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Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals

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Abstract

Two models are proposed to explain Notch function during helper T (Th) cell differentiation. One argues that Notch instructs one Th cell fate over the other, whereas the other posits that Notch function is dictated by cytokines. Here we provide a detailed mechanistic study investigating the role of Notch in orchestrating Th cell differentiation. Notch neither instructed Th cell differentiation nor did cytokines direct Notch activity, but instead, Notch simultaneously regulated the Th1, Th2, and Th17 cell genetic programs independently of cytokine signals. In addition to regulating these programs in both polarized and non-polarized Th cells, we identified *Ifng* as a direct Notch target. Notch bound the *Ifng* CNS-22 enhancer, where it synergized with Tbet at the promoter. Thus, Notch acts as an unbiased amplifier of Th cell differentiation. Our data provide a paradigm for Notch in hematopoiesis, with Notch simultaneously orchestrating multiple lineage programs, rather than restricting alternate outcomes.

Naïve CD4⁺ T cells are responsible for controlling both intracellular and extracellular infections. Although developmentally mature, naïve CD4⁺ T cells require activation in order to adopt one of several effector programs, including: the interferon- γ (IFN- γ) producing T helper 1 (Th1) cell, the interleukin-4 (IL-4) producing T helper 2 (Th2) cell, and the interleukin-17 (IL-17) producing T helper 17 (Th17) cell. These three Th subsets serve different functions. Th1 cells are necessary to combat intracellular pathogens and mediate autoimmune diseases, such as graft-versus-host disease (GVHD). Th2 cells are essential effectors during parasitic helminth infection and also mediate airway hypersensitivity and allergic inflammation. Th17 cells are critical for controlling extracellular bacterial and fungal infections and are also responsible for autoimmunity (Coghill et al., 2011).

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The T helper cell program adopted by a naïve CD4⁺ T cell is instructed both by extracellular molecules, such as cytokines, and intracellular molecules, such as the Th1, Th2, and Th17 cell transcription factors, Tbet, Gata3, and Ror γ t respectively. Notch has also been proposed to mediate Th cell differentiation, where it functions to relay intercellular signals from the membrane to the nucleus in order to instruct Th cell differentiation (Amsen et al., 2009).

Notch signaling initiates when a Notch ligand interacts with a Notch receptor leading to a series of proteolytic cleavages that release the Notch intracellular domain (ICN) from the cell membrane; whereupon it translocates to the nucleus and forms a transcriptional activation complex with the transcription factor RBPJ and a member of the Mastermind-like (MAML) family (Kopan and Ilagan, 2009). Compelling cases have been made for Notch involvement in both Th1 and Th2 cell differentiation. Manipulating Notch ligand mediated stimulation of CD4⁺ T cells preferentially instructed Th1 or Th2 cell programs, suggesting that individual Notch ligands have different instructive capacities (Amsen et al., 2004; Maekawa et al., 2003; Okamoto et al., 2009).

Loss of function studies also demonstrated that Notch instructed the Th1 cell program *in vitro* and promoted the CD4⁺ T cell IFN γ response in a murine GVHD model (Minter et al., 2005; Skokos and Nussenzweig, 2007; Zhang et al., 2011). In contrast, other reports showed that Notch was required to instruct the Th2 but not the Th1 cell program (Amsen et al., 2009; Amsen et al., 2004; Fang et al., 2007; Kubo, 2007; Tu et al., 2005). More recently, Notch was found to regulate the Th17 cell signature genes *Il17a* and *Rorc*, suggesting the bi-potential instructional model may not be sufficient to explain Notch function in Th cell differentiation (Keerthivasan et al., 2011; Mukherjee et al., 2009). While the instructional model posits that ligands direct Notch function during Th cell differentiation, an alternative model argues that Notch target gene selectivity is dictated by upstream cytokine signals (Ong et al., 2008). Despite the differences between these models, both contend that Notch has the capacity to discriminately activate different Th cell programs. Thus, the paradox remains: how can such a basic signaling module selectively drive the differentiation of multiple distinct lineages?

In order to address these controversies, we investigated the molecular mechanisms by which Notch orchestrates Th cell differentiation. We find that Notch neither initiates a single helper T cell program nor do cytokine signals dictate the outcome of Notch signaling. Instead, Notch simultaneously regulates the Th1, Th2, and Th17 genetic programs independently of cytokine signals. Even under strong polarizing conditions, Notch directly regulates critical effectors of Th1 Th2, and Th17 cell differentiation. In addition to *Il4*, *Tbx21*, *Gata3-1a*, *Il17a*, and *Rorc*, we identify *Ifng* as a direct Notch target. Notch regulates *Ifng* by binding to a highly conserved RBPJ motif in the *Ifng* CNS-22 and synergizes with Tbet activity at the *Ifng* promoter. These data support a model in which Notch integrates and amplifies cytokine-derived signals, instead of acting as a transcriptional driver or a downstream accessory of cytokines. Not only do our data unify the disparate data on Notch and Th cell differentiation but they also offer an alternative view of Notch function in the hematopoietic system, whereby Notch reinforces multiple fates rather than restricting alternate outcomes.

Results

Notch signaling is dispensable for Th2 cell initiation during *Trichuris muris* infection

We previously showed that CD4⁺ T cells expressing the pan-Notch inhibitor dominant negative mastermind (DNMAML), which binds the Notch:RBPJ dimer but fails to transactivate, do not mount an effective Th2 cell response against the intestinal helminth *Trichuris muris* and fail to clear infection with normal kinetics (Tu et al., 2005). The

outcome of *T. muris* infection depends on the balance of Th1 cells, which are responsible for chronic infection, and Th2 cells, which are required for parasite expulsion and resistance to infection (Artis et al., 2002; Blackwell and Else, 2001; Cliffe and Grecis, 2004; Cliffe et al., 2005; Else et al., 1994). While Notch was necessary for optimal Th2 cell-dependent immunity in this infection model, it remained unclear whether Notch was essential to initiate Th2 cell differentiation or instead, was required to generate the optimal balance of Th1 and Th2 cells. To test this, *Cd4-Cre* (CC) and *Cd4-Cre* x *DNMAML^{FL/FL}* (CCD) mice were infected with *T. muris* and CCD mice were treated with neutralizing anti-IFN γ mAbs for the duration of infection. If Notch were required to initiate Th2 cell differentiation, anti-IFN γ treated CCD mice should remain susceptible to *T. muris* infection. Alternatively, if Notch played a greater role in generating an optimal Th2 cell response, then IFN γ blockade should be sufficient to relieve any inhibitory effects of a suboptimal Th1:Th2 ratio on infection-induced Th2 differentiation.

As expected, mesenteric lymph node cells from the control CC mice displayed robust IL-4, IL-5 and IL-13 responses upon restimulation (Figure 1A). Consistent with our previous findings, CCD mice demonstrated impaired Th2 cell cytokine responses (Figure 1A). In contrast, CCD mice receiving anti-IFN γ mAb treatment restored IL-4, IL-5, and IL-13 production and diminished IFN γ production, suggesting that Notch was not required to initiate Th2 responses (Figure 1A). In agreement with the cytokine data, CCD mice displayed an impaired protective *T. muris* specific IgG1 response and an elevated non-protective *T. muris* specific IgG2c response. In contrast, anti-IFN γ mAb treated CCD mice recovered parasite specific IgG1 and showed a trend towards decreased IgG2c (Figure 1B). Similarly, while CCD mice displayed decreased serum IgE, anti-IFN γ mAb treatment restored this response (Figure 1C). Furthermore, histologic analysis of intestinal sections revealed that anti-IFN γ mAb treatment rescued the goblet cell mucin response in CCD mice (Figure 1D). Finally, anti-IFN γ mAb treatment restored the ability of CCD mice to expel parasites with kinetics comparable to infected CC control mice by 21 days post infection (Figure 1E). Collectively, these data demonstrate that Notch is not essential to initiate Th2 cell responses *in vivo*, and instead suggest that Notch functions to optimize the response.

