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Non-canonical Notch signaling drives activation and differentiation of peripheral CD4\(^+\) T cells

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ABSTRACT

Cleavage of the Notch receptor via a γ-secretase, results in the release of the active intracellular domain of Notch that migrates to the nucleus and interacts with RBP-Jκ, resulting in the activation of downstream target genes. This canonical Notch signaling pathway has been documented to influence T-cell development and function. However, the mechanistic details underlying this process remain obscure. In addition to RBP-Jκ, the intracellular domain of Notch also interacts with other proteins in the cytoplasm and nucleus, giving rise to the possibility of an alternate, RBP-Jκ independent Notch pathways. However, the contribution of such RBP-Jκ independent, “non-canonical” Notch signaling 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 T1 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.

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 intracellular domain of Notch by γ-secretase. This active, intracellular 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 intracellular 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 (T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17, T-reg) depending on the cytokine milieu. Interleukin-12 and IFN-γ polarize CD4\textsuperscript{+} T cells to the T\textsubscript{H}1 phenotype. T\textsubscript{H}1 cells express the lineage specific transcription factor T-bet, secrete the signature cytokine IFN-γ, and provide protection against intra-cellular pathogens (7). T\textsubscript{H}2 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 T\textsubscript{H}17 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 (iT-regs), which are also induced by TGF-β, are characterized by the expression of FoxP3 and exhibit immunosuppressive functions (12-14).

Ligation of the TCR accompanied by co-stimulation, generates intra-cellular Notch in CD4\textsuperscript{+} T cells while inhibition of Notch activation with γ-secretase inhibitors (GSI) decreases T cell activation 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 T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17 and iTreg lineages (4, 18-20). Pharmacological inhibition of Notch using GSIs dampens the ability to adopt a T\textsubscript{H}1 fate by attenuating the expression of T-bet (4). However, inhibition of signaling downstream of RBP-J\textsubscript{K} via genetic deletion or by using dominant negative MAML inhibits adoption of a T\textsubscript{H}2 fate in vivo while preserving a T\textsubscript{H}1 phenotype (21-23). Given the ability of intra-cellular Notch to interact with proteins different from RBP-J\textsubscript{K}, it is possible that disparate results could be attributed to RBP-J\textsubscript{K} independent 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\textsuperscript{+} T-cells specifically requires Notch1 but not RBP-J\textsubscript{K} since conditional deletion of Notch1 impaired these processes while conditional deletion of RBP-J\textsubscript{K} had no effect. Such non-canonical, RBP-J\textsubscript{K} independent regulation of these processes likely occurs via NF-κB. Conditional deletion of Notch1 also impaired polarization to T\textsubscript{H}1 and induction of regulatory T cells in vitro. Nevertheless, RBP-J\textsubscript{K} deficiency did not impair T\textsubscript{H}1 or iTreg cell fate in vitro once again supporting a novel role of non-canonical Notch signaling in controlling differentiation towards these lineages. In vitro polarization to T\textsubscript{H}2 was not affected in the absence of either Notch1 or RBP-J\textsubscript{K}. Our in vitro observations demonstrate a cell intrinsic function of RBP-J\textsubscript{K} 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

1. 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 hrs. after activation and the amount of N1IC increased over time (Fig 1A). This increase was abrogated after GSI treatment (Fig 1A). Inhibition of Notch activation did not alter proximal signaling events as evidenced by intact phosphorylation of Zap 70 even in GSI treated cells (Fig 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 (Fig 1C-F). This decrease was most prominent close to 48 hrs 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 upto 6 hrs after activation and peaks at distal time points (Supp. Fig 1A,B). 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 (Supp. Fig 1 C-H). 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 (Supp. Fig. 1 I, K). Collectively, these data show that N1IC is generated in CD4+ T cells after stimulating via the TCR and influences distal TCR signaling events.

2. 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 ligation 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 (Fig 2A). This was also accompanied by an increase in the percentage of cells expressing N1IC and this increase could be inhibited by GSI treatment (Fig 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 (Fig 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 (Fig 2D,E). This increase could be blocked after GSI treatment (Fig 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. (Fig 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 4hrs and 24hrs 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 48hrs post stimulation (Fig 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 (Fig 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.

3. 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 Notch1fl/fl Mx Cre+/- mice (abbreviated as cN1KO) and Notch1fl/fl 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
N1IC expression. (Supp. Fig 2A, D)(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 (Supp. Fig 2C).
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 (Supp. Fig 2E, F) To investigate the specific contributor of Notch1 in
influencing activation of peripheral T cells, CD4+ T cells from cN1KO or control animals
were stimulated in vitro with anti-CD3ε 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 (Fig. 3A,B,D,E). Since
cN1KO mice were knocked-down for Notch1, as an internal control we gated on the
small percentage of residual Notch1 positive cells in the cN1KO animals. These cells
continued to express high levels of CD25 and CD69. However, cells deficient for Notch1
expressed lower levels of both activation markers (Fig 3C,F). This decrease was
accompanied by a significant reduction in the levels of IL-2 and IFN-γ secreted post-
activation (Fig 3G, H). Whether CD4+ T cells from cN1KO animals were also impaired
in their ability to proliferate was determined by measuring the incorporation of ³H-
thymidine. Proliferative capability was significantly diminished in the absence of Notch1
and could not be rescued by adding exogenous IL-2 (Fig 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 hrs
after activation (Supp. Fig 3A,B). 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 (Fig 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.

