Non-canonical Notch signaling drives activation and differentiation of peripheral CD4+ T cells

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INTRODUCTION

The Notch receptor protein plays a crucial role in embryonic development and specification of cell fates (1). There are four Notch receptors (Notch1–4), which can be activated by ligands that belong to either the Delta-like (DLL1, 3, 4) or Jagged family (Jagged 1 and 2) of proteins (2). Binding of a Notch ligand to the receptor triggers a series of proteolytic cleavages that culminate in the release of the intra-cellular domain of Notch by γ-secretase. This active, intra-cellular fragment of Notch migrates to the nucleus and interacts with the transcriptional repressor – recombination signal– binding protein-Jκ (RBP-Jκ). Following recruitment of co-activators such as p300 and master-mind-like (MAML), RBP-Jκ is converted to a transcriptional activator leading to the expression of downstream target genes. Such RBP-Jκ dependent or canonical Notch signaling has been long thought to regulate T cell responses. Recent reports suggest that the intra-cellular domain of Notch also interacts with other proteins besides RBP-Jκ in the cytoplasm and nucleus suggesting that Notch could possibly use an alternate route to exert some of its effects in an RBP-Jκ – independent or “non-canonical” fashion (3–6). However, whether or not such non-canonical Notch signaling is involved in regulating peripheral T cell responses is unknown.

T cell receptor (TCR) mediated activation of peripheral T cells is a fundamental process of the adaptive immune system. Activation of CD4+ T cells is accomplished by binding of an antigen to the TCR presented by an MHC Class II molecule on the antigen-presenting cell. A co-stimulatory signal between B7 (CD80/CD86) on the antigen-presenting cell and CD28 on the T cell stimulates the onset of multiple downstream signaling events, which result in T cell activation and proliferation. Helper T cells can differentiate into at least four different lineages (TH1, TH2, TH17, T-reg) depending on the cytokine milieu. Interleukin-12 and IFN-γ polarize CD4+ T cells to the TH1 phenotype. TH1 cells express the lineage specific transcription factor T-bet, secrete the signature cytokine IFN-γ, and provide protection against intra-cellular pathogens (7). TH2 cells, which are induced by IL-4, are primarily involved in asthma and allergies, and protect against extra-cellular parasites. They require the transcription factor GATA3 to secrete IL-4, IL-5, and IL-13 (8). Interleukin-6 and TGF-β generate TH17 cells, which provide protection against nematodes and fungal infections, secrete signature cytokines IL-17 and IL-23 and express ROR-γt (9–11). Induced regulatory T cells (iTregs), which are also induced by TGF-β, are characterized by the expression of FoxP3 and exhibit immuno-suppressive functions (12–14).

Ligation of the TCR accompanied by co-stimulation, generates intra-cellular Notch in CD4+ T cells while inhibition of Notch activation with γ-secretase inhibitors (GSI) decreases T cell activation via the TCR. However, whether or not such non-canonical Notch signaling is involved in regulating peripheral T cell responses is unknown. In this report, we specifically demonstrate the requirement of Notch1 for regulating signal strength and signaling events distal to the T cell receptor in peripheral CD4+ T cells. By using mice with a conditional deletion in Notch1 or RBP-Jκ, we show that Notch1 regulates activation and proliferation of CD4+ T cells independently of RBP-Jκ. Furthermore, differentiation to TH1 and iTreg lineages although Notch dependent, is RBP-Jκ independent. Our striking observations demonstrate that many of the cell-intrinsic functions of Notch occur independently of RBP-Jκ. Such non-canonical regulation of these processes likely occurs through NF-κB. This reveals a previously unknown, novel role of non-canonical Notch signaling in regulating peripheral T cell responses.

Keywords: Notch1, CD4+ T cell, non-canonical, activation, differentiation

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as well as proliferation (15, 16). Since GSIs have multiple substrates and inhibit all Notch receptors (17), whether such a decrease in T cell activation is precisely due to Notch1, or a GSI induced effect, is ambiguous and needs to be investigated. Additionally, precisely the point in the TCR signaling cascade Notch at which Notch is involved remains to be determined. Several studies using various approaches to inhibit Notch activity have reported conflicting functions of Notch in regulating T cell activation as well as differentiation. Notch signaling has been shown to regulate differentiation to TH1, TH2, TH17, and Treg lineages (4, 18–20). Pharmacological inhibition of Notch using GSIs dampens the ability to adopt a TH1 fate by attenuating the expression of T-bet (4). However, inhibition of signaling downstream of RBP-Jk via genetic deletion or by using dominant negative MAML inhibits adoption of a TH1 fate in vivo while preserving a TH1 phenotype (21–23). Given the ability of intra-cellular Notch to interact with proteins different from RBP-Jk, it is possible that disparate results could be attributed to RBP-Jk dependent functions of Notch. Furthermore, whether canonical and non-canonical Notch signaling affects T cell activation and differentiation processes differently requires further investigation.

