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NON-CANONICAL NOTCH SIGNALING REGULATES ACTIVATION AND DIFFERENTIATION OF PERIPHERAL CD4+ T CELLS

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NON-CANONICAL NOTCH SIGNALING REGULATES ACTIVATION AND DIFFERENTIATION OF PERIPHERAL CD4$^+$ T CELLS

A Dissertation Presented

By

ANUSHKA DONGRE

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DEDICATION

To my loving husband, Chiraag Bhadana

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ABSTRACT

NON-CANONICAL NOTCH SIGNALING DRIVES ACTIVATION AND DIFFERENTIATION OF CD4$^+$ T CELLS

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Cleavage of the Notch receptor via a γ-secretase, results in the release of the active intra-cellular domain of Notch that migrates to the nucleus and interacts with RBP-$J_{K,}$ 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-J_K, the intra-cellular domain of Notch also interacts with other proteins in the cytoplasm and nucleus, giving rise to the possibility of an alternate, RBP-J_K independent Notch pathway. However, the contribution of such RBP-J_K independent, "non-canonical" Notch signaling in regulating peripheral T-cell responses is unknown. 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-J_K, we show that Notch1 regulates activation and proliferation of CD4$^+$ T cells independently of RBP-J_K. Furthermore, differentiation towards T_H1 and *i*Treg

lineages is also Notch dependent but RBP-J_K independent. Our data provide evidence that non-canonical regulation of these processes likely occurs through NF-$_K$B. Additionally, we also provide evidence suggestive of cross-talk between Notch and the mTOR pathway. Notch1, but not RBP-J_K, is required for phosphorylation of several substrates directly downstream of mTORC2. Collectively, these striking observations demonstrate that many of the cell intrinsic functions of Notch occur independently of RBP-J_K. This reveals a previously unknown, novel role of non-canonical Notch signaling in regulating peripheral T-cell responses.

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CHAPTER 1

INTRODUCTION

1.1 The Notch signaling pathway

1.1.1 The Notch receptor and its ligands

The Notch protein was first discovered in *Drosophila melanogaster*, when a loss-of-function mutation produced a "notched" wing phenotype (Morgan *et al.,*1917). Mammals express four Notch receptors (Notch 1,2,3 and 4), which differ in the composition of their structural components (Fig 1.1A) (Baron, 2003). The Notch receptor consists of an extra-cellular domain, a trans-membrane domain and an intra-cellular domain. The extra-cellular domain is comprised of epidermal growth factor (EGF)-like repeats. Notch1 and Notch2 have 36 repeats while Notch3 and Notch4 have 34 and 29 respectively. The trans-membrane domain comprises of a cysteine rich lineage (LIN) domain that prevents ligand-independent activation and a hetero-dimerization domain. The intra-cellular domain is cytosolic and is comprised of an RBP-J_K - associated molecule domain (RAM) that binds the transcription factor RBP-J_K, an ankyrin repeat domain (ANK) that mediates protein-protein interactions, two nuclear localization sequences (NLS), a transcriptional activation domain (TAD) and a proline-glutamate-serine and threonine-rich domain (PEST) that regulates protein stability

and turn-over. The TAD domain is present only in Notch1 and Notch2 (Osborne and Minter, 2007). The Notch receptor can be activated by five different ligands that belong to two different families of proteins. These include Delta-like (DLL1, DLL3 and DLL4) and Jagged (Jagged1 and Jagged 2) ligands (Fig 1.1B) (Baron, 2003). All ligands contain a conserved sequence Delta/Serrate/Lag2 (DSL) at the amino terminus, which is involved in receptor binding, followed by EGF-like repeats. Jagged ligands also contain an additional cysteine rich (CR) domain located close to the plasma membrane. The extra-cellular domain of the Notch receptor can be glycosylated by the Fringe glycosyltransferases (Manic fringe, Lunatic fringe, Radical fringe) (Radtke *et al*., 2010), which in turn dictate ligand binding. Glycosylation by Fringes usually promotes binding of the Notch receptor to DLL ligands.