4. 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 T_{H1} versus T_{H2} lineage decisions is unclear. While
some studies have shown that inhibiting Notch using GSIs diminishes the ability of CD4+
T cells to adopt a T_{H1} 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 polarized in vitro to T_{H1}, T_{H2} or iTreg lineages. Absence of Notch1 impaired T_{H1}
differentiation in vitro. CD4+ T cells from cN1KO mice had significantly fewer cells that
stained positive for intra-cellular IFN-γ (Fig 4A, B). Secreted IFN-γ was also reduced
significantly in the absence of Notch1 (Fig 4C). This decrease was accompanied by a reduction in the amount of the master T\(_h\)1 transcription factor T-bet (Fig 4D, E, F). In contrast, no marked effect was observed in T\(_h\)2 differentiation. Although, the amount of GATA3 was reduced in the absence of Notch1 (Fig 4G, H, 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 (Fig 4A, B) and expressed comparable levels of secreted IL-4 (Fig 4C). Whether CD4\(^+\) T cells from cN1KO mice proliferated differently under different polarizing conditions, was determined by \(^3\)H-thymidine uptake under non-polarized (NP), T\(_h\)1 and T\(_h\)2 conditions. Proliferative capability of CD4\(^+\) T cells from cN1KO mice was the same under different polarizing conditions (Supp. Fig 4C,D), despite of differences in cytokine secretion. In addition to T\(_h\)1, Notch1 deficiency significantly reduced induced T-reg populations as observed by a significant decrease in the frequency of CD25\(^+\)FoxP3\(^+\) cells in cN1KO animals (Fig 4J, K). These results show that Notch1 is required for T\(_h\)1 and iTreg differentiation but is dispensable for T\(_h\)2 cell fate acquisition in vitro. Furthermore, these data demonstrate an intrinsic role of Notch1 in regulating helper T cell differentiation.

5. 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-Jk in the cytoplasm and nucleus. 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 differ 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-Jk loxp-flanked sites to mice expressing Cre recombinase under the control of the Mx promoter. CD4\(^+\) T cells from RBP-Jk \(^{fl/fl}\) Mx Cre \(^{+/-}\) mice (abbreviated as cRBP-Jk -KO) expressed substantially reduced transcript and protein levels of RBP-Jk in comparison to to CD4\(^+\) T cells from RBP-Jk \(^{fl/fl}\) Mx Cre \(^{-/-}\) mice. (abbreviated as controls). (Supp Fig 4A,B). Additionally, cRBP-Jk -KO mice had significantly fewer peripheral CD4\(^+\) and CD8\(^+\) T cells in their spleens (Supp Fig 4C,D). To determine how canonical Notch signaling influenced activation, CD4\(^+\) T cells from control and cRBP-Jk -KO mice were stimulated in vitro with anti-CD3e and anti-CD28. Absence of RBP-Jk did not alter the production of intra-cellular Notch1 after TCR stimulation (Fig 5A, B, C). In contrast to impaired activation observed in the absence of Notch1 (Fig 3), RBP-Jk deficiency did not alter the activation of CD4\(^+\) T cells. On the contrary, CD4\(^+\) T cells from cRBP-Jk -KO animals produced marginally higher numbers of CD4\(^+\) T cells expressing activation the markers CD25 and CD69 and displayed slightly elevated amounts of each marker (Fig D-I). CD4\(^+\) T cells from both cRBP-Jk –KO mice secreted IL-2 and IFN-γ just as efficiently as Controls. (Fig 5K,L). Proliferation of CD4\(^+\) T cells was unaffected by the absence of RBP-Jk irrespective of addition of recombinant IL-2 (Fig 5J). These results suggest that activation and proliferation of CD4\(^+\) T cells is not impaired in the absence of RBP-Jk. However,
since these processes required Notch1, our data show that activation and proliferation occurs independently of canonical Notch signaling.