In this study, we report that Notch is required for controlling signaling events distal to the T cell receptor and also acts as a critical regulator of TCR signal strength. We also show that activation and proliferation of peripheral CD4+ T cells specifically requires Notch1 but not RBP-Jk since conditional deletion of Notch1 impaired these processes while conditional deletion of RBP-Jk had no effect. Such non-canonical, RBP-Jk independent regulation of these processes likely occurs via NF-κB. Conditional deletion of Notch1 also impaired polarization to TH1 and induction of regulatory T cells in vitro. Nevertheless, RBP-Jk deficiency did not impair TH1 or Treg cell fate in vitro once again supporting a novel role of non-canonical Notch signaling in controlling differentiation toward these lineages. In vitro polarization to TH2 was not affected in the absence of either Notch1 or RBP-Jk. Our in vitro observations demonstrate a cell-intrinsic function of RBP-Jk independent Notch signaling in regulating peripheral T cell responses. Such non-canonical regulation of these processes may serve to explain some of the differential, pleiotropic effects of Notch.

RESULTS
Notch IS REQUIRED FOR DISTAL TCR SIGNALING EVENTS
Activation of T cells via the TCR accompanied by co-stimulation leads to the production of the active, intra-cellular domain of Notch1 (N1IC) and its inhibition via γ-secretase inhibitors (GSI), decreases activation, and proliferation of T cells (15, 16). While Notch has been demonstrated to influence T cell activation, precisely where Notch exerts its influence downstream of the TCR is obscure. Furthermore, whether Notch affects signaling events proximal or distal to the TCR is unclear. To address these questions, we determined the kinetics of Notch activation over time and asked how inhibition of Notch activation via GSI treatment influences downstream TCR signaling events at early and late time points after stimulation. We detected N1IC in CD4+ T cells activated with plate-bound anti-CD3ε and anti-CD28 4 h after activation and the amount of N1IC increased over time (Figure 1A). This increase was abrogated after GSI treatment (Figure 1A). Inhibition of Notch activation did not alter proximal signaling events as evidenced by intact phosphorylation of Zap 70 even in GSI treated cells (Figure 1B). On the contrary, GSI treatment significantly decreased distal TCR signaling events such as the expression of activation markers CD25, CD69, IL-2, and IFN-γ (Figures 1C–F). This decrease was most prominent close to 48 h after TCR stimulation suggesting that Notch activation is critical for signaling events distal to the TCR, but could be dispensable for proximal events. Since we observed that activating cells via the TCR also triggered the activation of Notch, we determined whether CD4+ T cells themselves express Notch ligands. We observed that surface expression of DLL1 and Jagged1 is minimal up to 6 h after activation and peaks at distal time points (Figures S1A,B in Supplementary Material). Based on this observation, we determined whether stimulating T cells in the presence of recombinant Notch ligands alters the generation of N1IC downstream of the TCR. Activation in the presence of recombinant DLL1 or Jagged1 did not alter the generation of N1IC nor did it impact T cell activation (Figures S1C–H in Supplementary Material). Finally, stimulating T cells via the TCR in the presence of DLL1 or Jagged1 did not significantly influence the acquisition of helper T cell fate, although DLL1 enhanced IFN-γ production under pre-existing TH1 conditions (Figures S1I,K in Supplementary Material). Collectively, these data show that N1IC is generated in CD4+ T cells after stimulating via the TCR and influences distal TCR signaling events.

Notch AS A REGULATOR OF SIGNAL STRENGTH
A possible role of Notch as a regulator of signal strength has been observed in thymocytes since constitutive expression of N1IC in DP thymocytes prevented their maturation into single positive CD4+ and CD8+ T cells by interfering with TCR signal strength (24). However, whether Notch can influence the strength of TCR signaling in peripheral CD4+ T cells is unknown. Given the importance of Notch signaling in regulating activation, we asked whether it also influenced the threshold of signaling via the TCR. Since ligations of the TCR accompanied by co-stimulation was sufficient for the expression of N1IC, we first determined if altering signal strength could also influence the generation of N1IC. We stimulated CD4+ T cells with increasing concentrations of anti-CD3ε keeping the amount of anti-CD28 constant. Increasing signal strength resulted in a corresponding increase in the amount of N1IC expression (Figure 2A). This was also accompanied by an increase in the percentage of cells expressing N1IC and this increase could be inhibited by GSI treatment (Figure 2B). To determine if Notch is required for regulating signal strength, we stimulated cells with increasing amounts of anti-CD3ε after inhibiting Notch activation via GSI treatment. While DMSO treated cells secreted higher levels of IL-2 with increasing signal strength, GSI treated cells secreted significantly low amounts of IL-2 even at the highest signal strength of 10 μg/ml anti-CD3ε (Figure 2C) suggesting that Notch influenced the threshold of signaling via the TCR. To confirm this observation, we stimulated D0.11.10-TCR transgenic CD4+ T cells with antigen-presenting cells pulsed with increasing concentrations of ova-peptide. Once again, increasing antigen concentrations lead to a dose-dependent increase in the amount of N1IC as well as the percentage of N1IC positive cells.
FIGURE 1 | Notch is required for distal TCR signaling events. Splenocytes from C57BL/6J mice were pretreated with DMSO or GSI and stimulated with plate-bound anti-CD3ε and anti-CD28 for the indicated times. Cells were harvested and analyzed by flow cytometry after gating on CD4+ T cells. Mean fluorescent intensity (MFI) values were plotted for (A) N1IC, (C) CD25, and (D) CD69. (E) IL-2 and (F) IFN-γ ELISA from supernatants of cells stimulated as described. (B) Western Blot for phosphorylated and Total Zap70. Splenocytes from C57BL/6J mice were pretreated with DMSO or GSI and stimulated with anti-CD3ε and anti-CD28 for the indicated time points. Whole cell lysates were made at for each time point and analyzed by Western Blotting. Data are representative of three independent experiments. Data represent the mean ± SEM n = 3. *p < 0.05, **p < 0.005, and ***p < 0.001.