1.1.2 Canonical Notch signaling

Activation of the Notch pathway is a multi-step process that involves several proteolytic cleavage events (Fig 1.3). The Notch receptor in the endoplasmic reticulum transits to the golgi after fucosylation by O-fucosyltransferase (Logeat, F *et al*., 2008). In the golgi, the newly synthesized Notch receptor is cleaved at the S1 site by a furin-like protease resulting in a non-covalently linked , heterodimeric receptor. This mature form of the receptor is then expressed on the cell surface where it can bind to Notch ligands. Ligand binding presumably induces a conformational change that exposes the S2 site,

which is subsequently cleaved by an ADAM (a disintegrin and a metalloproteinase) protease. The receptor is then endocytosed following ubiquitylation of the intra-cellular domain. The final cleavage is then mediated by a γ-secretase at the S3 site (Mumm JS *et al.,* 2000). This cleavage releases the active, intra-cellular domain of Notch (N^{IC}), which translocates into the nucleus and interacts with its canonical-binding partner RBP-J_K (murine homolog) also called as CSL (CBF-1 in mammals, Suppressor of hairless in *Drosophila melanogaster* and Lag 1 in *Caenorhabditis elegans*). RBP-J_K, is a transcriptional repressor. However, following binding to N^{IC}, several co-activators such as MAML (Mastermind-like) and p300 are recruited to the N^{IC}- RBP-J_K complex. This results in conversion of RBP-J_K from a repressor to an activator of transcription. Activation of RBP-J_K leads to the transcription of several Notch target genes, such as those belonging to the *Hes* (Hairy/enhancer-of-split) or *Hey* (Hairy/enhancer-of-split related) families. Such RBP-J_K dependent signaling is called canonical Notch signaling.

1.1.3 Non-Canonical Notch signaling

In addition to canonical Notch signaling described above, any Notch signaling that occurs independently of RBP-J_K is defined as "non-canonical" Notch signaling. Recent reports in the literature suggest that N^{IC} can interact with several proteins besides RBP-J_K in the cytoplasm and nucleus (Minter *et al.,* 2005; Perumalsamy *et al.,* 2009; Shin *et al.,* 2006), suggesting an RBP-J_K –

independent mechanism for propagation of Notch signals. Some of these alternative binding partners of N^{IC} include (but are not limited to) NF-κB, PI3K, Akt, T-bet and GATA3. We have shown previously that Notch and NF-κB exist in a complex on the *Ifn-γ* promoter even in the absence of RBP-J_K (Shin *et al.,* 2006; Cho *et al.,* 2009). Additionally, all NF-κB binding sites contain within them a consensus binding sequence for RBP-$J_{K,}$ while the reverse may not be true (Minter and Osborne, 2012). Notch has also been shown to bind to PI3K and is required for the phosphorylation of Akt (Sade *et al*., 2004). Furthermore Notch exerts a protective, anti-apoptotic effect in an mTORC2-dependent but RBP-J_K independent fashion in cell lines (Perumalsamy *et al.*, 2009). These data also imply a cytosolic function of N^{IC} apart from its well-documented nuclear function. Evidence of non-canonical Notch signaling has also been observed during axon guidance as well as dorsal closure during embryonic development in Drosophila (Crowner *et al.,* 2003; Zecchini *et al.,* 1999)In addition, a cytosolic function of Notch is required for survival of neural stem cells (Androutsellis-Theotokis *et al.,* 2006). 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 (Jin *et al.,* 2013). Additionally, mammary tumor development has been shown to occur independently of RBP-Jκ.

1.1.4 Inhibitors of Notch signaling

Pharmacological inhibition of Notch signaling is often accomplished by the use of γ-secretase inhibitors (GSI), which inhibit γ-secretase mediated cleavage at the S3 site and prevent the release of N^{IC}. γ-secretase is a multi-protein protease complex that processes type I membrane proteins. Besides Notch, γ-secretase has 89 other substrates (Beel and Sanders, 2008). Hence GSIs are not always Notch-specific and may have several off-target effects. This drawback is often compensated by the use of Notch-sparing GSIs (NS-GSI) that inhibit all γ-secretase substrates except Notch.