6. 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 (Fig 6A) and significantly reduced the expression of the activation markers CD25 and CD69 (Fig 6B,C). This was accompanied by a significant decrease in the cytokines IL-2 and IFN-γ (Fig 6D,E). Importantly, NS-GSI treatment rescued Notch activation as well as CD25, CD69 and IL-2 (Fig 6A-D). A partial rescue was observed with IFN-γ (Fig 6E). Furthermore, a decrease in proliferation of CD4+ T cells from cRBP-Jκ-KO mice after GSI treatment was also rescued by the NS-GSI (Fig 6L-M). These data suggest that while canonical Notch signaling is dispensable for the activation and proliferation of peripheral CD4+ T cells, these processes require intra-cellular Notch. The role of Notch and NF-κB in regulating T cell activation and differentiation processes has been well documented (3, 15, 36, 37). We have shown that the intra-cellular domain of Notch1 binds to and exerts some of its effects via NF-κB suggestive of cross talk between the two pathways (3, 15). Hence we asked whether these RBP-Jκ-independent processes, were also dependent on NF-κB. This was determined by examining activation markers after inhibiting NF-κB in CD4+ T cells lacking RBP-Jκ using a pharmacological inhibitor, Dehydroxymethyl-epoxyquinomicin (DHMEQ) (38). DHMEQ has been used to block NF-κB activity in different types of solid tumors, malignant cells and T cells (39, 40). DHMEQ treatment effectively inhibited nuclear translocation of NF-κB (Supp. Fig 6A,B). Although DHMEQ treatment did not alter the levels of N11γ (Fig 6F), DHMEQ treated CD4+ T cells from cRBP-Jκ-KO mice showed a significant reduction in the amounts of CD25, CD69, IL-2 and IFN-γ (Fig 6G-J). Furthermore, DHMEQ treatment of CD4+ T cells from cRBP-Jκ-KO animals significantly impaired proliferation (Fig 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.

7. 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 by canonical and non-canonical Notch signaling. Whether the absence of RBP-Jκ influenced polarization of CD4+ T cells in vitro was determined by skewing cells from control or cRBP-Jκ -KO animals towards T1H1 and T1H2 cell fates. While the number of cells secreting IFN-γ was reduced in the CD4+ T cells from cRBP-Jκ-KO mice (Fig 7A, B), the amount of IFN-γ secreted under T1H1 conditions was
unaffected (Fig 7C). Levels of T-bet remained unchanged in the absence of RBP-Jκ in comparison to controls (Fig 7D, E, F). Similarly, RBP-Jκ -deficiency did not alter polarization towards T\(_{H2}\). Although CD4\(^+\) T cells from RBP-Jκ-KO animals had lower amounts of GATA-3 (Fig 7G, H, I) this decrease did not influence the number of IL-4 positive cells (Fig 7A, B) or the amount of secreted IL-4 under T\(_{H2}\) conditions in vitro (Fig 7C). In addition to T\(_{H1}\) and T\(_{H2}\) phenotypes, the absence of RBP-Jκ did not impair the ability to induce regulatory T- cells. The number of CD25\(^+\)Foxp3\(^+\) cells was significantly increased in cRBP-Jκ-KO animals (Fig 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, intracellular 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 hrs 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-CD3\(\varepsilon\) or antigen pulsed APCs, increased the amount of N1\(^{IC}\) in proportion to increasing signal strength. Although we did observe basal levels of N1\(^{IC}\) 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 N1\(^{IC}\). 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 only 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 cNIKO 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 cNIKO 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 Th1 versus Th2 lineage decisions remains controversial partially due to the disparate methods used to attenuate Notch signaling. Inhibition of Notch signaling via GSIs blocked Th1 cell fate in vitro and in vivo by preventing Notch mediated up-regulation of T-bet but did not alter Th2 responses in vitro (4). On the contrary, inhibition of RBP-Jk function either by genetic deletion or by using dominant negative co-activators of RBP-Jk such as dnMAML (dominant negative Master-mind-Like) which mimics a loss-of-Notch function phenotype, preserved Th1 responses but dampened Th2 responses in vivo (18, 21-23, 33). In models of RBP-Jk deletion, the generation of N1ic is preserved (Fig 5). This is particularly important since N1ic has also been documented to interact with other proteins besides RBP-Jk (3-5). Therefore we reasoned that since Notch1 is known to interact with proteins other than RBP-Jk, N1ic may be capable of functioning in an RBP-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 RBP-Jk 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 Th1 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 Th1 decisions. Another study has shown that deleting Notch1 under the control of a CD4 Cre promoter does not dampen Th1 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 Th1, 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 Notch in regulating these responses. Although we observed a decrease in both Th1 and iTreg populations in the absence of Notch1, we did not see any decrease in Th2 responses in vitro. We have shown previously that inhibition of Notch via GSIs under Th2 conditions does not alter IL-4 production (4). Corroborating these
observations, another study showed that genetic deletion of presenilin, a component of the γ-secretase complex, did not alter T_{H2} responses in vitro (41). Our T_{H2} data concur with these reports suggesting that Notch1 is dispensable for intrinsic acquisition of a T_{H2} cell fate in vitro.