(Figures 2D,E). This increase could be blocked after GSI treatment (Figure 2E). In addition to N1IC, increasing signal strength also led to an increase in IL-2. However, in the absence of Notch activation, even high concentrations of ova-peptide were not sufficient for maximal IL-2 production (Figure 2F). These data demonstrate that not only can the levels of N1IC be influenced by the amount of signal via the TCR, but also Notch itself can regulate signal strength by decreasing the threshold of signaling. In addition to Notch, the cell cycle regulator c-Myc is also influenced by TCR signal strength as strong peptide agonists cause a greater induction and nuclear translocation of c-Myc in T cells (25). c-Myc is a downstream target of Notch and is suspected to be important in thymocyte development and T cell function (25–28). Thus, we asked whether or not the regulation of c-Myc in response to signal strength was Notch dependent. We first confirmed Notch dependency of c-Myc in T cells. In concurrence with previous reports, we observed that phosphorylated and total c-Myc had a biphasic appearance in T cells and peaked at 4 and 24 h after TCR stimulation (28). However in addition to these results, we also observed that inhibition of Notch activation abrogated both phosphorylated and total c-Myc with the most prominent reduction at 48 h post stimulation (Figures 2G,H). Additionally, increasing signal strength lead to an increase in the expression of c-Myc, which was abrogated in the absence of Notch activation (Figure 2I). Thus, these data show that Notch is required for sustained c-Myc induction in peripheral CD4+ T cells. Furthermore, the induction of c-Myc in response to signal strength is Notch dependent.

Notch1 IS REQUIRED FOR ACTIVATION AND PROLIFERATION OF CD4+ T CELLS

Inhibition of Notch activation using GSIs has been demonstrated to abrogate activation and proliferation of CD4+ T cells (15, 16). However, GSIs inhibit all isoforms of Notch and have multiple substrates (17). Hence, the specific role of Notch1 in controlling these processes requires further investigation. To determine the specific function of Notch1 in T cell activation and differentiation, we conditionally knocked-out Notch1 in peripheral T cells by crossing mice with loxp-flanked Notch1 alleles, to mice expressing Cre recombinase under the control of the interferon responsive Mx promoter (29). The Mx Cre promoter enables acute deletion of Notch1 in peripheral T cells. Since Notch1 is required for development of T cells (30–32), this deletion strategy enables cells to develop normally in the presence of Notch,
before its deletion in the periphery. Both Notch1\(^{+/+}\) Mx Cre\(^{\pm}\) mice (abbreviated as cN1KO) and Notch1\(^{+/+}\) Mx Cre\(^{-/-}\) mice (abbreviated as control) were injected with equivalent amounts of Poly I:Poly C and rested for 3 weeks before use (29). CD4\(^{+}\) T cells from cN1KO animals showed a significant decrease in Notch1 transcript as well as a marked reduction in the amount of Notch1IC expression (Figures S2A,D in Supplementary Material) (29). We also confirmed the expression of other Notch receptors in CD4\(^{+}\) T cells from control and cN1KO animals and observed an increase in the expression of Notch2 and Notch3 transcripts in the absence of Notch1 (Figures S2B,C in Supplementary Material). We could not detect Notch4 in cells from either control or cN1KO mice. In addition, both cN1KO and control mice expressed similar percentages of CD4\(^{+}\) and CD8\(^{+}\) peripheral T cells (Figures S2E,F in Supplementary Material). To investigate the specific contribution of Notch1 in influencing activation of peripheral T cells, CD4\(^{+}\) T cells from cN1KO or control animals were stimulated in vitro with anti-CD3e and anti-CD28 and activation markers were observed by flow cytometry. cN1KO animals had a significantly lower percentage as well as absolute number of cells expressing CD25 and CD69 (Figures 3C,F). This decrease was accompanied by a significant reduction in the levels of IL-2 and IFN-\(\gamma\) secreted post-activation (Figures 3G,H). Whether CD4\(^{+}\) T cells from cN1KO animals were also impaired in their ability to proliferate was determined by measuring the incorporation of \(^{3}\)H-thymidine.
Proliferative capability was significantly diminished in the absence of Notch1 and could not be rescued by adding exogenous IL-2 (Figure 3I). To determine if the observed decrease in proliferation was due to enhanced apoptosis in the absence of Notch1, Annexin V positive cells from control or cN1KO animals were analyzed by flow cytometry. CD4+ T cells from cN1KO animals had only marginally more apoptosis 48 h after activation (Figures S3A,B in Supplementary Material). Since Notch1 was required for the activation and proliferation of CD4+ T cells, we asked whether it also influenced the threshold of signaling via the TCR. CD4+ cells from control and cN1KO animals were stimulated with increasing concentrations of anti-CD3ε accompanied by co-stimulation. While cells from control mice secreted higher levels of IL-2 with increasing signal strength, cells from cN1KO mice had low levels of IL-2 even at the highest signal strength of 0.2 μg/ml anti-CD3ε (Figure 3J). These results demonstrate that activation and proliferation of CD4+ T cells specifically requires Notch1. Furthermore, Notch1 also acts to decrease the threshold for signaling via the TCR.