Persistent Notch signaling is required to maintain the Th1 and Th2 cell programs

To further test whether Notch is required to maintain an optimal Th2 cell response, we investigated the effect of inhibiting Notch subsequent to Th2 cell differentiation. For these studies, we developed an *in vitro* differentiation system in which Notch could be inhibited at different times by addition of a gamma secretase inhibitor (GSI) following activation of CD4⁺ T cells. This system also provided the opportunity to test the requirement for Notch in Th1 cell differentiation. Importantly, Notch inhibition by GSI did not affect T cell activation, proliferation, or cell numbers, even after prolonged exposure (Figure S1A–C), in contrast to a recent report (Helbig et al., 2012). To look at Notch specific effects and exclude autocrine cytokine effects, cells were cultured in the presence of neutralizing IL-4 and IFN γ antibodies.

To test whether Notch was required post-initiation to maintain both the Th1 and Th2 cell programs, naïve CD4⁺ T cells were activated in the presence of irradiated splenocytes. After 5 days, cells were restimulated and treated with either DMSO or GSI and left in culture for an additional 2 days (day 7) before harvest. When looking at Th1 cell signature genes, both mRNA and protein for Tbet and IFN γ were significantly lower after 2 days of GSI treatment (Figure 2A–C). Similarly, GSI decreased mRNA and protein expression of the Th2 signature genes, *Gata3* and *Il4* (Figure 2A–C).

While our data show that persistent Notch signaling is required to maintain the Th1 and Th2 cell programs post-initiation, it remained unclear whether the activity of Notch on Th1 and

Th2 cell targets was restricted to early patterning events or if Notch was capable of re-activating target genes late after T cell stimulation. To address this, naïve CD4⁺ T cells were activated in the presence of either DMSO or GSI, under neutralizing conditions. After 5 days, cells were washed, restimulated, and returned to either DMSO or GSI to test whether cells previously treated with GSI could recover cytokine and transcription factor expression. While cells treated with GSI for all 7 days of culture showed impaired Tbet and IFN γ induction, cells treated with GSI only for the first round of stimulation recovered Tbet and IFN γ responses (Figure 2D–F). Similarly, IL-4 and GATA3 expression decreased when cells were treated with GSI for all 7 days and recovered when GSI was removed (Figure 2D–F). Our findings indicate that Notch is capable of promoting Th1 and Th2 signature gene expression both at early and late time points following stimulation and that inhibiting Notch at either the beginning of T cell stimulation or at later time points represses both Th1 and Th2 cell differentiation. Furthermore, we repeatedly observed that expression of both Th1 and Th2 cell program genes and proteins were suppressed in the same population upon Notch inhibition, suggesting that Notch concurrently regulates both programs. Collectively, these data show that Notch functions in Th cell differentiation do not require upstream signals from polarizing cytokines and demonstrate that the activity of Notch on its targets is not kinetically restricted.

Notch concurrently regulates both the Th1 and Th2 programs

Although both Th1 and Th2 cell signature genes were sensitive to GSI, it was important to show that the effects were Notch-specific as GSI has Notch-independent effects. Furthermore, Notch-independent, Presenilin-dependent effects on cytokine production were reported for both Th1 and Th2 cell types (Ong et al., 2008). To test whether the changes we observed were Notch-specific, we utilized mice containing two floxed alleles of dominant negative mastermind (DNMAML^{FL/FL}), a potent and specific GFP-tagged pan-Notch inhibitor.

Tat-Cre treated YFP^{FL/FL} and DNMAML^{FL/FL} naïve CD4⁺ T cells were stimulated under neutralizing conditions. Use of the *Tat*-Cre peptide to acutely induce DNMAML expression minimized potential compensation for Notch signaling by other pathways. As expected, YFP⁺ T cells produced IL-4 and IFN γ (Figure 3A). Consistent with observations using GSI treatment, DNMAML expressing T cells displayed a marked reduction in the fraction of cells that produced IL-4 and IFN γ confirming that Notch regulates both IL-4 and IFN γ production in the same cell population (Figure 3A). In contrast to the GSI data, analysis of Tbet and GATA3 protein in DNMAML expressing cells revealed reduced expression of Tbet, but not GATA3 (Figure 3B). Notch had been shown to regulate *Tbx21* in a GSI-dependent manner and directly regulate *Il4* via a 3' enhancer; however, the GATA3 result was unexpected as Notch directly regulates *Gata3* transcription by binding to the *Gata3-1a* promoter in primary CD4⁺ T cells (Amsen et al., 2007; Amsen et al., 2004; Fang et al., 2007; Minter et al., 2005). When transcripts for both Th1 and Th2 signature genes were analyzed by qPCR, all four (*Il4*, *Gata3-1a*, *Ifng* and *Tbx21*) were expressed at lower levels in DNMAML expressing cells (Figure 3C). The observed differences in GATA3 protein expression between GSI and DNMAML treatment were likely due to the finding that GSI suppressed transcripts from both *Gata3-1a* and *Gata3-1b*, whereas DNMAML only suppressed the *Gata3-1a* transcript, which accounts for a minor fraction of total *Gata3* transcripts (Yu et al., 2009) (Figure S2A–B). These data indicate that Notch likely exerts its primary effect on the Th2 program through its regulation of *Il4*. It is important to note that while DNMAML expression had no impact on cell proliferation (Figure S2C), we did observe a minor increase in apoptosis as measured by Annexin-V (Figure S2D). While this subtle increase cannot account for the marked reduction in cytokine transcript and protein

observed in DNAM1L expressing cells, it suggests that a role for Notch signaling in cell survival may also contribute to these defects.

Our observation that both Th1 and Th2 cell signature genes were sensitive to Notch inhibition in the same population suggested that Notch does not instruct one program over the other, but instead acts as a global regulator. This raised the possibility that Notch could influence both Th1 and Th2 cell signature genes, even in strongly polarizing conditions. In order to assay direct Notch effects, we utilized the GSI-washout assay (Weng et al., 2006). Notch targets are identified as transcripts that demonstrate GSI sensitivity under mock wash conditions and recover upon washout in the presence of cycloheximide.

Naïve CD4⁺ T cells were activated as described and cultured under strong Th1 or Th2 cell polarizing conditions. As published, both *Ii4* and *Gata3-1a* behaved as direct Notch targets under Th2 cell polarizing conditions (Amsen et al., 2009; Amsen et al., 2004; Fang et al., 2007) (Figure 3D). Unexpectedly, both of these genes behaved as direct Notch targets under Th1 cell polarizing conditions, although the magnitude of their expression was greatly reduced, likely due to suppression by Th1 cell culture conditions (Figure 3D). Consistent with others (Minter et al., 2005), we identified *Tbx21* as a direct Notch target in Th1 cell conditioned cells, but surprisingly also observed that *Tbx21* behaved as a direct Notch target even under Th2 cell polarizing conditions (Figure 3D). To confirm the unbiased behavior of Notch under polarizing conditions, chromatin immunoprecipitation (ChIP) assays were performed. Consistent with GSI-washout results, Notch1 bound the previously reported *Ii4* HS-V, *Gata3-1a*, and *Tbx21* Notch1 binding sites (Figure 3E). The magnitude of Notch binding was similar in both Th1 and Th2 cell polarizing conditions suggesting that the polarizing conditions do not bias Notch binding to these critical Th1 and Th2 cell targets. This also shows endogenous Notch1 binds *Tbx21* in primary cells, confirming previous studies (Minter et al., 2005). Together with GSI-washout data, our ChIP findings confirm that Notch concurrently regulates both Th1 and Th2 programs, independent of cytokine signals.

Notch simultaneously orchestrates multiple Th cell programs by sensitizing cells to exogenous cytokine

Having observed that Notch regulates both Th1 and Th2 cell programs, we hypothesized that Notch could regulate these targets in other Th cell populations, such as Th17 cells. To test this, naïve CD4⁺ T cells were activated as described, cultured under strong Th17 cell differentiating conditions, and subjected to the GSI-washout assay. Consistent with Th1 and Th2 cell observations, *Ii4* and *Tbx21* both behaved as direct Notch targets in Th17 polarized cells (Figure 4A); *Gata3-1a* transcript was undetectable under these conditions (data not shown). To test if Notch1 binding was conserved between Th1, Th2, and Th17 cells, ChIP analysis was performed. In keeping with our GSI washout observations, Notch1 bound to the *Ii4*, *Gata3-1a*, and *Tbx21* loci in Th17 polarized cells, demonstrating that even though *Gata3-1a* was expressed at levels below the limit of detection, Notch still occupied this region. These data illustrate that the concurrent regulation of Th cell programs by Notch is not a unique feature of Th1 and Th2 cells, but rather a conserved function of Notch signaling in mature, activated CD4⁺ T cells.