To determine the contribution of RBP-J_K dependent, canonical Notch signaling in regulating activation and differentiation of peripheral CD4^+ T cells, we conditionally deleted RBP-J_K once again under the control of the Mx promoter facilitating acute deletion. CD4^+ T cells from cRBP-J_K−KO animals expressed N1^K upon TCR stimulation. Strikingly, contrary to CD4^+ T cells from cN1KO animals, CD4^+ T cells from cRBP-J_K−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 RBP-J_K 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 RBP-J_K, can activation and proliferation be decreased. Additionally, an NS-GSI could “rescue” activation and proliferation in the absence of RBP-J_K. 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 N1^K 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-J_K 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 N1^K could compensate for the absence of RBP-J_K.

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-J_K. 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-J_K−KO animals NF-κB inhibition also attenuated proliferation in the absence of RBP-J_K. Furthermore, attenuation of NF-κB in the absence of RBP-J_K attenuated a T_{H1} response in vitro since DHMEQ treatment reduced the expression of IFN-γ and T-bet in cells lacking RBP-J_K (Supp. Fig 6C, D), suggesting that RBP-J_K 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 48hrs and abolished the expression of c-Myc suggesting that Notch may require NF-κB to control these processes. (Supp. Fig 5). 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 T_{H1}, T_{H2} and iTreg responses in vitro in the absence of RBP-J_K. In stark contrast to CD4^+ T cells from cN1KO animals which showed a markedly dampened T_{H1} response, CD4^+ T cells from cRBP-J_K-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 in vitro. A recent study has also suggested such RBP-Jk independent Notch signaling in regulating Tg1 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 Tg1, 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 (Fig. 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 Tg2 cytokines in vitro. Our data do not support an intrinsic role for Notch1 signaling in Tg2 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 Tg2 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 Tg1 and iTreg fates in vitro. Such non-canonical Notch signaling most likely involves NF-kB. 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-kB (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-kB 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). cN1KO and cRBP-Jk-KO mice were generated by breeding Notch1fl/fl (notch1tm2Rko/Gridd1) or Rbp-Jkfl/fl (Rbpjtm1Hon) mice to mx1Cre+/-.
(B6.Cg-Tg(mx1-cre)1Cgn/J) mice from the Jackson Laboratory (Bar Harbor, ME). All mice - Notch1^{fl/fl} x Mx Cre^{+/−} mice (cNIKO), Notch1^{fl/fl} x Mx Cre^{−/−} (Control), RBP-Jk^{fl/fl} x Mx Cre^{+/−} (cRBPP-Jk-KO) and RBP-Jk^{fl/fl} x 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 x 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.

T₃₁: 10 µg/ml anti-IL-4 and 1 ng/ml recombinant mouse IL-12 (BD Pharmingen). T₃₂: 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 2ng/ml of recombinant human TGFβ1 (R& D systems). Cytokines from supernatants were determined using a standard ELISA assay (BD Pharmingen). For thymidine uptake assays, 3 x 10⁵ cells/ml were activated in 96-well plates pre-coated with anti-CD3ε 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 1µci/rxn in the final 16 hours 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 1µM CFSE using the manufacturer’s protocol (Molecular Probes). Cells were stimulated for 48 hrs 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 mins before activation.

**Strength of Signal Assays**

CD4⁺ T cells were isolated from the spleens of C57BL/6J as described and stimulated with the indicated concentrations of anti-CD3ε and 1 µg/ml of anti-CD28 (BD Pharmingen) for 48 hrs. Cells were analyzed by flow cytometry and IL-2 was detected in the supernatants by an ELISA. For co-culture experiments, empty vector Chinese Hamster Ovary – Antigen presenting cells (CHO-APCs) were fixed by treating with Mitomycin C (Sigma) 37°C for 45 mins. 6 x 10⁵ cells/ml of CHO-APCs were pulsed with the indicated concentrations of Ova peptide 323-339 (Genscript). CD4⁺ T cells were isolated from spleens of BALB/c-Tg(DO11.10)10Loh/J and mixed with peptide pulsed APCs at 2.5 x 10⁶ cells/ml in each well of a 12 well plate. Cells were harvested after 48 hrs and analyzed by flow cytometry after gating on CD4⁺ T cells expressing the DO.11.10 TCR. Supernatants were used for detection of IL-2 by an ELISA.
Flow Cytometry