**Notch1 IS REQUIRED FOR T H1 DIFFERENTIATION AND PRODUCTION OF iTREGS IN VITRO**

Although Notch has been implicated in influencing differentiation of T cells, the precise role of Notch1 in favoring Th1 versus Th2 lineage decisions is unclear. While some studies have shown that inhibiting Notch using GSIs diminishes the ability of CD4+ T cells to adopt a Th1 fate, other studies using different strategies to inhibit Notch signaling have reported conflicting observations (21, 22, 33). To determine the precise role of Notch1 in helper T cell differentiation, CD4+ T cells from control or cN1KO mice were stimulated in vitro to Th1, Th2, or iTreg lineages. Absence of Notch1 impaired Th1 differentiation in vitro. CD4+ T cells from cN1KO mice had significantly fewer cells that stained positive for intra-cellular IFN-γ (Figures 4A,B). Secreted IFN-γ was also reduced significantly in the absence of Notch1 (Figure 4C). This decrease was accompanied by a reduction in the amount of the master Th1 transcription factor T-bet (Figures 4D–F). In contrast, no marked effect was observed in Th2 differentiation. Although, the amount of GATA3

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**FIGURE 3 | Notch1 is required for activation and proliferation of CD4+ T cells.** CD4+ T cells were isolated from Control or cN1KO mice and stimulated with plate-bound anti-CD3ε and anti-CD28. Cells were harvested after 48 h (A–F) or at indicated times (G–I). Cells were surface stained for CD4, CD25, and CD69 and intra-cellular stained for N1IC+ and analyzed by flow cytometry. (A,D) Dot plots obtained after flow cytometry. Data are representative of four independent experiments. Numbers in each quadrant represent percentage of cells. (B) Percentage of cells and cell counts for cells positive for CD25 and CD69. (C,F) Histograms for CD25 and CD69 after gating on Notch negative cells from cN1KO mice (dashed line), Notch positive cells from Control mice (solid line). CD4+ T cells from Control mice secreted higher levels of IL-2 with activation and proliferation of CD4+ T cells, we asked whether it also influenced the threshold of signaling via the TCR. CD4+ cells from control and cN1KO animals were stimulated with increasing concentrations of anti-CD3ε accompanied by co-stimulation. While cells from control mice secreted higher levels of IL-2 with increasing signal strength, cells from cN1KO mice had low levels of IL-2 even at the highest signal strength of 0.2 μg/ml anti-CD3ε (Figure 3J). These results demonstrate that activation and proliferation of CD4+ T cells specifically requires Notch1. Furthermore, Notch1 also acts to decrease the threshold for signaling via the TCR.

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was reduced in the absence of Notch1 (Figures 4G–I), both control as well as cN1KO mice had similar percentages and absolute number of CD4⁺ T cells that were positive for intra-cellular IL-4 (Figures 4A,B) and expressed comparable levels of secreted IL-4 (Figure 4C). Whether CD4⁺ T cells from cN1KO mice proliferated differently under different polarizing conditions, was determined by ³H-thymidine uptake under non-polarized (NP), T₃₁ and T₃₂ conditions. Proliferative capability of CD4⁺ T cells from cN1KO mice was the same under different polarizing conditions but is dispensable for T₃₂ cell fate acquisition in vitro. Furthermore, these data demonstrate an intrinsic role of Notch1 in regulating helper T cell differentiation.

**CANONICAL Notch SIGNALING IS NOT REQUIRED FOR ACTIVATION AND PROLIFERATION OF CD4⁺ T CELLS**

Several studies have highlighted an emerging role of non-canonical Notch signaling in controlling helper T cell differentiation (5, 34). This is potentiated by interactions of the intra-cellular domain of Notch with other proteins besides RBP-Jκ. Several studies have highlighted an emerging role of non-canonical Notch signaling in controlling helper T cell differentiation (5, 34). This is potentiated by interactions of the intra-cellular domain of Notch with other proteins besides RBP-Jκ. Some of these alternate binding partners include (but are not limited to) NF-κB, T-bet, GATA3, PI3K, and Akt (3–5, 15, 21–23, 35). However, whether canonical and non-canonical Notch signaling differs in their ability to influence T cell activation and differentiation is not well defined. Hence, to determine the importance of RBP-Jk-dependent, canonical Notch signaling, we conditionally knocked-out RBP-Jk in peripheral T cells by breeding mice carrying RBP-Jkfl/fl Mx Cre⁻/⁻ mice expressing Cre recombinase under the control of the Mx promoter. CD4⁺ T cells from RBP-Jkfl/fl Mx Cre⁻/⁻ mice (abbreviated as cRBP-Jk) expressed substantially reduced transcript and protein levels of RBP-Jk in comparison to cN1KO mice expressing Cre recombinase under the control of the Mx promoter.
mice (abbreviated as controls) (Figures S4A,B in Supplementary Material). Additionally, cRBP-Jκ-KO mice had significantly fewer peripheral CD4+ and CD8+ T cells in their spleens (Figures S4C,D in Supplementary Material). To determine how canonical Notch signaling influenced activation, CD4+ T cells from control and cRBP-Jκ-KO mice were stimulated in vitro with anti-CD3ε and anti-CD28. Absence of RBP-Jκ did not alter the production of T cells in their spleens (Figures S4C,D in Supplementary Material). T o determine how canonical Notch signaling is not required for activation and proliferation of CD4+ T cells, CD4+ T cells were isolated from control or cRBP-Jκ-KO mice and stimulated with plate-bound anti-CD3ε and anti-CD28. Cells were harvested after 48 h (A-I) or at indicated times. (J-L) Surface staining was performed on CD4, CD25, CD69, and intracellular staining for N1κ and analyzed by flow cytometry. (A,D,G) Dot plots obtained from flow cytometry showing CD4+ T cells positive for (A) N1κ, (D) CD25, and (G) CD69. Numbers in each quadrant represent percentage of cells. Data represent three independent experiments. [B,E,H] Percentage and absolute numbers of cells positive for (B) N1κ, (E) CD25, and (H) CD69 obtained from dot plots n = 4. [C,F,I] Histograms for (C) N1κ, (F) CD25, and (I) CD69. Data represent three independent experiments. [J] Counts per minute (CPM) obtained after 1H-thymidine uptake in CD4+ T cells from control and cRBP-Jκ-KO mice stimulated as described above with and without rmIL-2 (20 ng/ml). Data represent three independent experiments. [K] IL-2 and [L] IFN-γ ELISA from supernatants obtained from control and cRBP-Jκ-KO mice stimulated as described above n = 6. Data represent mean ± SEM, ns, not significant.