To further demonstrate that Notch functions to regulate multiple cell programs from a common progenitor, we assayed whether the Th17 cell signature genes *Ii17a* and *Rorc* behaved as direct Notch targets, regardless of the cytokine environment. Naïve CD4⁺ T cells were activated as described, cultured under Th1, Th2, or Th17 cell conditions, and subjected to the GSI washout assay. Although the expression varied by condition, *Ii17a* and *Rorc* both behaved as direct Notch targets in all three Th cell types (Figure 4C).

To confirm that the observed effects on Th17 cell signature gene transcription were biologically significant, we expressed DNAM1L in naïve CD4⁺ T cells using Tat-Cre, as described, and activated them under Th17 differentiating conditions. We observed at day 3 that DNAM1L expression resulted in a marked reduction in the frequency of IL-17A producing cells (Figure 4D).

In light of reports showing that exogenous cytokine has the capacity to rescue cytokine production in Notch loss of function models (Amsen et al., 2004; Tu et al., 2005), we hypothesized that an important function of Notch was to sensitize CD4⁺ T cells to polarizing factors, especially when these factors may be limiting. To test this, CD4⁺ T cells were activated in the presence of serially diluted Th1, Th2, or Th17 polarizing cytokines, with media containing either DMSO or GSI. If Notch played a role in sensitizing cells to exogenous cytokine, we would predict that Notch inhibition would minimally affect differentiation at high levels of differentiating factors, but exert a negative effect on differentiation when exogenous cytokines were diluted. When cells were cultured in the presence of DMSO, secretion of IFN γ , IL-4, and IL-17A displayed an expected dose dependent response to polarizing cytokine, with maximal cytokine secretion observed in undiluted differentiating media (Figure 4E). Consistent with our hypothesis, when cells were cultured with GSI, cells displayed similar levels of cytokine secretion to DMSO control cells when differentiating cytokine was undiluted, but a much more rapid and marked decay in cytokine secretion when differentiating factors became limiting (Figure 4E). Altogether, these findings demonstrate that Notch simultaneously orchestrates multiple Th cell programs by sensitizing cells to exogenous differentiating factors.

Notch directly regulates IFN γ expression, independently of Tbet

As Notch regulates a *Gata3:Il4* axis in Th2 cells and an *Il17a:Rorc* axis in Th17 cells, we hypothesized that Notch might similarly regulate a *Tbx21:Ifng* axis in Th1 cells. To confirm that *Ifng* is a direct Notch target, we performed a GSI washout assay on cells cultured under strong Th1, Th2, and Th17 polarizing conditions. Similar to *Gata3-1a*, *Il4*, and *Tbx21*, *Ifng* behaved like a direct Notch target under all three culture conditions (Figure 5A).

To exclude the possibility that effects of Notch on *Ifng* resulted from indirect effects on *Tbx21*, we inhibited Notch signaling in *Tbx21*^{-/-} cells. Naïve CD4⁺ T cells from WT and *Tbx21*^{-/-} mice were activated and retrovirally transduced with vector control (MigR1) or DNAM1L. To obtain sufficient IFN γ expression in Tbet deficient cells, cells were cultured in the presence of IL-12 and neutralizing anti-IFN γ and anti-IL-4 antibodies, conditions that allow Tbet-independent IFN γ production (Schulz et al., 2009; Usui et al., 2006) and exclude confounding paracrine effects between transduced and untransduced cells. Transduction of either WT or *Tbx21*^{-/-} cells with DNAM1L decreased the fraction of cells producing IFN γ as well as the total amount of IFN γ secreted (Figure 5B–C). Collectively, these data suggest that Notch directly regulates *Ifng* expression, independently of its role in regulating *Tbx21*, and identify *Ifng* as a Notch target.

Notch1 binds to the *Ifng* CNS-22

To determine the mechanism by which Notch directly regulates *Ifng*, we searched for RBPJ binding sites in regions known to be critical for *Ifng* transcription (Hatton et al., 2006). Three RBPJ elements were identified in the *Ifng* CNS-22, a conserved enhancer that is required for *Ifng* expression in T and NK cells (Figure 6A) (Hatton et al., 2006). Primers were designed for ChIP that flanked the strongest and most conserved RBPJ binding site in the region. Two days after stimulation, CD4⁺ T cells exhibited Notch1 binding at the *Ifng* CNS-22 (Figure 6B). Similar to *Il4*, *Gata3-1a*, and *Tbx21* (Figure 3F), Notch1 bound the *Ifng* CNS-22 site in Th1, Th2, and Th17 cells (Figure 6B).

Notch1 and Tbet synergize to drive transcription from the *Ifng* CNS-22

The observation that Notch1 binds the *Ifng* CNS-22 suggested that Notch directly regulates *Ifng* expression through this element. To test this, we utilized reporter constructs for the *Ifng* CNS-22 region (Hatton et al., 2006). These constructs contain either the minimal 468 bp *Ifng* promoter (Pro^{WT}) or this promoter with the *Ifng* CNS-22 placed upstream (CNS^{WT}Pro^{WT}). Two putative T-box half-sites were identified in the *Ifng* CNS-22 fragment and mutant constructs were generated to both the upstream (CNS^{MT1}Pro^{WT}) and downstream (CNS^{MT2}Pro^{WT}) T-box half-sites (Figure 7A) (Hatton et al., 2006). Although the CNS^{MT2}Pro^{WT} construct was originally described as a Tbet binding site mutant, this same mutation also ablates the most highly conserved RBPJ binding site in *Ifng* CNS-22. To test if Notch was capable of driving transcription through the *Ifng* CNS-22 at this site in cells capable of endogenous IFN γ production, activated Jurkat cells were transfected with vector control (pcDNA) or the constitutively active Notch1 intracellular domain ICN1 (Aster et al., 2000), as well as a reporter containing pGL3, Pro^{WT}, CNS^{WT}Pro^{WT}, or CNS^{MT2}Pro^{WT} (Figure 7B). Addition of CNS^{WT}Pro^{WT} resulted in slightly increased reporter activity that was markedly enhanced upon the addition of ICN1, suggesting that Notch has the capacity to act on this enhancer element. When CNS^{MT2}Pro^{WT} was used, the increased activity observed from the CNS^{WT}Pro^{WT} was lost and the addition of ICN1 failed to enhance reporter activity (Figure 7B). These data suggest that Notch1 is capable of enhancing transcription through the *Ifng* CNS-22.

Although the Jurkat cell data established that Notch1 can drive transcription from the *Ifng* CNS-22 in a mature T cell line, it remained possible that Notch1 indirectly enhanced luciferase activity by promoting *Tbx21* transcription. In addition, the loss of luciferase activity observed when CNS^{MT2}Pro^{WT} was expressed may have resulted from a loss of Tbet binding, rather than the loss of RBPJ binding at the same site. To test these alternate hypotheses, we utilized U2OS cells, which do not express endogenous *Tbx21* and express very low amounts of Notch1 (data not shown). U2OS cells were transfected with the reporter constructs described above as well as CNS^{MT1}Pro^{WT}, which ablates the upstream T-box half-site without disrupting the RBPJ binding site. Tbet overexpression in U2OS cells increased luciferase activity in all constructs (Figure 7C). This effect appeared primarily due to Tbet activity on the *Ifng* minimal promoter, as the addition of the *Ifng* CNS-22 and mutation of either T-box half-site had no additional effect on reporter activity (Figure 7C).

In contrast to Jurkat cells, ICN1 did not increase luciferase activity in U2OS cells, suggesting that other factors endogenous to Jurkat cells, such as Tbet, may be required to cooperate with Notch1 activity. Accordingly, co-expression of Tbet and ICN1 synergistically increased luciferase activity from CNS^{WT}Pro^{WT}. Moreover, the enhanced luciferase activity was specific to CNS^{WT}Pro^{WT} and was not observed with Pro^{WT}, suggesting that the synergy between Notch1 and Tbet resulted from Notch1 activity at the *Ifng* CNS-22 (Figure 7C).

To directly address this possibility, we utilized the construct in which the upstream T-box half-site was mutated (CNS^{MT1}Pro^{WT}). In cells expressing this reporter, luciferase activity was similar to the CNS^{WT}Pro^{WT} under all conditions, suggesting that the primary effect of Tbet was through its activity on the promoter. In contrast, when the conserved RBPJ binding site was mutated (CNS^{MT2}Pro^{WT}), luciferase activity was comparable to the Pro^{WT} construct (Figure 7C). Collectively, these data suggest that ICN1 acts at the *Ifng* CNS-22 enhancer and Tbet acts at the *Ifng* promoter.