1.2 Notch and T cells

1.2.1 T cell activation and differentiation

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 (Fig 1.4). Helper T cells can differentiate into several different lineages (T_H1, T_H2, T_H17, T_H9, T-reg, Tfh) depending on the cytokine

milieu (Fig 1.4). IL-12 and IFN- γ polarize $CD4^+$ T cells towards the T_H1 phenotype. T_H1 cells express the lineage specific transcription factor T-bet, secrete the signature cytokine IFN-γ, and provide protection against intra-cellular pathogens (Szabo et al., 2002). T_H2 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 (Zheng and Flavell, 1997). Il-6 and TGF-β generate T_H17 cells, which provide protection against nematodes and fungal infections, secrete signature cytokines IL-17 and IL-23 and express ROR-γt (Langrish et al., 2005; Murphy et al., 2003; Yang et al., 2008). *i*T-regs which are also induced by TGF-β, are characterized by the expression of FoxP3 and exhibit immuno-suppressive functions (Chen *et al*., 2003; Fu et al., 2004; Rao et al., 2005).

1.2.2 The role of Notch in T cell function.

Notch signaling is plays a critical role in specification of the T cell fate. Deletion of Notch in developing thymocytes abrogates the production of T cells at the expense of B cells (Pui *et al.,* 1999)On the other hand, over-expression of Notch in bone-marrow progenitors elicits the onset of T cell leukemia (Radtke *et al.,* 1999). Apart from its role in thymocyte development, several studies have demonstrated a role of Notch in regulating peripheral T cell function. We have shown that activation of peripheral T cells with anti-CD3ε and anti-CD28 triggers activation of the Notch pathway (Adler *et al.,* 2003; Palaga *et al.,* 2003) and its

inhibition via GSI treatment abrogates activation, proliferation and IFN- γ production by peripheral T cells (Palaga *et al*., 2003). Additionally, Notch is also required for IL-2 secretion and expression of the high affinity IL-2 receptor, CD25 (Adler *et al*., 2003).

The role of Notch activation in regulating helper T cell differentiation is controversial. Notch signaling has been shown to regulate differentiation towards T_H1, T_H2, T_H17 and *i*Treg lineages. Pharmacological inhibition of Notch using GSIs dampens the ability to adopt a T_H1 fate (but not T_H2) by attenuating the expression of T-bet (Minter *et al*., 2005). However, inhibition of signaling downstream of RBP-J_K dampens the ability to adopt a T_H2 fate *in vivo* by regulating the expression of IL-4 and GATA3 while preserving a T_H1 phenotype. (Amsen *et al*., 2007; Fang *et al*., 2007)Another study showed that mice lacking Notch signaling were unable to mount a protective T_H2 response to *Trichuris muris*, but effectively mounted a T_H1 response against *Leishmania major in vivo* (Tu *et al*., 2005). We have also shown that inhibition of Notch activation attenuates the adoption of a T_H17 cell fate (Keerthivasan *et al*., 2011) and also impairs the induction of regulatory T cells by influencing the expression of FoxP3 (Samon *et al*., 2008). Additionally, Notch ligands can also influence instruction of helper T cell fates (Radtke *et al*., 2010). Thus, while several studies have demonstrated a role for Notch in specification of several helper T cell fates, a clear understanding of these pleiotropic effects of Notch is lacking.

1.3 NF-κB

1.3.1 The NF-κB pathway

NF-κB is a transcription factor that allows cells to respond to extra-cellular stimuli such as stress, DNA damage and cytokines (Hoffmann and Baltimore, 2006). In the context of T cells, NF-κB has been implied in regulating T cell activation, proliferation and differentiation (Vallabhapurapu and Karin, 2009)The NF-κB family of transcription factors consist of five members – Rel A, Rel B, c-Rel, p50 and p52, the later two being mature forms generated after processing of larger precursors p105 and p100 respectively (Fig 1.5A). All members contain a Rel homology domain at the N-terminus responsible for homo and hetero-dimerization and sequence specific DNA binding (Gilmore, 2006). Furthermore, Rel A, Rel B and c-Rel have an additional transcriptional activation domain. NF-κB exists as a homo or hetero-dimer that is sequestered in the cytoplasm by its association with the Inhibitor of κB (IκB). Upon receiving an activation signal, the IκB kinase (IKK) phosphorylates IκB, leading to its subsequent ubiquitylation and proteasomal degradation. This releases NF-κB dimers, which migrate to the nucleus and gain access to transcriptional machinery (Fig 1.5B).