Surface staining was performed in PBS with 1% BSA using the indicated antibodies - CD25-APC, CD69-FITC (eBioscience), CD4-PerCP (BD Pharmingen), Anti-mouse DO.11.10-TCR Biotin (Invitrogen), Streptavidin PerCP (BD Pharmingen). Intra-cellular staining was performed for detection of intra-cellular Notch1, T-bet, GATA3 and FoxP3 using the FoxP3 staining buffer set (eBioscience) and the following antibodies: anti-Human/Mouse Notch1-PE, anti-Human/Mouse T-bet PE-Cy7, anti-Human/Mouse GATA-3 eFluor 660, anti-Mouse/Rat FoxP3 Alexa 488 (eBioscience). For detection of intra-cellular cytokines, cells were harvested at indicated time points and re-stimulated with plate bound anti-CD3ε in the presence of Golgi Plug (IFN-γ) or Golgi Stop (IL-4) (BD Pharmingen) for 5 hrs. Intra-cellular cytokine staining was performed using the BD Cytofix/CytoPerm plus kit and cytokines detected using anti-Mouse IFN-γ FITC and anti-Mouse IL-4 PE (BD Pharmingen). Flow cytometry data was acquired on a FACS LSR II (BD) and analyzed using FlowJo software (Trestar) after gating on CD4+ T cells or as indicated.

Western Blot

Whole cell lysates were made in RIPA buffer (150mM NaCl, 1% IgeCal-CA 360, 0.1% SDS, 50mM 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 a transferred on a nitro-cellulose membrane and blocked in Blotto (5% milk powder, 0.2% Tween-20 in PBS). Membranes were probed over-night 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 Zap 70 (Y319), anti-Zap70, anti-HDAC (Cell signaling), anti-cRel (Santa Cruz Biotechnology) anti-Actin (Sigma), anti-cMyc (9E10) was obtained from Dr. Dominique Alfandari. 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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CONFLICT OF INTEREST

The authors declare no competing financial and commercial conflict of interest

AUTHOR CONTRIBUTIONS

A.D., L.S., R.G.L. performed experiments and analyzed data. A.D., L.S., R.G.L. and B.A.O. designed experiments with contributions from L.M.M., L.M. and T.E.G. A.H.F. synthesized GSIs and DHMEQ. B.A.O. conceived the study, supervised experimental design and interpretation of data. A.D. and B.A.O. wrote the manuscript.

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FIGURE LEGENDS

**Fig 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 (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, ***, p < 0.001.

**Fig 2: Notch as a regulator of signal strength**

(A) Histogram for N1IC expressed in CD4+ T cells stimulated with the indicated concentrations of anti-CD3ε and 1 μg/ml of anti-CD28 for 48 hrs. Data represent three independent experiments. (B-C) CD4+ T cells were pretreated with DMSO or GSI and activated with the indicated concentrations of anti-CD3ε and 1 μg/ml of anti-CD28 for 48 hrs. (B) Percentage of cells expressing N1IC as determined by flow cytometry. (C) IL-2 ELISA from supernatants. n = 3-5. (D-F) CD4+ T cells were isolated from DO.11.10 – TCR transgenic mice and pretreated with DMSO or GSI prior to co-culture with CHO-APCs expressing MHC-II and B7. They were pulsed with the indicated concentrations of ova peptide 323-339. Cells were harvested after 48 hrs and analyzed by flow cytometry after gating on D0.11.10 TCR positive CD4+ T cells. (D) Histogram for N1IC expressed in CD4+ T cells expressing the D0.11.10 TCR stimulated with the indicated concentrations of ova-peptide. Data represent three independent experiments. (E) Percentage of cells expressing N1IC as determined by flow cytometry. n = 3. (F) IL-2 ELISA from supernatants. n = 3. (G) Western Blot for phosphorylated and total c-Myc. 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. Whole cell lysates were made at each time and analyzed by Western Blotting. Data represent three independent experiments. (H) Integrated density values obtained after normalizing phospho and total c-Myc to Actin. (I) Western Blot for c-Myc expressed in CD4+ T cells stimulated as described in (A). Data represent three independent experiments. Data represent the mean ± SEM, *, p < 0.05, ** p < 0.005, ***, p < 0.001.

**Fig 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 48hrs (3A-F, J) or at indicated
times (3G-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 % of cells. (B) % of cells and cell counts for cells positive for CD25
and (E) CD69. (A, D). n=4. (C, F) Histograms for CD25 and CD69 after gating on Notch
negative cells from cN1KO mice (dashed line), Notch positive cells from cN1KO mice
(dotted line) or CD4+ T cells from Control mice (solid line). Data represent four
independent experiments. (G) IL-2 and (H) IFN-γ ELISA from supernatants obtained
from control and cN1KO mice stimulated as described above. n = 4. (I) Counts per
minute (CPM) obtained after 3H-thymidine uptake in CD4+ T cells from control and
cN1KO mice stimulated as described above with and without rmIL-2 (20ng/ml). Data
represent three independent experiments. (J) IL-2 ELISA from supernatants of CD4+ T
cells from Control or cN1KO mice stimulated with the indicated concentrations of anti-
CD3ε and 1 µg/ml of anti-CD28 for 48 hrs. n=3-5. Data represent mean ± SEM. *, p <
0.05, ** p < 0.005.