To directly assay the site of Tbet activity, we mutated the T-box site in the *Ifng* promoter leaving the putative Tbox sites in the CNS-22 intact (Tong et al., 2005) (Figure 7A). When U2OS cells were transfected with Pro^{MT}, neither Tbet alone nor Tbet plus ICN increased

reporter activity to that observed in Pro^{WT} (Figure 7D). Moreover, mutation of the promoter in the context of CNS-22 (CNS^{WT}Pro^{MT}) ablated the ability of either Tbet alone or Tbet plus ICN1 to increase reporter activity (Fig 7D). Overall, these data demonstrate that binding of Notch at the *Ifng* CNS-22 is insufficient to activate transcription by itself and that Tbet binding at the promoter leads to weak activation; however, the combination of Notch binding to the CNS-22 and Tbet binding to the *Ifng* promoter leads to a synergistic increase in *Ifng* transcription (Figure 7E).

Discussion

Within the lymphoid compartment, Notch is understood to selectively promote one lineage outcome at the expense of alternate fates (Radtke et al., 2010). This instructive paradigm was proposed to explain Th cell differentiation, however, the emerging data are difficult to reconcile with this model as Notch promotes mutually exclusive cell fates from a multipotential cell (Amsen et al., 2004; Keerthivasan et al., 2011; Maekawa et al., 2003; Minter et al., 2005; Mukherjee et al., 2009; Tu et al., 2005). Here, we present data demonstrating that Notch acts as an unbiased amplifier of the Th1, Th2, and Th17 cell programs by sensitizing cells to environmental signals.

By acutely inhibiting Notch signaling under neutralizing conditions, we reveal a role for Notch in simultaneously orchestrating both Th1 and Th2 programs. GSI treatment and acute DNAM1 expression synchronously reduced IL-4, IFN γ , and Tbet protein and mRNA for *Il4*, *Ifng*, *Tbx21*, and *Gata3-1a*, demonstrating that Notch lacks selectivity in regulating critical Th cell program targets. We further observed that Notch regulates Th17 target genes even when their expression is suppressed under Th1 and Th2 conditions and *vice versa*. Although the polarizing conditions influence the magnitude of gene expression, the ability of Notch to bind these key loci was unchanged, illustrating that the cytokine environment does not impact the ability of Notch to regulate its targets. Accordingly, our work suggests that Notch plays a critical role in reinforcing Th cell differentiation at physiologic levels of cytokine signaling, which would be important early during immune responses when differentiating cytokine cues are limiting. In addition, these findings help reconcile conflicting reports in the literature that emphasized the ability of Notch to preferentially regulate specific Th programs.

In related work, Ong et al. contended that Notch signaling itself had minimal impact on Th differentiation, but rather upstream cytokine signals directed Notch to selectively enhance individual Th responses (Ong et al., 2008). Consistent with our findings, these studies argued that Notch lacks instructive capacity. Our *T. muris* studies provide the first *in vivo* loss-of-function data confirming that Notch is not required for instruction, however our GSI-washout and ChIP data provide a distinct mechanistic view of Notch function during Th differentiation. Rather than requiring cytokine signals to condition Notch selectivity, we find that Notch binds and regulates target loci without regard to cytokine signals. Moreover, we further show that Notch concurrently regulates Th cell programs even under neutralizing conditions. Thus, our work suggests that the activity of Notch is not dictated by cytokine signaling, but rather that Notch simultaneously facilitates transcription of multiple programs regardless of polarizing cues.

In addition to providing a unifying model for Notch in Th cell differentiation, we present definitive genetic loss-of-function and molecular data evincing a role for Notch in the Th1 program. Not only do our data demonstrate for the first time that endogenous Notch1 binds *Tbx21* and that *Tbx21* is a direct Notch target in primary CD4⁺ T cells, but we also show that *Ifng* is a novel, direct Notch target, independent of Notch's role in regulating *Tbx21*. While the Notch effects on *Ifng* are independent of its activity on *Tbx21*, Notch does not

appear capable of driving *Ifng* transcription by itself, consistent with a model in which Notch lacks the capacity to instruct Th differentiation. Both factors are needed for optimal *Ifng* expression, where they bind different regulatory elements. Moreover, the *Tbx21*^{-/-} studies suggest that Notch is capable of collaborating with factors other than Tbet, as DNMA1L suppressed *Ifng* expression in its absence. The original report using these luciferase constructs found that Tbet overexpression was capable of enhancing luciferase activity of the WT CNS-22 construct when cells were stimulated with PMA and ionomycin (Hatton et al., 2006), which also induces NFκB binding to the *Ifng* CNS-22 (Balasubramani et al., 2010). As the *Ifng* CNS-22 contains multiple regulatory motifs, these data suggest that Tbet activity at the *Ifng* promoter synergizes with multiple factors regulating the *Ifng* CNS-22, including Notch and NFκB. Altogether, these data illustrate the dynamism of the *Ifng* promoter and CNS-22 and demonstrate how these elements have the potential to integrate inputs from multiple pathways.

The results of our reporter assays are reminiscent of the synergy between Gata3 and Notch1 at the *Ii4* locus and suggest that Notch and cytokine signaling collaborate in both Th1 and Th2 differentiation (Fang et al., 2007). Furthermore, Notch appears to participate in a feed-forward loop, promoting *Tbx21* transcription and in turn synergizing with Tbet protein to enhance *Ifng* transcription. Work by Flavell and colleagues suggests that this Th1 feed-forward loop must be stabilized by other factors (Amsen et al., 2007). In their study, constitutive Notch signaling was insufficient to enforce Th1 differentiation in the presence of endogenous Gata3, demonstrating that low levels of Gata3 act as a failsafe against runaway Th1 differentiation in response to Notch activation. In addition to transcriptional regulation, Notch can regulate IFNγ secretion in an RBPJ-independent manner, suggesting that Notch regulates the CD4⁺ T cell IFNγ response at multiple, mechanistically distinct levels (Auderset et al., 2012). Additionally, a recent human T cell study implicated a role for Notch in Th1 differentiation (Le Friec et al., 2012). Together, these data firmly establish Notch as a key regulator of the Th1 program.

As well as revealing a definitive role for Notch in promoting Th1 differentiation, our data further clarify the mechanism by which Notch regulates Th2 differentiation. The findings from anti-IFNγ mAb treated *T. muris* infected CCD mice indicate that the role of Notch during *in vivo* Th2 inflammation is similar to what has been reported for NFκB2, IL-25, and TSLP (Artis et al., 2002; Owyang et al., 2006; Taylor et al., 2009). Like Notch, mice deficient for each of these factors displayed susceptibility to helminth infection and impaired Th2 immune responses, however blockade of Th1 responses in these mouse models resulted in restoration of the Th2 response and worm expulsion. Moreover, unlike the role for Notch in regulating Th1 differentiation, we observe a major role for Notch in promoting IL-4 production but minimal impact on total Gata3, suggesting that additional factors, such as the Notch target Tcf1, may be required to fully engage the Th2 program (Yu et al., 2009). These studies collectively illustrate a clear distinction between Th2 initiating factors, such as NFκB1 (Artis et al., 2002), and the multiple inputs that maintain an optimal Th2 response *in vivo*, but are dispensable for Th2 program initiation, such as Notch.

In addition to regulating Notch-dependent IL-4 production in Th2 cells, recent work demonstrated that the IL-4 HS-V region is critical for T follicular helper cell (Tfh) production of IL-4 (Harada et al., 2012; Vijayanand et al., 2012). These findings raise the possibility that Notch inhibition in the *T. muris* studies may impact both Th2 and Tfh subsets during infection and the combined effects on these two populations contribute to the phenotype observed. Importantly, anti-IFNγ treatment restored productive immunity against helminth infection; and therefore, the role that Notch signaling plays in Tfh biology is either redundant with its function in Th2 cells or not essential for Tfh differentiation, similar to what we observe for Th1, Th2, and Th17 subsets.

