L FITC, anti-NK1.1 PE (all Pharmingen), recombinant mouse IL4, recombinant mouse IL2 (both Pharmingen), recombinant mouse IL12 (a generous gift from Wyeth Research).

Mice

Five- to eight-week-old B6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and maintained in the Yale University Animal Resources Center. Bred in our colony under SPF conditions: STAT6$^{-/-}$ (Shimoda et al., 1996), AND TCR transgenic x STAT6 $^{-/-}$, RBPJκ$^{+/-}$ (Oka et al., 1995), RBPJκ flox/+ (Tanigaki et al., 2002), Myd88$^{-/-}$ (Adachi et al., 1998) TLR4$^{-/-}$ mice (Hoshino et al., 1999). RBPJκ flox/+ mice were bred with CD4-Cre transgenic mice (Wolfer et al., 2001). Offspring was bred with RBPJκ$^{+/-}$ mice.

RT-PCR

cDNA was generated from DNase1-treated (DNA free, Ambion) RNA (isolated using Trizol reagent, Invitrogen, Life Technologies).

Primers:

Notch1: TGTTAATGAGTGCATCTCCAA and CATTCGTAGCCAT CAATCTTGTCC

Notch2: TGGAGGTAAATGAATGCCAGAGC and TGTAGCGATTG ATGCCGTCC

Notch3: ACACTGGGAGTTCTCTGT and GTCTGCTGGCATGGG ATA

Notch4: CACCTCCTGCCATAACACCTTG and ACACAGTCATCT GGGTTCATCTCAC

HPRT:GTTGGATACAGGCCAGACTTTGTTG and GAGGGTAGGC TGGCCTATAGGCT

Primers and probes used for real-time PCR: β-actin primers (from S.W. Kim): GAAGTCCCTCACCCTCCCAA and GGCATGGACGCG ACCA, β-actin probe: 6-FAM-AGCCACCCCCACTCCTAAGAGG AGG-BHQ

Jagged1 primers: AGAAGTCAGAGTTCAGAGGCGTCC and AGT AGAAGGCTGTCACCAAGCAAC, Jagged1 probe: 6-FAM-CTGAAA AACAGAACAGATTTCCTGGT-BHQ

Jagged2 primers: AGCCACGGAGCAGTCATTTG and TCGGATT CCAGAGCAGATAGCG, Jagged2 probe: 6-FAM-TCAAGTGCCTCA GGGCACCACC-BHQ

Delta4 primers: AGGTGCCACTTCGGTTACACAG and CAATCAC ACACTCGTTCCTCTCTTC, Delta4 probe: 6-FAM-TCGGTTACACAG TGAGAAGCCAGA-BHQ

Primers and probes for GATA3, IL4, and T-Bet were described (Grogan et al., 2001).

Fluorogenic probes were obtained from Biosearch Technologies, Novato, California. Quantitative PCR was performed for 40 cycles using an icycler iQ (Bio-Rad, Hercules, California). Samples were normalized for β-actin contents. Concentrations were determined on basis of standard curves of plasmid DNA using software provided by the manufacturer.

Nuclear and Whole-Cell Extracts

Nuclear extracts were made by lysis in hypotonic lysis buffer (10 mM Hepes, pH 7.9, 3 mM $MgCl_2$ 10 mM NaCl, 0.1 mM EDTA, 300 mM Sucrose, 0.5 mM DTT, complete protease inhibitors [Roche]). After 10 min incubation on ice, 1/10th volume 1% NP40 solution was added followed by centrifugation, a wash in hypotonic buffer, and lysis in nuclear extract buffer (20 mM Hepes, pH 7.9, 3 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, protease inhibitors) on ice for 15 min.

Whole-cell extract buffer: 50 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, 1% TritonX 100.

Bone Marrow Dendritic Cell Generation

Bone marrow was isolated from tibia and femur. 1×10^6 bone marrow cells were cultured in RPMI, supplemented with 5% heat-inactivated FCS, 2-mercaptoethanol, glutamine, penicillin, streptomycin, and 2% L929 supernatant containing GMCSF. Fresh medium was added every two days. After 5 days, stimuli were added to the culture. Cells were harvested by gentle pipetting after 6 hr for mRNA determinations and 24 hr for Western blotting and use as APC. Concentrations of stimuli: 50 ng/ml of LPS, 1 μg/ml cholera toxin, 10^{-6} M Prostaglandin E2.

In Vitro T Cell Differentiation Experiments

CD62L^{hi}, CD44^{low}, NK1.1$^-$ CD4$^+$ T cells from spleen and peripheral lymph nodes were isolated by MACS sorting using anti-CD4 coupled beads and collums (Miltenyi Biotec) followed by FACS sorting. Cells were cultured in Bruff's medium (10% FCS, penicillin, streptomycin and L-glutamine).