Fig 4: Notch1 is required for T H1 differentiation and production of iTregs in vitro
CD4+ T cells from control and cN1KO mice were differentiated under T H1, T H2 or iTreg
inducing conditions for 3 days followed by re-stimulation with plate bound anti-CD3ε.
Cells were analyzed by flow cytometry. Supernatants were used for ELISA. (A) Dot plots
obtained from flow cytometry showing intra-cellular staining for IFN-γ and IL-4.
Numbers in each quadrant represent % of cells. Data represent 3-5 independent
experiments. (B) % and absolute cell numbers of IFN-γ or IL-4 positive cells determined
by flow cytometry. n=3-5. (C) IFN-γ and IL-4 production under T H1 and T H2 conditions
respectively determined by an ELISA. Each data point represents one animal. (D, G) Dot
plots showing intra-cellular staining for (D) T-bet and N1IC under T H1 conditions (G) or
GATA3 and N1IC under T H2 conditions. Numbers in each quadrant represent % of cells.
Data represent 3-5 independent experiments. (E, H) % and absolute numbers of double
positive cells as determined by flow cytometry. n = 3-5. (F) Histograms for T-bet and (I)
GATA3 expression under T H1 and T H2 conditions respectively. Data represent 3-5
independent experiments. (J) Dot plots for CD25+ and FoxP3+ cells. Numbers in each
quadrant represent % of cells. Data represent 3 independent experiments. (K) % and
absolute cell numbers of double positive cells determined by flow cytometry. n=3. Data
represent mean ± SEM. *, p < 0.05, ** p < 0.005, ***, p < 0.001, ns – not significant.

Fig 5: Canonical Notch signaling is not required for activation and proliferation of
CD4+ T cells
CD4+ T cells were isolated from control or cRBPGK-KO mice and stimulated with plate
bound anti-CD3ε and anti-CD28. Cells were harvested after 48hrs (A-I) or at indicated
times. (J-L). Cells were surface stained for CD4, CD25, CD69 and intra-cellular stained
for N1IC and analyzed by flow cytometry. (A, D, G) Dot plots obtained from flow
cytometry showing CD4+ T cells positive for (A) N1IC (D) CD25 and (G) CD69.
Numbers in each quadrant represent % of cells. Data represent three independent
experiments. (B, E, H) Percentage and absolute numbers of cells positive for (B) N1IC (E)
CD25 and (H) CD69 obtained from dot plots. n=4. (C, F, I) Histograms for (C) N1IC (F)
CD25 and (I) CD69. Data represent three independent experiments. (J) Counts per
minute (CPM) obtained after $^3$H-thymidine uptake in CD4$^+$ T cells from control and cRBPJ$\kappa$-KO mice stimulated as described above with and without rmIL-2 (20ng/ml). Data represent three independent experiments. (K) IL-2 and (L) IFN-γ ELISA from supernatants obtained from control and cRBPJ$\kappa$-KO mice stimulated as described above. n = 6. Data represent mean ± SEM, ns - not significant.

**Fig 6:** Activation and Proliferation of CD4$^+$ T cells is RBPJ$\kappa$ independent but Notch and NF-κB dependent.

CD4$^+$ T cells were isolated from cRBPJ$\kappa$-KO mice, pretreated with DMSO, GSI or NS-GSI (A-E, L, M) or DHMEQ (F-J) and stimulated with plate bound anti-CD3ε and anti-CD28 for 24 hrs. Cells were surface stained for CD4, CD25 and CD69 and intra-cellular stained for N1$^{\text{IC}}$ and analyzed by flow cytometry. Supernatants were used to detect IL-2 and IFN-γ by an ELISA Mean Fluorescent Intensity (MFI) values were plotted for (A, F) N1$^{\text{IC}}$ (B, G) CD25 (C, H) CD69. n =3-5. Histograms to the right of (A-C) show expression of N1$^{\text{IC}}$, CD25 and CD69. Data represent three independent experiments. (D, I) IL-2 and (E, J) IFN-γ as determined by an ELISA. (K) Counts per minute (CPM) obtained after $^3$H-thymidine uptake in CD4$^+$ T cells from cRBPJ$\kappa$-KO mice treated with DMSO or DHMEQ and stimulated for 48hrs. (L-M) Histograms representing a CFSE Proliferation Assay. CD4$^+$ T cells from cRBPJ$\kappa$-KO mice pretreated with DMSO, GSI, or NS-GSI were labeled with CFSE and activated with plate bound anti-CD3ε and anti-CD28 for 48 hrs followed by flow cytometry analysis. Data represent three independent experiments. (M) Bar graph showing the % of CFSE negative cells obtained by flow cytometry. Data represent three independent experiments. Data represent mean ± SEM. * p < 0.05, ** p < 0.005, *** p < 0.001, ns - not significant.