With the recent recognition of Th cell plasticity, particularly at early time points, our data suggest that Notch functions to ensure that activated CD4⁺ T cells overcome a Th cell commitment threshold (Murphy and Stockinger). In this manner, Notch tunes the responsiveness of an activated CD4⁺ T cell to a specific Th cell program by sensitizing cells to limiting environmental differentiation cues. Thus, depending on the inflammatory environment for a given immune response, the requirement for Notch will vary depending on whether the strength of the differentiating signals a T cell receives are sufficient to achieve a signaling threshold for Th cell commitment. For example, during *T. muris* infection, our model argues that Notch sensitizes T cells to limiting Th2 differentiating cues, while residual IFN γ signals destabilize the Th2 circuitry when Notch signaling is abrogated. This model would also explain why Th1 differentiation occurs independently of Notch during *Leishmania* infection, as we would predict that the environmental differentiating signals are sufficient to overcome a commitment threshold (Amsen et al., 2004; Tu et al., 2005). Alternatively, in the context of GvHD, which is characterized by a mixed Th cell response, Notch is required to sensitize cells to subthreshold signals and achieve optimal IFN γ production (Zhang et al., 2011). While our data favor a model in which Notch regulates Th differentiation by sensitizing cells to their environment, Notch likely plays additional roles in other aspects of Th biology, such as survival and metabolism, that warrant future study.

Overall, these findings offer a paradigm for Notch in the immune system. In addition to its roles as an arbiter of alternate fate decisions and a key regulator of cell survival, proliferation, and metabolism, we reveal that Notch also acts to potentiate multiple fates from a single progenitor. Not only does this paradigm reconcile previously conflicting studies, but it also suggests that manipulating the amounts of Notch signaling in Th cell mediated pathologies may have therapeutic benefit.

Experimental Procedures

Mice

ROSA26-DNMAML mice were previously described (Tu et al., 2005). C57Bl/6 mice were obtained from the National Cancer Institute (Frederick, MD). YFP mice were obtained from Jackson Labs (Bar Harbor, ME). *Tbx21*^{-/-} mice were provided by John Wherry. All mice were housed in specific pathogen-free facilities at the University of Pennsylvania. Experiments were performed according to the guidelines from the National Institutes of Health with approved protocols from the University of Pennsylvania Animal Care and Use Committee.

TAT-Cre

Expression of TAT-cre was induced with IPTG in bacteria during log phase of growth at 37 °C in the presence of chloramphenicol and carbenicillin. The Children's Hospital of Pennsylvania Protein Core carried out purification of TAT-Cre protein. 10⁷ CD4⁺ T cells from DNAML^{FL/FL} or YFP^{FL/FL} mice were MACS purified by positive selection with CD4 Microbeads (Miltenyi). Subsequently, cells were washed, resuspended in 1 ml serum-free OPTI-MEM (Life Technologies), and incubated with 1 ml of 100 mg/ml of TAT-Cre in OPTI-MEM for 12 min at 37°C. Cells were then washed and cultured in IMDM (Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 100ng/mL IL-7. Twenty-four hours later, naïve YFP⁺ or GFP⁺ CD4⁺ T cells were FACS sorted

Quantitative PCR

RNA was extracted using the QIAGEN RNeasy Mini Kit. cDNA was synthesized from RNA with the Superscript II kit (Invitrogen). Transcripts were amplified with Sybr Green PCR Master Mix (ABI) and quantitative PCR was performed on an ABI ViiA 7 Real-Time PCR System. (Primers, Table S1)

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed as described previously (Yashiro-Ohtani et al., 2009). Briefly, chromatin samples were prepared from fixed 6 million cells and immunoprecipitated with rabbit immunoglobulin G (#sc-3888; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Notch1 antibodies (Aster et al., 2000). Purified DNA was subjected to real-time polymerase chain reaction (PCR) with primers flanking RBPJ binding sites at *Gata3-1a*, *Ii4 HS-V*, *IFNg CNS-22*, or *Tbx21* promoter (primers, Table S2). CD4⁺ T cells from Notch1 null mice were used for an immunoprecipitation negative control. *Nanog* was used as an internal negative control. The DNA quantity recovered from each ChIP sample is shown as the relative value to the DNA input sample. Supplementary Table 1 lists primer sequences.

Luciferase assay

Ifng luciferase constructs are described (Hatton et al., 2006). Constructs included a reporter control (pGL3), the reporter plus a 468 bp *Ifng* minimal promoter (Pro^{WT}), the minimal promoter construct containing a mutated T-box binding site (Pro^{MT}), the minimal promoter construct with a WT *Ifng* CNS-22 upstream (Pro^{WT}CNS^{WT}), the WT CNS-22 construct containing a mutated T-box binding site in the promoter region (Pro^{MT}CNS^{WT}), the *Ifng* CNS-22 construct with the upstream CNS-22 T-box half-site mutated (Pro^{WT}CNS^{MT1}), and the *Ifng* CNS-22 construct with the downstream CNS-22 T-box half-site mutated (Pro^{WT}CNS^{MT1}). Point mutations to T-box binding sites in the *Ifng* promoter were made using the Promega QuikChange Site Directed Mutagenesis Kit. Constructs were sequenced to prove authenticity. 2×10^5 Jurkat cells were plated in triplicate and transfected with 50 ng of the indicated promoter/reporter constructs, 25 ng pRL-TK, and 100 ng pcDNA-ICN1 or vector control using DMRIE-C (Invitrogen). Transfected cells were cultured overnight and then stimulated for 4 hours with 20 ng/mL PMA and 200 ng/mL ionomycin. 2×10^4 U2OS cells were plated in triplicate and transfected with 50 ng of the indicated promoter/reporter constructs, 10 ng pRL-TK, 10 ng pcDNA-ICN1 or vector control, and 25 ng Mig-Tbet or vector control using FuGENE 6 (Promega). After stimulation, firefly substrate activity was measured using Britelite Plus (PerkinElmer) and renilla substrate activity measured using Stop & Glo (Promega). Firefly values were normalized to Renilla and then all normalized values set relative to the pGL3 control vector. All measurements were performed using a GloMax-96 Microplate Luminometer (Promega).

In vitro T cell culture

Lymph nodes from WT mice were CD4⁺ MACS purified and then naïve CD4⁺ T cells were FACS sorted. Naïve CD4⁺ T cells were co-cultured with irradiated, Thy1.2 depleted splenocytes at a 1:5 ratio and stimulated using anti-CD3e (1 µg/mL), and anti-CD28 (1 µg/mL) mAb. Cells were cultured under either neutral (5 ng/mL IL-2, 20 µg/mL anti-IL-4, and 20 µg/mL anti-IFNγ), Th1 (5 ng/mL IL-2, 5 ng/mL IL-12, and 20 µg/mL anti-IL-4), Th2 (20 ng/mL IL-4, 20 µg/mL anti-IFNγ, and 20 µg/mL anti-IL-12), or Th17 (20 ng/mL IL-6 and 5 ng/mL TGF-β) culture conditions. For GSI experiments, cells were treated with either DMSO or 1 µM GSI.

Measurement of cytokines

For intracellular cytokine staining, cells were restimulated, fixed, and stained as described (Tu et al., 2005). Cells were stained with anti-CD4 (RM4-5, Biolegend), anti-IFN γ (XMG1.2, BD), anti-IL4 (11B11, eBioscience), anti-IL17A (ebio17B7, eBioscience), anti-Tbet (eBio4B10, eBioscience), and anti-GATA3 (L50-823, BD). Cells were acquired on a LSRII (Becton Dickinson) and data was analyzed with FlowJo (TreeStar). ELISA was performed as described (Tu et al., 2005).

GSI-washout assay

WT CD4⁺ T cells were stimulated as above and 24 hours post-stimulation cells were treated with either DMSO or GSI (1 μ M) for 20 hours. Subsequently, T cells were CD4⁺ MACS purified and cells cultured in media containing cycloheximide (20 μ g/mL) and cells previously cultured DMSO were replated in DMSO and cells cultured in GSI were either replated in 1 μ M GSI or in DMSO for 4 hours. RNA was then harvested and analyzed by qPCR.