**ACTIVATION AND PROLIFERATION OF CD4+ T CELLS IS RBP-Jκ-INDEPENDENT BUT NOTCH AND NF-κB DEPENDENT**

To confirm that RBP-Jκ-independent activation and proliferation was in fact Notch dependent but RBP-Jκ-independent, we used the following strategies. We first inhibited activation of Notch in CD4+ T cells from cRBP-Jκ-KO by treating these cells with GSI. To control for the off-target effects of GSIs, we also treated cells with a Notch sparing GSI (NS-GSI) that inhibited all GSI substrates except Notch. GSI treatment of CD4+ T cells from cRBP-Jκ-KO mice inhibited intra-cellular Notch (Figure 6A) and significantly reduced the expression of the activation markers CD25 and CD69 (Figures 6B,C). This was accompanied by a significant decrease in the cytokines IL-2 and IFN-γ (Figures 6D,E). Importantly, NS-GSI treatment rescued Notch activation as well as CD25, CD69, and IL-2 (Figures 6A–D). A partial rescue was observed with IFN-γ.
We have shown that the intra-cellular Notch. The role of Notch and NF-κB activation markers after inhibiting NF-κB-dependent on NF-κB-dependent processes, were also impaired proliferation (Figures 6G–J). Furthermore, DHMEQ treatment did not alter the levels of N1IC (Figure 6F), DHMEQ treated CD4+ T cells from cRBP-Jκ-κ-κKO mice showed a significant reduction in the amounts of CD25, CD69, IL-2, and IFN-γ (Figures 6G–J). Furthermore, DHMEQ treatment of CD4+ T cells from cRBP-Jκ-κ-κKO animals significantly impaired proliferation (Figure 6K). These data show that activation and proliferation of CD4+ T cells is an RBP-Jκ-independent but Notch dependent process. Furthermore, our data suggest that non-canonical Notch signaling may control these processes, at least in part through NF-κB.

**RBP-Jκ-DEFICIENCY DOES NOT ALTER CD4+ T CELL DIFFERENTIATION IN VITRO**

Deletion of either Notch1 or RBP-Jκ has been shown to have different outcomes on helper T cell differentiation, suggesting that acquisition of helper T cell fate may be differentially influenced through canonical and non-canonical Notch signaling pathways.
by canonical and non-canonical Notch signaling. Whether the absence of RBP-Jk influenced polarization of CD4+ T cells in vitro was determined by skewing cells from control or cRBP-Jk-KO animals toward Th1 and Th2 cell fates. While the number of cells secreting IFN-γ was reduced in the CD4+ T cells from cRBP-Jk-KO mice (Figures 7A,B), the amount of IFN-γ secreted under Th11 conditions was unaffected (Figure 7C). Levels of T-bet remained unchanged in the absence of RBP-Jk in comparison to controls (Figures 7D–F). Similarly, RBP-Jk-deficiency did not alter polarization toward Th12. Although, CD4+ T cells from RBP-Jk-KO animals had lower amounts of GATA3 (Figures 7G–I) this decrease did not influence the number of IL-4 positive cells (Figures 7A,B) or the amount of secreted IL-4 under Th12 conditions in vitro (Figure 7C). In addition to Th11 and Th12 phenotypes, the absence of RBP-Jk did not impair the ability to induce regulatory T cells. The number of CD25+Foxp3+ cells was significantly increased in cRBP-Jk-KO animals (Figures 7J,K). These data show that differentiation of CD4+ T cells in vitro does not require canonical Notch signaling.