**Fig 7:** RBPJ$\kappa$ deficiency does not alter CD4$^+$ T cell differentiation in vitro

CD4$^+$ T cells from control and cRBPJ$\kappa$-KO mice were differentiated under T$_{\text{H}1}$, T$_{\text{H}2}$ or iTreg inducing condition for 3 days followed by re-stimulation with plate bound anti-CD3. Cells were analyzed by flow cytometry. Supernatants were used for ELISA. (A) Dot plots showing intra-cellular staining for IFN-γ and IL-4. (B) % and absolute numbers of IFN-γ or IL-4 positive cells determined by flow cytometry. Numbers in each quadrant represent % of cells. n>3. (C) IFN-γ and IL-4 production under T$_{\text{H}1}$1 and T$_{\text{H}2}$ conditions respectively determined by an ELISA. Each data point represents one animal. (D, G) Dot plots showing intra-cellular staining for (D) T-bet and N1$^{\text{IC}}$ under T$_{\text{H}1}$ conditions and (G) GATA3 and N1$^{\text{IC}}$ under T$_{\text{H}2}$ conditions. (E, H) Percentages and absolute numbers of double positive cells determined by flow cytometry. n = 5. (F, I) Histograms for T-bet and GATA3 expression under T$_{\text{H}1}$ and T$_{\text{H}2}$ conditions respectively. Data represent at least 3 independent experiments. (J) Flow cytometry plots for CD25$^+$ and FoxP3$^+$ cells. (K) Percentages and absolute numbers of double positive cells determined by flow cytometry. n= 3. Data represent mean ± SEM. * p < 0.05, ns - not significant. Data represent three to five independent experiments.
Supplementary Figures: LEGENDS

Figure S1: (A,B) Percentage of DLL1 and Jagged1 positive cells. CD4+ T cells from C57Bl/6 mice were stimulated in vitro with plate bound anti-CD3ε and anti-CD28 under T_H1 or T_H2 conditions. Cells were harvested at the indicated times, surface stained for DLL1 and Jagged1 and analyzed by flow cytometry. n = 3. (C-H) CD4+ T cells were stimulated as described in the presence of 1 μg/ml of recombinant DLL1 or Jagged1. Cells were harvested after 48hrs, surface stained for CD4, CD25 and CD69 and intracellular stained for N1IC and analyzed by flow cytometry. Percentage of cells positive for (C) N1IC (D) CD25 (E) CD69. Histograms for (F) N1IC (G) CD25 (H) CD69. Data represent at least 3 independent experiments. n = 3-5. (I, J) CD4+ T cells were isolated from DO.11.10-TCR transgenic mice and co-cultured with CHO-APCs expressing either DLL1, Jagged1 or empty vector in addition to MHC II and B7. These were pulsed with 2.5 μg/ml ova peptide 323-339 under T_H1, T_H2 or non-polarized (NP) conditions. Cells were harvested after 4 days and re-stimulated with plate-bound anti-CD3ε. Supernatants were used for detecting cytokine levels by an ELISA. (I) IFN-γ and (J) IL-4 levels as determined by an ELISA. Data represent mean ± SEM. * p < 0.05, ns - not significant.

Figure S2: CD4+ T cells were isolated from control or cN1KO mice and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48hrs. (A, B, C) RNA was isolated and reverse transcribed to cDNA. Expression of (A) Notch1 (B) Notch2 and (C) Notch3 transcripts was determined by real time PCR and analyzed by the \(2^{-\Delta\DeltaCT}\) method. Results are presented as fold change in gene expression after normalization to actin and expressed relative to controls. (D) Histogram for N1IC expressed in stimulated CD4+ T cells from C57BL/6J wild type, control or cN1KO mice. Data represent at least 5 independent experiments. (E, F) Splenocytes from control and cN1KO mice were surface stained for CD4 and CD8 and analyzed by flow cytometry. (E) Dot plots for CD4+ and CD8+ cells obtained by flow cytometry. Numbers in each quadrant represent % of cells. Data represent five independent experiments. (F) Percentage of CD4+ and CD8+ T cells obtained from the dot plots. n = 5. Data represent the mean ± SEM. * p < 0.05, ** p < 0.005, *** p < 0.001, ns - not significant.

Figure S3: (A, B) CD4+ T cells were isolated from control or cN1KO mice and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48hrs. (A, B, C) Histograms showing Annexin V expression obtained by flow cytometry, representative of 3 independent experiments. (B) % and absolute cell numbers of Annexin V+ cells obtained by flow cytometry. n = 3. (C) Counts per minute (CPM) obtained after \(^{3}\)H-thymidine uptake. CD4+ T cells were isolated from control or cN1KO mice and stimulated in with plate bound anti-CD3ε and anti-CD28 under Non-Polarized (NP), T_H1 and T_H2 conditions for 72hrs. Data represent three independent experiments. ns – not significant.