T. muris infection and antigen

T. muris eggs were prepared as described (Tu et al., 2005). Mice were infected on day 0 with 150–200 embryonated eggs, and parasite burdens were assessed on day 21 after infection. Mesenteric LN (MLN) cell suspensions were prepared and resuspended in IMDM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine, and 50 M 2-mercaptoethanol. Cells were plated at 4 X 10⁶ cells/well and cultured in the presence of anti-CD3e and anti-CD28 for 72 hours. Levels of IL-4, -5, and -13 were assayed by sandwich ELISA. For histology, segments of mid-cecum were removed, washed in sterile PBS, and fixed for 24 hours in paraformaldehyde. Tissues were processed and paraffin embedded using standard histological techniques. For detection of intestinal goblet cells, 5 μ m sections were cut and stained with hematoxylin and eosin or Alcian blue periodic acid Schiff. Analysis of parasite-specific IgG1 and IgG2c production was performed by antigen capture

Statistical Analysis

Significance was determined using a Student's T-test. A *P* value of less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Notch concurrently regulates Th1, Th2, and Th17 cell differentiation
- Notch activity is unbiased and is not affected by the cytokine environment
- Notch regulates *Ifng* at the *Ifng* CNS-22 and synergizes with Tbet at the promoter
- Notch can simultaneously orchestrate multiple lineage programs

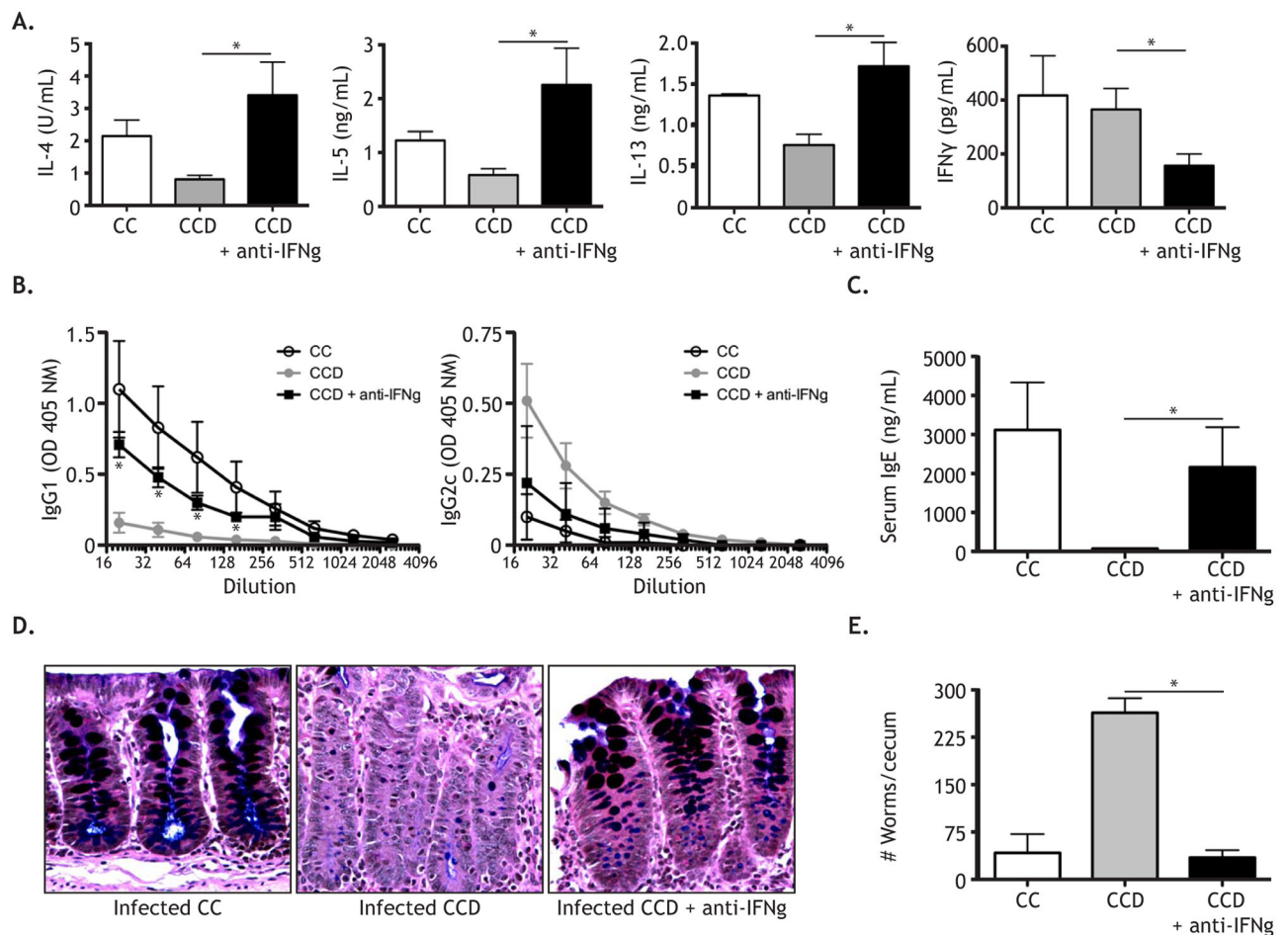


Figure 1. Notch signaling is dispensable for Th2 initiation during *Trichuris muris* infection (A) At day 21, MLN from *T. muris* infected CC, CCD, and CCD + anti-IFN γ mice were stimulated with anti-CD3 for 72 hours and cytokine levels were measured by ELISA. (B) Serum *T. muris* specific IgG1 and IgG2c and (C) total IgE were measured by ELISA from infected mice, at day 21. (D) Goblet cells, dark staining, in gut sections were detected by mucin staining. (E) Cecal worm burdens at day 21 post-infection. *, $P < 0.05$. Data are represented as mean \pm SEM.