**DISCUSSION**

Several studies have demonstrated the generation of the active, intra-cellular domain of Notch in T cells stimulated via the TCR accompanied by co-stimulation (15, 16). In this report, we address precisely where Notch exerts influence downstream of the TCR signaling cascade. We specifically outline the kinetics of Notch activation in peripheral T cells and suggest that Notch is most important for regulating signaling events distal to the TCR. We show that inhibition of Notch activation had no effect on phosphorylation of Zap70, a proximal TCR signaling event. However, GSI treatment effectively abolished the expression of T cell activation markers – CD25, CD69, IL-2, and IFN-γ, events that occur several hours following TCR stimulation. In addition, GSI treatment also abolished the expression of c-Myc most prominently at 14–48 h post TCR stimulation. While these data do not exclude a role of Notch in affecting other early TCR events besides phosphorylation of Zap70, they suggest that Notch has a very significant influence on distal events. Furthermore, our data also reveal a critical influence of Notch activation on TCR signal strength. We
show that stimulating T cells with increasing concentrations of either anti-CD3ε or antigen pulsed APCs, increased the amount of N1IC in proportion to increasing signal strength. Although, we did observe basal levels of N1IC expression in CD4+ T cells stimulated with CHO-APCs in the absence of ova-peptide, CHO-APCs express low levels of Jagged 1 and likely contribute to the basal expression of N1IC. Most importantly, abrogating Notch activation either via GSI treatment or conditional deletion significantly increased the threshold of signaling via the TCR. While Notch has been implicated in influencing strength of signal in thymocytes (23), our data demonstrate for the first time a role of Notch as a regulator of signal strength in peripheral CD4+ T cells. We also observed a concomitant increase in c-Myc in response to increasing signal. While a similar response has been recently documented in peripheral T cells (24), our data add to these data by showing that an increase in c-Myc in response to increasing signal strength is, in fact, Notch dependent. These data also suggest that Notch may likely exert control over signal strength via c-Myc. However, further experimentation is required to investigate the precise mechanism that underlies Notch dependent regulation of signal strength.

Many studies have implicated a role of Notch in regulating peripheral T cell responses using GSIs to inhibit Notch activity (4, 15, 16). However, the use of GSIs obscures the specific contribution of Notch1 in regulating these processes since GSIs inhibit all isoforms of the Notch receptor and have multiple substrates, some of which are important in immune function (17). Thus, GSI treatment may not inhibit Notch but other type I transmembrane proteins as well suggesting that attenuation of T cell responses post GSI treatment may not necessarily be due to Notch1. Here, we specifically address such concerns by conditionally deleting Notch1 using the Mx Cre system, which produces “acute” deletion of Notch1 in peripheral T cells. CD4+ T cells from cN1KO animals showed a significant reduction in CD25, CD69, IL-2, and IFN-γ coupled with impaired proliferation that could not be rescued in the presence of exogenous IL-2. In addition, although CD4+ T cells from cN1KO animals expressed Notch2 and Notch3, it was not sufficient to rescue activation or proliferation. Thus, our data show that activation and proliferation are in fact Notch dependent processes that specifically require Notch1.

The precise function of Notch signaling in determining T1H versus T1H2 lineage decisions remains controversial partially due to the disparate methods used to attenuate Notch signaling. Inhibition of Notch signaling via GSIs blocked T1H1 cell fate in vitro and in vivo by preventing Notch mediated up-regulation of T-bet but did not alter T1H2 responses in vitro (4). On the contrary, inhibition of RB-Jk function either by genetic deletion or by using dominant negative co-activators of RB-Jk such as dnMAML (dominant negative MAML), which mimics a loss-of-Notch function phenotype, preserved T1H1 responses but dampened T1H2 responses in vivo (18, 21–23, 33). In models of RB-Jk deletion, the generation of N1IC is preserved (Figure 5). This is particularly important since N1IC has also been documented to interact with other proteins besides RB-Jk (3–5). Therefore, we reasoned that since Notch1 is known to interact with proteins other than RB-Jk, N1IC may be capable of functioning in an RB-Jk independent fashion and such “non-canonical” signaling could serve to reconcile existing differences about the precise role of Notch in influencing T cell differentiation. To this end, we generated mice with a conditional deletion of either Notch1 or RB-Jks and determined whether deleting different components of the Notch pathway produced distinct phenotypes. Furthermore, we specifically chose to study how the absence of Notch signaling affects T cell differentiation in vitro to delineate a cell-intrinsic role of Notch in controlling effector T cell responses in contrast to previously used in vivo approaches, which cannot distinguish between extrinsic and intrinsic effects.

We show that conditionally deleting Notch1 attenuates T1H1 responses in vitro as observed by a significant decrease in the percentage of cells secreting IFN-γ, the amount of secreted IFN-γ and the amount of T-bet expressed suggesting that Notch1 is in fact required for T1H1 decisions. Another study has shown that deleting Notch1 under the control of a CD4 Cre promoter does not dampen T1H1 responses in vitro. We suggest that the differences between this study and ours may most likely be due to the different approaches used to delete Notch since deletion under the control of the CD4 Cre promoter, deletes Notch during thymic development. Apart from T1H1, we also show that deletion of Notch1 impaired the ability to generate induced regulatory T cells. Inhibition of Notch via GSIs has also been shown to decrease iTreg populations, suggestive of a requirement for Notch1 in regulating these responses. Although, we observed a decrease in both T1H1 and iTreg populations in the absence of Notch1, we did not see any decrease in T1H2 responses in vitro. We have shown previously that inhibition of Notch via GSIs under T1H2 conditions does not alter IL-4 production (4). Corroborating these observations, another study showed that genetic deletion of preselinin, a component of the γ-secretase complex, did not alter T1H2 responses in vitro (41). Our T1H2 data concur with these reports suggesting that Notch1 is dispensable for intrinsic acquisition of a T1H2 cell fate in vitro.