1.3.2 Inhibitors of the NF-κB pathway

Several pharmacological inhibitors have been employed to inhibit activation of the NF-κB pathway. One of these includes the compound Dehydroxymethyl-epoxyquinomicin (DHMEQ). DHMEQ is a new NF-κB inhibitor that is a 5-dehydroxymethyl derivative of a novel compound epoxyquinomicin (Matsumoto *et al.,* 2009). DHMEQ prevents nuclear translocation of NF-κB by sequestering it in the cytoplasm. To this end, it has been used to block NF-κB activity in different types of solid tumors, malignant cells and T cells (Horie *et al.,* 2006)

1.3.3 NF-κB and T cell function

Activation of T cells via the TCR can trigger the activation of NF-κB. Following T cell activation, the scaffolding molecules Bcl-10, CARMA1 and Malt1 recruit and induce the activity of IKK, which in turn activates NF-κB by phosphorylation and subsequent degradation of IκB. NF-κB is critical for T cell activation as it is primarily thought to regulate survival and proliferative signals downstream of the TCR (Vallabhapurapu and Karin, 2009)NF-κB controls the expression of IL-2, the high affinity receptor for IL-2, IL-2Rα and IFN-γ (Vallabhapurapu and Karin, 2009). Additionally, NF-κB has also been documented to instruct acquisition of helper T cell fate. Mice lacking p50 were unable to mount airway inflammation due to downregulation of GATA3 but were

unimpaired in their ability to express T-bet. Conversely, mice lacking c-Rel had a defect in T-bet expression and secretion of IFN-γ(Das *et al.,* 2001). Thus different NF-κB sub-units may have unique roles in specification of particular helper T cell fates.

1.3.4 Cross talk between the Notch and NF-κB pathways in T cells.

Several studies have demonstrated cross-talk between the Notch and NF-κB pathways. One study demonstrated that activation of the Notch pathway via Jagged1, induces maturation of keratinocytes via NF-κB (Nickoloff *et al.,* 2002). We have demonstrated that inhibition of Notch activation via GSIs reduces NF-κB activity in peripheral T cells (Palaga et al., 2003). Furthermore, constitutive NF-κB activity is observed in Notch3 transgenic mice (Barbarulo *et al.,* 2011; Vacca *et al.,* 2006)Furthermore, Notch has been shown to physically interact with NF-κB and regulate its activity by sustaining its nuclear localization (Shin *et al.,* 2006). Specifically, $N1^{IC}$ could be immuno-precipitated with p50 and c-Rel. Furthermore, complexes of $N1^{IC}$ and NF-κB were also found on the *Ifn-γ* promoter suggesting that Notch may regulate the expression of IFN- γ via NF-κB (Shin *et al.,* 2006). In addition to $CD4^+$ T cells, cross-talk between the two pathways has also been observed in $CD8^+$ T cells. Notch could be complexed with NF-κB from the promoters of *EOMES*, *perforin* and *granzyme B* and this interaction was abolished after GSI treatment (Cho *et al.,* 2009).

1.4 mTOR

1.4.1 The mTOR pathway

The mechanistic target of Rapamycin (mTOR) functions as an environmental sensor that integrates several extrinsic stimuli to ultimately regulate cell growth and metabolism. mTOR was discovered during genetic experiments in yeast and mammals as a target of the macrolide Rapamycin (Sabatini *et al*., 1994) and belongs to the phosphoinositide 3- kinase (PI3K) family. mTOR can exist in two different complexes – mTORC1 and mTORC2 depending on the association of mTOR with unique proteins (Fig 1.6). In the mTORC1 complex, mTOR associates with the regulatory associated protein of mTOR (RAPTOR) in addition to mLST8, Deptor and Pras40. In the mTORC2 complex, mTOR associates with the Rapamycin insensitive companion of TOR (RICTOR) along with PROTOR, mSIN1, mLST8 and Deptor (Fig 1.6). RAPTOR containing mTORC1 is sensitive to Rapamycin. In contrast, RICTOR containing mTORC2 is insensitive to Rapamycin, although recent studies have demonstrated that prolonged treatment with Rapamycin can make it sensitive as well well (Zoncu *et al.,* 2011).