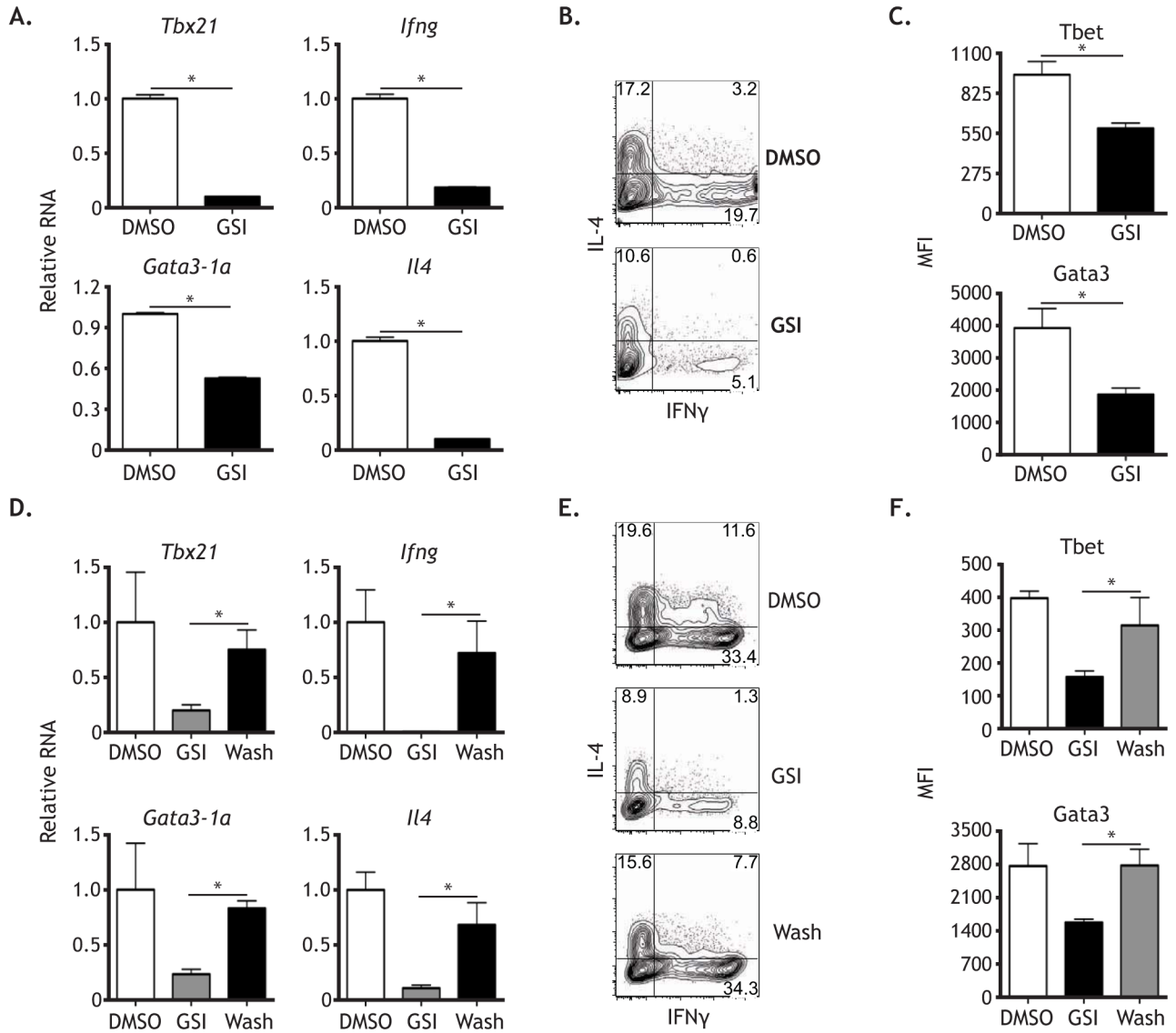


Figure 2. Persistent Notch signaling is required to maintain the Th1 and Th2 programs
 WT naive CD4⁺ T cells were stimulated with irradiated splenocytes, anti-CD3 ϵ , and anti-CD28, and cultured under neutral conditions. After 5 days, cells were restimulated as above and replated in media containing either DMSO or 1 μ M GSI. After 2 days (day 7 post-activation), (A) RNA was then harvested and analyzed by qPCR. Cells were analyzed for (B) cytokine production and (C) transcription factor expression by intracellular FACS. WT naive CD4⁺ T cells were stimulated and cultured as above for 5 days in media containing either DMSO or GSI. Cells were then washed and restimulated under the same conditions, with cells previously cultured in DMSO being replated in media containing DMSO and cells previously treated with GSI being replated in either DMSO or 1 μ M GSI. After 2 days of restimulation, (D) RNA was then harvested and analyzed by qPCR. Cells were analyzed for (E) cytokine production and (F) transcription factor expression by intracellular FACS. *, P < 0.05. Data are represented as mean \pm SEM. See also Figure S1.

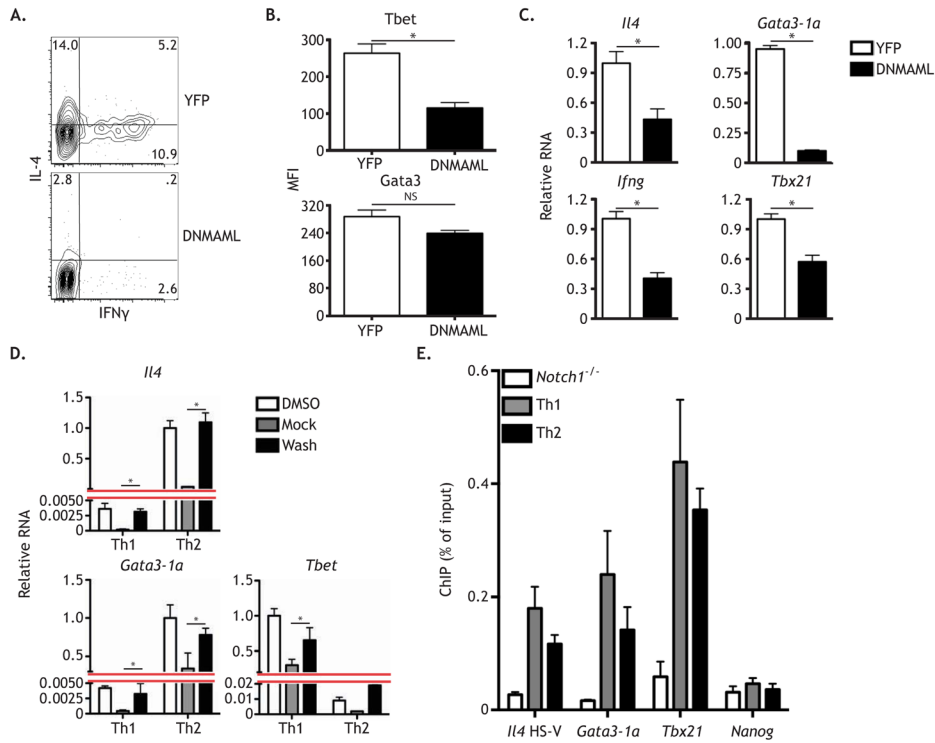


Figure 3. Notch concurrently regulates both the Th1 and Th2 programs

YFP^{FL/FL} or DNAMAML^{FL/FL} CD4⁺ T cells were Tat-cre treated and rested for 24 hours in media containing 100 ng/mL IL-7. Naive CD4⁺ T cells were then FACS sorted and stimulated with irradiated splenocytes, anti-CD3ε, and anti-CD28, and cultured under neutral conditions. After 5 days, (A) cytokine production was measured by intracellular FACS, (B) Tbet and Gata3 protein was measured by intracellular FACS, and (C) RNA was analyzed by qPCR. WT CD4⁺ T cells were stimulated as above and cultured under either Th1 or Th2 culture conditions. After 24 hours, cells were treated with either DMSO or GSI for 20 hours. Subsequently, T cells were CD4⁺ MACS purified and cells cultured in DMSO were replated in DMSO and cells cultured in GSI were either replated in GSI (Mock) or in DMSO (Washout) for 4 hours; all cells were placed in media containing CHX. (D) RNA was then harvested and analyzed by qPCR. (E) WT CD4⁺ T cells were stimulated as above. After 2 days, cells were fixed and ChIP was performed using anti-Notch1 antibody, with Nanog serving as an internal negative control. As a negative control for the assay and antibody, anti-Notch1 ChIP was performed on CD4-Cre x Notch1^{FL/FL} CD4⁺ T cells. *, P < 0.05. Data are represented as mean ± SEM. See also Figure S2, Table S1, and Table S2.

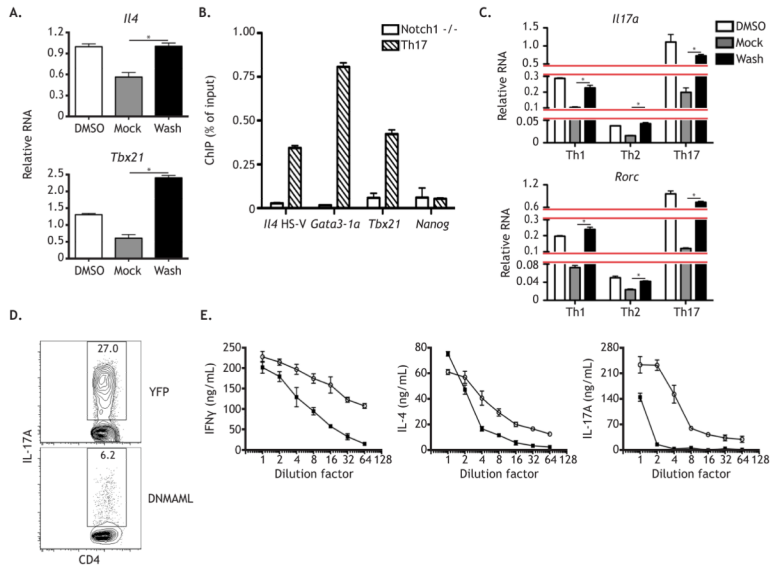


Figure 4. Notch simultaneously orchestrates multiple Th cell programs
 WT CD4⁺ T cells were stimulated as described under Th17 culture conditions. (A) After 24 hours, cells were treated with either DMSO or GSI for 20 hours and then subjected to a GSI washout assay. Cells cultured in DMSO were replated in DMSO and cells cultured in GSI were either replated in GSI (Mock) or in DMSO (Washout) for 4 hrs. RNA was then harvested and analyzed by qPCR. (B) After 2 days, cells were fixed and ChIP was performed using anti-Notch1 antibody, with Nanog serving as an internal negative control. Anti-Notch1 ChIP was performed on CD4-Cre x Notch1^{FL/FL} CD4⁺ T cells as an experimental and antibody negative control. (C) A GSI washout assay was performed as above on activated CD4⁺ T cells cultured under either Th1, Th2, or Th17 polarizing conditions. At the end of the assay (48 hrs), RNA was harvested and analyzed by qPCR. (D) Naive CD4⁺ T cells were FACS sorted from Tat-Cre treated YFP^{FL/FL} or DNMAAML^{FL/FL} CD4⁺ T cells. Cells were then stimulated as above, and cultured under Th17 conditions. After 3 days, cytokine production was measured by intracellular FACS. (E) WT CD4⁺ T cells were stimulated as described and then cultured in a titration series of two-fold serially diluted Th1 (left panel), Th2 (middle panel), or Th17 (right panel) conditioned media, in the presence of either DMSO or GSI. After 5 days, cells were restimulated and supernatants were analyzed by ELISA two days later. *, P < 0.05. Data are represented as mean +/- SEM. See also Table S1 and Table S2.