To determine the contribution of RB-Jk dependent, canonical Notch signaling in regulating activation and differentiation of peripheral CD4+ T cells, we conditionally deleted RB-Jks once again under the control of the Mx promoter facilitating acute deletion. CD4+ T cells from cRB-Jk-KO animals expressed N1IC upon TCR stimulation. Strikingly, contrary to CD4+ T cells from cN1KO animals, CD4+ T cells from cRB-Jk-KO animals were not deficient in activation or proliferation and expressed all activation markers at identical levels to CD4+ T cells from control mice suggesting an RB-Jk independent role of Notch signaling in regulating these processes. We confirmed this by showing that only after intra-cellular Notch is inhibited in CD4+ T cells lacking RB-Jk, activation and proliferation can be decreased. Additionally, an NS-GSI could “rescue” activation and proliferation in the absence of RB-Jk. The NS-GSI only partially rescued IFN-γ. A possible explanation for this observation is that Notch may regulate IFN-γ production via an intermediary molecule, which is a GSI target and is hence not spared by the NS-GSI. While these data suggest only a partial requirement of N1IC for IFN-γ production, determining the precise molecular players that interact with Notch to regulate IFN-γ, requires further experimentation. Our data concur with previously reported...
observations that showed no overt effect of RBP-Jk deletion on T cell activation or proliferation (23, 41–43). However, our study is the first to suggest that this is due to non-canonical Notch signaling as N1IC could compensate for the absence of RBP-Jk.

The mechanism by which non-canonical Notch signaling regulates activation, proliferation, and differentiation requires further investigation. Our data along with that of others suggest NF-κB to be the most likely candidate (3, 15, 36, 37). Thus, to determine if some of the effects of non-canonical Notch signaling require NF-κB, we inhibited NF-κB in the absence of RBP-Jk. Inhibition of NF-κB did not alter the levels of intra-cellular Notch1 but still decreased the expression of CD25, CD69, IL-2, and IFN-γ in CD4+ cells from cRBP-Jk-KO animals NF-κB inhibition also attenuated proliferation in the absence of RBP-Jk. Furthermore, attenuation of NF-κB in the absence of RBP-Jk attenuated a T111 response in vitro since DHMEQ treatment reduced the expression of IFN-γ and T-bet in cells lacking RBP-Jk (Figures S6C,D in Supplementary Material), suggesting that RBP-Jk independent but Notch1 dependent regulation of these responses may require NF-κB. Additionally, inhibiting NF-κB significantly affected T cell activation only at a distal time point of 48 h and abolished the expression of c-Myc suggesting that Notch may require NF-κB to control these processes (Figure S5 in Supplementary Material). However, deciphering the precise mechanism of such NF-κB mediated non-canonical Notch signaling is an ongoing area of investigation that requires further experimentation.

To extend the contribution of non-canonical Notch signaling to helper T cell differentiation, we validated T112, and iTreg responses in vitro in the absence of RBP-Jk. In stark contrast to CD4+ T cells from cN1KO animals which showed a markedly dampened T111 response, CD4+ T cells from cRBP-Jk-KO mice secreted IFN-γ and expressed T-bet just as efficiently as controls. These data once again showcase RBP-Jk independent, but Notch1 dependent regulation of effector T cell responses. A recent study has also suggested such RBP-Jk independent Notch signaling in regulating T111 responses in vivo by showing that CD4+ T cells lacking RBP-Jk could mount a protective response to Leishmania major but those lacking both the Notch1 and Notch2 receptors could not (34). In addition to T111, we observed an increase in the number of CD25 FoxP3+ double positive cells in the absence of RBP-Jk suggesting that induction of regulatory T cells may also rely on non-canonical Notch signaling. This observation was in contrast to a significant decrease of the same subset seen in the absence of Notch1 (Figures 4J,K). These differences can be reconciled by the fact that cRBP-Jk-KO mice continue to express elevated levels of N1IC. Since N1IC is required for T-reg lineage determination, an increase in N1IC expression in the absence of RBP-Jk, could be responsible for a significant increase in the regulatory T cell population. Additionally, since RBP-Jk is a transcriptional repressor, deletion of RBP-Jk could de-repress FoxP3, resulting in an increase in the number of CD25 FoxP3+ T-regs. Finally, we did not observe an effect of RBP-Jk deficiency on the secretion of T112 cytokines in vitro. Our data do not support an intrinsic role for Notch1 signaling in T112 responses. However, since Notch1 acts upstream of IL-4 and Notch1 has been shown to regulate IL-4 secretion by NKT cells, we suggest that Notch1 regulates T112 responses extrinsically and may instead regulate in vivo IL-4 production.