mTORC1 is activated by several different stimuli such as nutrients, amino acids, growth factors, stress and changing energy requirements. Activation of mTORC1 is mediated by its association with Ras homologue enriched in brain (RHEB) in its GTP bound form (Fig 1.7). RHEB is an essential activator of

mTORC1 and is controlled by growth factor inputs (Laplante and Sabatini, 2009). Upon activation, mTORC1 phosphorylates its downstream substrates - S6 kinase 1 (S6K1) and eLF4e binding protein 1 (4E-BP1), which associate with mRNAs and control the rate of protein synthesis (Ma and Blenis, 2009). In contrast to mTORC1, the upstream regulators of mTORC2 are incompletely defined. However, upon activation, mTORC2 phosphorylates the AGC family of kinases. These include phosphorylation of residue S473 in the hydrophobic motif of Akt, the Protein kinase C (PKC) family of kinases and the SGK1 kinase. Of these, phosphorylation of residue S473 of Akt is thought to be mediated exclusively by mTORC2. This phosphorylation event is one way in which the mTOR pathway merges with the PI3K pathway (Fig 1.7).

The PI3K pathway is activated by several growth factors leading to the phosphorylation of the cognate growth factor receptors creating a docking site for PI3K. PI3K then converts phosphatidylinositol – 4,5-biphosphate (PIP2) to phosphatidylinositol – 3,4,5-triphosphate (PIP3). Akt contains a pleckstrin homology domain to which PIP3 can bind. Activation of Akt is accomplished by phosphorylation at S473 by mTORC2 and T308 by PDK1. Activated Akt then activates several downstream substrates, which regulate cell survival, growth and metabolism. In addition, Akt can also feedback onto the mTOR pathway by phosphorylating the tuberous sclerosis complex TSC1/TSC2, which normally represses mTORC1 by converting RHEB-GTP to an inactive RHEB-GDP bound form. Phosphorylation of TSC1/TSC2 by Akt inactivates the complex. Inactive TSC1/TSC2 can no longer convert RHEB to its inactive GDP associated state,

ultimately leading to the activation of mTORC1 (Fig 1.7) (Laplante and Sabatini, 2009).

1.4.2 Inhibitors of mTOR pathway

In addition to Rapamycin, which can individually target Raptor and in some cases Rictor, several new pharmacological inhibitors have been designed to inhibit the activity of mTOR itself. One of these is Pp242, an ATP competitive inhibitor of mTOR. Pp242 showed a better therapeutic response than Rapamycin in a mouse model of leukemia (Janes NR *et al*., 2010). Pp242 is also more selective towards mTOR compared to other PI3K family kinases. Another small molecule catalytic inhibitor of mTOR is Ku-0063794. This inhibitor suppresses the activity of mTOR but preserves the activity of 76 other protein and lipid kinases, making it even more specific than Pp242 Pp242 (Garcia Martiner JM *et al*., 2009). While these inhibitors efficiently suppress mTOR activity and phosphorylation of the downstream substrates of mTORC1 and mTORC2, they do not suppress phosphorylation of Akt –T308 that is mediated by the PDK1 kinase in certain cells (Zoncu *et al*., 2011). Thus it is likely that Akt may retain partial activity in these circumstances.

1.4.3 mTOR and T cell function

Several recent studies have outlined a role for mTOR in regulating T cell function. Activation of T cells accompanied by co-stimulation activates the PI3K and mTOR pathways. Consistent with its role as a regulator of metabolism, TSC1/TSC2 mediated control of mTOR enforces quiescence of thymocytes by regulating cell size, cell cycle entry and metabolic machinery (Chi *et al*., 2012).. mTOR may also be partially involved in regulating peripheral T cell function as mTOR deficient T cells have delayed proliferative capacity, although they do not exhibit any defect in the expression of activation markers or secretion of IL-2 (Delgoffe *et al*., 2011).

Furthermore, the mTOR pathway has also been shown to regulate helper T cell differentiation. One study showed that mTOR is required for acquisition of T_H1, T_H2 and T_H17 fate but represses regulatory T cells, as $CD4^+$ T cells deleted for mTOR showed a spontaneous up-regulation of FoxP3 positive cells but were impaired in their ability to differentiate to T_H1, T_H2 and T_H17 cells. This was attributed to a defect in up-regulation of lineage associated transcription factors. Deletion of either mTORC1 or mTORC2 individually, also affects helper T cell differentiation. mTORC2 is thought to regulate T_H1 fate via Akt and T_H2 fate via PKCθ (Lee *et al*., 2010). Another study also demonstrated that Rictor deficient $CD4^+$ T cells were unable to polarize to T_H2 cells due to enhanced expression of the suppressor for cytokine signaling (SOCS5) (Delgoffe *et al*., 2011). This study also demonstrated that mTORC1 was required for T_H1 and T_H17 cell fates but

repressed T_H2 fate since inhibition of mTORC1 activity by deletion of RHEB, dampened T_H1 and T_H17 responses but enhanced the number of GATA3 positive cells. (Delgoffe *et al.,* 2011). Thus, although an understanding of the precise mechanism underlying the regulation of T cell differentiation by mTOR is lacking, several studies firmly implicate mTOR in this process.