Figure S4: (A) RNA was isolated from CD4+ T cells from control and cRBPJKO mice and reverse transcribed to cDNA. Expression of RBPJK transcript was determined by real time PCR and analyzed by the \(2^{-\Delta\DeltaCT}\) method. Results are presented as fold change in
gene expression after normalization to actin and expressed relative to controls. (B) Western Blot for lysates obtained from CD4+ T cells from control and cRBPJκ-KO mice. (C) Splenocytes from control and cRBPJκ-KO mice were surface stained for CD4 and CD8 and analyzed by flow cytometry. Numbers in each quadrant represent % of cells. Data represent five independent experiments. (D) Percentage of CD4+ and CD8+ T cells. n = 5. Data represent the mean ± SEM, *, p < 0.05.

Figure S5: Splenocytes from C57BL/6J mice were pretreated with DMSO or DHMEQ 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. Supernatants were used for detecting cytokines by an ELISA. Mean Fluorescent Intensity (MFI) values were plotted for (A) N1IC (B) CD25 (C) CD69. (D) IL-2 and (E) IFN-γ as detected by an ELISA. n = 3-5 (F, G) Whole cell lysates were made at the indicated time points and analyzed by western blotting for the expression of (F) phosphorylated and (G) total c-Myc. Data represent two independent experiments. Data represent the mean ± SEM, *, p < 0.05, ** p < 0.005, ***, p < 0.001.

Figure S6: (A, B) CD4+ T cells isolated from cRBPJκ-KO mice were pretreated with DMSO, DHMEQ or GSI and stimulated with plate bound anti-CD3ε and anti-CD28 in vitro. Cytoplasmic and nuclear extracts were obtained from cells harvested after 24 hrs. These lysates were used to determine the expression of c-Rel by (A) Western blot. C - cytoplasmic extract; N - nuclear extract. (B) Bar graph showing integrated density values obtained after normalizing c-Rel expression to Histone deacetylase (HDAC). Data represent two independent experiments. (C, D) CD4+ T cells isolated from cRBPJκ-KO mice were pretreated with DMSO or DHMEQ under Th1 polarizing conditions for 24 hrs. Cells were analyzed by flow cytometry and supernatants were used for detecting cytokine levels by an ELISA. (C) IFN-γ detected by an ELISA. Each data point represents one animal. (D) Mean Fluorescent Intensity (MFI) of T-bet expressed in CD4+ T cells. n = 5. Data represent the mean ± SEM, *, p < 0.05.
Figure 1

(A) N1\textsuperscript{c} (MFI)

Time of activation (hrs)

(B) DMSO

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GSI

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P-Zap70 (Y319)

Zap70

(C) CD25 (MFI)

Time of activation (hrs)

(D) CD69 (MFI)

Time of activation (hrs)

(E) IL-2 (ng/ml)

Time of activation (hrs)

(F) IFN-\gamma (ng/ml)

Time of activation (hrs)
Figure 7

(A) - (D) Flow cytometry analysis of T_{H1} and T_{H2} cells in Control and cRBP-J_k-KO mice.

(E) - (H) Quantification of N1 IC binding to T-bet and GATA3 in Control and cRBP-J_k-KO mice.

(I) - (J) Comparison of T-bet expression in Control and cRBP-J_k-KO mice.

(K) - (L) Proportion of T_{H2} cells in Control and cRBP-J_k-KO mice.
Supplementary Figure 2

(A) **Notch1**

(B) **Notch2**

(C) **Notch3**

(D) Relative Expression (AU)

(E) **Control**

(F) % of max

(G) CD8

(H) CD4
Supplementary Figure 3

(A) Control vs. cN1KO Annexin V analysis showing 53.3% vs. 66.5% Annexin+ cells.

(B) Comparison of Annexin + (%) between Control and cN1KO with no significant difference (ns).

(C) Comparison of H3-Thymidine Uptake (CPM) between Non-polarized, TH1, and TH2 cells, with no significant differences (ns) between groups.
Supplementary Figure 5

(A) N1c (MFI) vs. Time of activation (hrs)
(B) CD25 (MFI) vs. Time of activation (hrs)
(C) CD69 (MFI) vs. Time of activation (hrs)

(D) IL-2 (ng/ml) vs. Time of activation (hrs)

(E) IFN-γ (ng/ml) vs. Time of activation (hrs)

(F) Western blot analysis for P-c-Myc and Actin with DMSO and DHMEQ treatment.

(G) Western blot analysis for c-Myc and Actin with DMSO and DHMEQ treatment.
Supplementary Figure 6

(A) 

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75 kD  
c-Rel

42 kD  
Actin

68 kD  
HDAC

(B) 

Integrated Density (c-Rel)

- DMSO
- DHMEQ
- GSI

(C) 

[Graph showing IFN-γ levels with DMSO and DHMEQ treatment]

(D) 

[Graph showing T-bet (MFI) levels with DMSO and DHMEQ treatment]