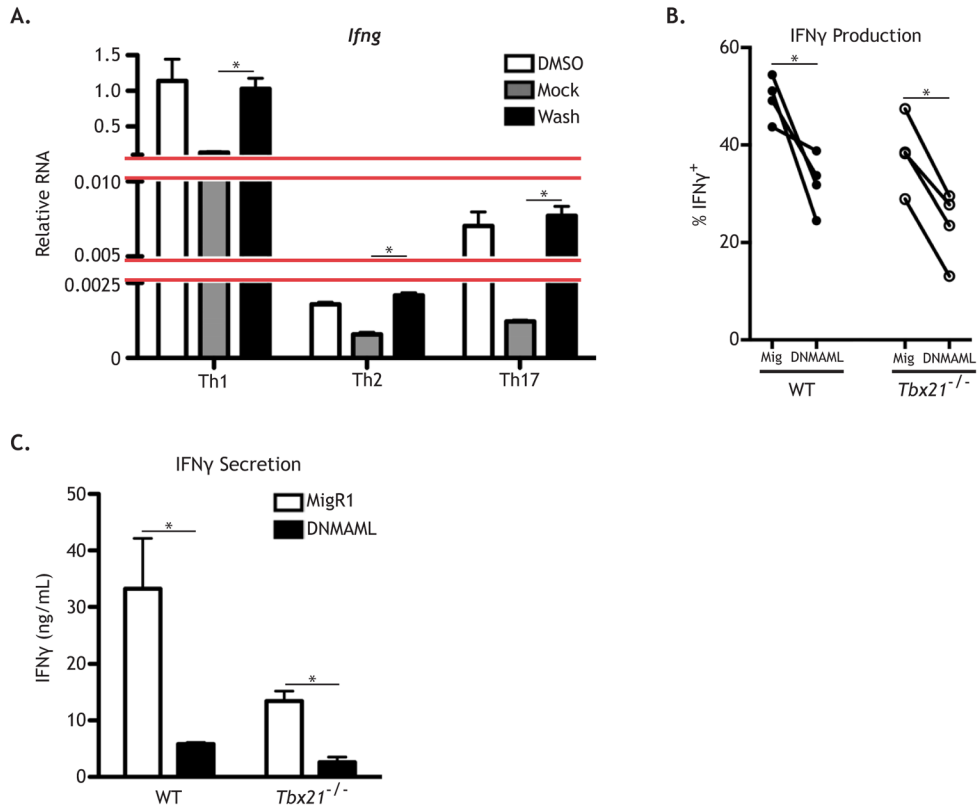


Figure 5. Notch directly regulates *Ifng* expression

(A) WT CD4⁺ T cells were stimulated as described under either Th1, Th2, or Th17 culture conditions. 24 hours later, cells were treated with either DMSO or GSI for 20 hours and then subjected to a GSI washout assay. Cells cultured in DMSO were replated in DMSO and cells cultured in GSI were either replated in GSI (Mock) or in DMSO (Washout) for 4 hours. RNA was then harvested and analyzed by qPCR. WT and Tbet KO naive CD4⁺ T cells were stimulated as above and cultured with IL-12 (5 ng/mL), anti-IL-4 (20 μ g/mL), and anti-IFN γ (20 μ g/mL). After 24 hours, cells were retrovirally transduced with either vector control (Mig) or DNMAAML. (B) Cytokine production was measured by intracellular FACS and (C) cytokine secretion by ELISA, 48 hours post-transduction. *, P < 0.05. Data are represented as mean \pm SEM.

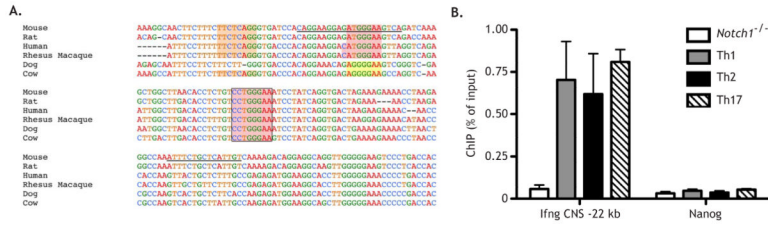


Figure 6. Notch binds to the *Ifng* CNS-22
 (A) Multiple species alignment of the *Ifng* CNS-22 region, using NCBI DCODE.org. Highly conserved strong (red), moderate (orange), and weak (yellow) RBPJ binding sites are highlighted. Primer sequences used for ChIP are underlined. (B) WT CD4⁺ T cells were stimulated as described and cultured under Th1, Th2, or Th17 conditions. After 2 days cells were fixed and ChIP was performed using anti-Notch1 antibody, with *Nanog* serving as an internal negative control. Anti-Notch1 ChIP on CD4-Cre x *Notch1*^{FL/FL} CD4⁺ T cells served as a negative control for the antibody and assay. Data are represented as mean +/- SEM.

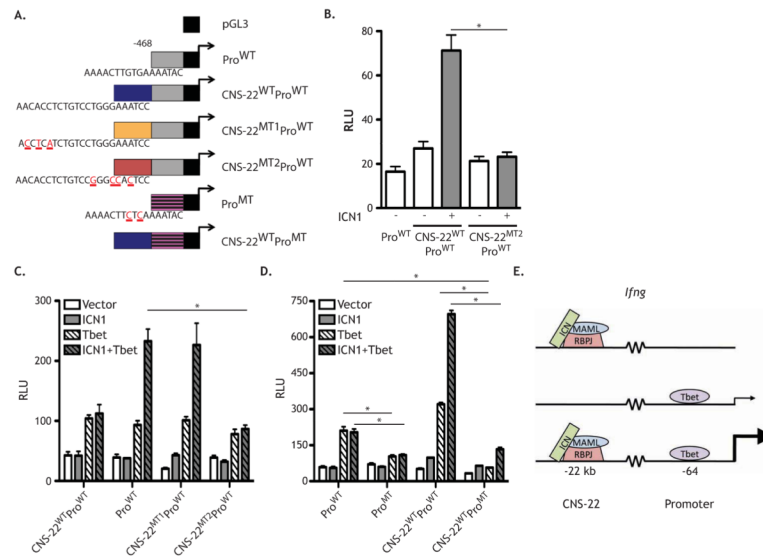


Figure 7. Notch1 and Tbet synergize to drive transcription from the *Ifng* CNS-22

(A) Schematic of the luciferase constructs used, as detailed in the methods. (B) 2×10^5 Jurkat cells were transfected with renilla control (pRLTK), either the Pro^{WT}, Pro^{WT}CNS^{WT}, or Pro^{WT}CNS^{MT2} luciferase reporter constructs, and either a vector control or ICN1, using DMRIE-C liposomes. After 20 hours, cells were stimulated with PMA and ionomycin for 4 hours. Cells were then washed and luciferase activity was measured. (C) 2×10^4 U2OS cells were transfected with pRLTK, either the promoter Pro^{WT}, Pro^{WT}CNS^{WT}, Pro^{WT}CNS^{MT1}, or Pro^{WT}CNS^{MT2} reporter constructs, ICN1 or vector control, and Tbet or vector control. After 48 hours, luciferase activity was measured. (D) 2×10^4 U2OS cells were transfected with pRLTK, either the Pro^{WT}, Pro^{MT}, Pro^{WT}CNS^{WT}, or Pro^{MT}CNS^{WT} reporter constructs, ICN1 or vector control, and Tbet or vector control. After 48 hrs, luciferase activity was measured. (E) Proposed model of *Ifng* loci regulation by Notch1 and Tbet. All values are normalized to the pGL3 control construct. Relative luciferase units were calculated by normalizing firefly activity to renilla activity and setting all values relative to the pGL3 control construct. *, $P < 0.05$. Data are represented as mean \pm SEM.