Plates were coated with anti-CD3 and anti-CD28 (10 μg/ml) in PBS. DCEK hi7 IE^k-expressing fibroblasts were transduced with retrovirus encoding IRES GFP-linked human Jagged1 or Delta1 (gift

from Dr. Parreira; Jaleco et al., 2001). IE^k and CD80 levels were similar on all lines. Lines were made twice and gave similar results. 2.5×10^5 DCEKhi7 cells (treated for 1 hr at 37°C with 100 μg/ml mitomycin C, Sigma) were incubated with 2×10^5 naïve AND CD4 T cells (24 well plates, Falcon) and the 88–103 moth cytochrome C peptide.

Skewed effector cells: CD4 T cells were stimulated with soluble antibodies to CD3 and CD28 (1 μg/ml) and 4×10^6 irradiated (2000 Gray) splenocytes (T cell depleted using anti-Thy1 and rabbit complement) and 10 U/ml recombinant mouse IL2 and (Th1) 3.5 ng/ml rIL12 and 10 μg/ml anti-IL4 or (Th2) 500 U/ml rIL4 and 10 μg/ml anti-γIFN. Effectors were used after 5 days of culture, or as indicated in the figure legends. Viable effector cells were isolated using ficoll (LSMOL Lymphocyte Separation Medium, Cappel) and restimulated at 1×10^5 cells per well (96 well flat bottom plate, Falcon) with plate bound anti-CD3. Cytokine concentrations (48 hr supernatants) were determined by ELISA (Pharmingen).

Luciferase assay

Lysate from 2.5×10^4 cell equivalents was assayed with the Promega luciferase assay substrate. Luciferase activity was measured by Lumat LB9507 luminometer (EG&G Wallac).

Transgenic Mice

pGL3-IL4P, pGL3-IL4P-HS5, and the minilocus were described previously (Lee et al., 2001). pGL3-IL4P-HS5 was mutated using the Quick Change Site Directed Mutagenesis Kit (Stratagene). Initial mutagenesis used the following oligos: CTCCTCACGATCACTTTG GCACACAGGGGAGGGAG and CTCCCTCCCCTGTGTGCCAAAGT GATCGTGAGGAG. The mutant product was subsequently mutagenized using these oligos: CTCTCCCCTCCCTTGCCAACTCCCTTTGG CAAGCCCTAAATAAAC and GTTTATTTAGGGCTTGCCAAAGGGA GTTGGCAAGGGAGGGGAGAG. The entire HS5 region was sequenced and a Sal1/Kpn1 fragment containing the mutagenized HS5 region was cloned together with a Kpn1/Sal1 fragment containing HM1 and HM4 into the Sal1 site of pGL3-IL4P-HSS-IE to create the mutated minilocus. For transgenesis, bacterial sequences were removed by partial digestion with Mlu1 and Sal1. Transgenesis was as described previously (Lee et al., 2001).

Retroviral Transductions

A BglII/Xho1 fragment encoding the entire intracellular tail of human Notch1 starting at amino acid 1701 was cloned into the BglII, Xho1 sites of pIRES-GFP-RV (Ouyang et al., 2000) as well as into the BglII/Sal1 sites of MSCV-Thy1.1 (Hildeman et al., 2002). The entire intracellular tail of human Notch2 was PCR amplified using the following primers: GATCAGGGATCCATGGCAAAACGAAAGCGTAAGC and GATCAGGGATCCTCACGCATAAACCTGCATGTTG. The PCR product was cloned into the BglII site of pIRES-GFP-RV and the BamH1 site of MSCV-Thy1.1.

CD4 T cells were stimulated with irradiated T-depleted splenic APC, 5 μg/ml anti-CD3, 2 μg/ml anti-CD28, and 50 U/ml rIL2 and transduced as described (Lee et al., 2001). Viable GFP-positive and -negative cells were separated by FACS. Viable Thy1.1-positive cells were isolated by ficoll gradient followed by MACS. Cells were stained with anti-Thy1.1 FITC (Pharmingen) followed by anti-FITC beads (Miltenyi). The Phoenix-ECO packaging cell was a gift of Dr. G. Nolan (Stanford University, Palo Alto, California).

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Note Added in Proof

A role for RBPJk in T helper differentiation has also recently been described by Dr. T. Honjo's group:

Tanigaki, K., Tsuji, M., Yamamoto, N., Han, H., Tsukada, J., Inoue, H., Kubo, M., and Honjo, T. (2004). Regulation of $\alpha\beta/\gamma\delta$ T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. Immunity *20*, in press.