In conclusion, our in vitro approach resolves discrepancies about the role of Notch signaling in CD4+ T cell function by showing that Notch1 regulates T cell activation, proliferation, and differentiation in a cell-intrinsic fashion. Importantly, our data demonstrate for the first time that RBP-Jk independent, non-canonical Notch signaling regulates activation, proliferation, and acquisition of T111 and iTreg fates in vitro. Such non-canonical Notch signaling most likely involves NF-κB. Evidence of non-canonical Notch signaling has been observed during axon guidance as well as dorsal closure during embryonic development in Drosophila (44, 45). In addition, a cytosolic function of Notch is required for survival of neural stem cells (46). Non-canonical Notch signaling has recently been shown to influence the IL-6/JAK/STAT pathway in breast tumors in a fashion that requires NF-κB (47). Additionally, mammary tumor development has been shown to occur independently of RBP-Jk. Further studies are required to delineate the precise molecular mechanisms underlying non-canonical Notch signaling however our data as well as others support a role for NF-κB in mediating non-canonical Notch signaling.

MATERIALS AND METHODS

ANIMALS

All animals were housed in animal facilities as per the guidelines approved by the Institutional Animal Care and Use Committee at the University of Massachusetts-Amherst. C57BL/6J mice and BALB/c-Tg (DO11.10)10Loh/J were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). cN1KO and cRBP-Jk-KO mice were generated by breeding Notch1ββ (Notch1tm2KosGru) or Rbp-jkββ (Rbpjktm2Har) mice to mx1Cre± (B6.Cg-Tg[mx1cre]1Cgn/J) mice from the Jackson Laboratory (Bar Harbor, ME, USA). All mice – Notch1ββ × Mx Cre± mice (cN1KO), Notch1ββ × Mx Cre−/− (Control), RBP-Jkββ × Mx Cre± (cRBP-Jk-KO) and RBP-Jkββ × Mx Cre−/− (Control) were injected with 12–15 µg/g body weight of Poly I:Poly C (GE Healthcare, Imgenex) every other day for 5 days. Animals were sacrificed after a 3-week resting period. Mice aged 7–12 weeks were used for all experiments.

T CELL ISOLATION AND IN VITRO ASSAYS

CD4+ T cells were isolated by magnetic separation using anti-CD4 magnetic particles (BD Pharmingen). Cells were activated in vitro by plating 3 × 10⁶ cells/ml on each well of a 12-well plate pre-coated with anti-CD3ε and anti-CD28 purified from 145-2c11 and 37N hybridoma cell lines respectively and cross-linked with anti-Hamster IgG (Sigma). Cells were activated in a half and half mixture of RPMI and DMEM (Lonza) supplemented with 10% Fetal Bovine Serum (GIBCO), l-Glutamine, Na-pyruvate, and Penicillin/Streptomycin (Lonza). The following conditions were used for polarization. T111: 10 µg/ml anti-IL-4 and 1 ng/ml recombinant mouse IL-12 (BD Pharmingen). T112: 10 µg/ml anti-IFN-γ and 1 ng/ml of recombinant mouse IL-4.
(BD Pharmingen). iTreg: CD4+CD25− cells were enriched from bulk splenocytes using the mouse CD4 T-lymphocyte enrichment set with 2.5 μg biotin conjugated anti-CD25 (BD Pharmingen). Cells were activated in the presence of 2 ng/ml of recombinant human TGFβ1 (R&D systems). Cytokines from supernatants were determined using a standard ELISA assay (BD Pharmingen). For cytokine uptake assays, 3 × 10^5 cells/ml were activated in 96-well plates pre-coated with anti-CD3e and anti-CD28 in the presence or absence of 20 ng/ml recombinant mouse IL-2 (BD Pharmingen). Cells were pulsed with ^3H-thymidine (Perkin Elmer) at a final concentration of 1 μCi/rxn in the final 16 h of activation. Cells were harvested on a mash harvester into scintillation vials. Counts per minute were obtained on a scintillation counter (Beckman). For CFSE assays, CD4+ T cells were labeled with 1 μM CFSE using the manufacturer’s protocol (Molecular Probes). Cells were stimulated for 48 h before analysis by flow cytometry. For drug treatments, CD4+ T cells were pretreated with 50 μM of the GSI z-IL-CHO, 5 μM NS-GSI JLK-6 (Tocris Bioscience), or 2 μM of DHMEQ at 37°C for 30 min before activation.

**WESTERN BLOT**

Whole cell lysates were made in RIPA buffer (150 mM NaCl, 1% IgeCal-CA 360, 0.1% SDS, 50 mM Tris, pH 8.0, 0.5% Sodium deoxycholate). Cytoplasmic and nuclear proteins were extracted as per the manufacturer’s instructions (Thermo Scientific). Lysates were resolved on an SDS-PAGE gel. Protein was transferred on a nitro-cellulose membrane and blocked in Blotto (5% milk powder, 0.2% Tween-20 in PBS). Membranes were probed overnight with primary antibody. Membranes were washed and incubated with horse-radish peroxidase (HRP) labeled secondary antibody. Membranes were developed using ECL reagents (Amersham). Primary Antibodies: anti-RBPSUH, anti-phospho Zap70 (Y319), anti-Zap70, anti-HDAC (Cell signaling), anti-c-Rel (Santa Cruz Biotechnology) anti-Actin (Sigma), anti-c-Myc (9E10) was obtained from Dr. Dominique Alfantari. Secondary Antibody: anti-Rabbit-HRP, anti-Mouse-HRP (Amersham).

**STATISTICAL ANALYSIS**

All data are represented as mean ± SEM. Statistical Analysis was performed using the GraphPad Prism 5 software. p Values were calculated using an unpaired two-tailed Student’s t-test.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fmmu.2014.00054/abstract

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