1.4.4 Cross-talk between Notch and mTOR

A few studies have demonstrated that the Notch pathway impinges on the mTOR/PI3K pathway (Ciofani and Zuniga-Pflucker, 2005; Lee *et al.,* 2012; Perumalsamy *et al.,* 2009). One study demonstrated that N^{IC} physically interacts with PI3K and $p56^{Lck}$ and protects a T cell hybridoma cell line from apoptosis by enhancing mTORC2 mediated phosphorylation of Akt (Sade *et al.,* 2004). Evidence of cross-talk between the two pathways in thymocytes, was first demonstrated by Ciofani and colleagues who showed that Notch promotes survival of pre-T cells at the β-selection checkpoint by regulating glucose metabolism. DN3 thymocytes cultured on OP9 cells expressing the Notch ligand DLL1, had greater cell size, phosphorylation of Akt-S473 and higher glycolytic rate compared to thymocytes that lacked on-going Notch signals. Furthermore, this increase in metabolism was dependent on PI3K/Akt (Ciofani *et al.,* 2005). Finally, a recent study showed that inspite of on-going Notch signaling, Rictor deficient thymocytes were unable to grow, proliferate or differentiate, suggesting that Notch may relay its signals via mTORC2 (Lee *et al.,* 2012). However, while

these studies imply cross-talk between the two pathways, whether these parallels can also be drawn in peripheral T cells is unknown.

1.5 Specific Aims and Hypothesis

Activation of T cells by cross-linking the TCR, triggers the generation of $N1^{IC}$. However, the precise mechanism that leads to the generation of $N1^{IC}$ downstream of the TCR is obscure. Whether such Notch activation requires activation by Notch ligands, or whether it is a ligand independent process is unknown. Not only have Notch ligands been shown to activate Notch, but some have also been suggested to influence specification of helper T cells (Amsen *et al.,* 2004). However, whether Notch ligands are able to instruct helper T cell differentiation by altering the levels of Notch itself is unanswered. Thus, the First Aim of this study is to determine whether Notch ligands influence Notch activation and peripheral T cell function.

$N1^{IC}$ generated downstream of the TCR, plays a crucial role in regulating peripheral T cell function, since inhibition of Notch activation via GSIs dampens activation and proliferation of T cells as well as decreases their ability to produce IFN- γ (Adler *et al.,* 2003; Palaga *et al.,* 2003). However, GSIs have two drawbacks. Firstly, they inhibit all isoforms of the Notch receptor, making it unclear to determine which specific Notch receptor is required for T cell activity. Secondly, they have multiple targets besides Notch. Hence, whether the observed

effects of GSIs on T cell function are indeed due to inhibition of Notch activation, or simply an off-target effect of GSIs, is obscure.

In addition to regulating activation of T cells, Notch is also required for regulating helper T cell differentiation. We have shown that GSI treatment abrogates the ability of cells to become T_H1 by down-regulating the expression of the transcription factor T-bet, suggesting that Notch is required for acquisition of T_H1 fate (Maekawa *et al*., 2003; Minter *et al*., 2005). However other studies have shown that inhibition of canonical Notch signaling by using dnMAML, inhibits the acquisition of T_H2 fate *in vivo*, suggesting that Notch regulates T_H2 fate but is dispensable for T_H1 fate (Amsen *et al*., 2007; Fang *et al*., 2007; Tu *et al*., 2005). Whether these differences can be attributed to the different approaches used to inhibit Notch signaling is unknown. This coupled with the fact that $N1^{IC}$ can interact with partners besides RBP-J_K, suggests that Notch may regulate some processes in a "non-canonical" fashion. However, the precise contribution of such "non-canonical" Notch signaling in regulating peripheral T cell responses is unknown. Importantly, whether such non-canonical signaling can indeed reconcile different results obtained by inhibiting different components of the Notch pathway, requires further investigation. Thus, the Second Aim of this study is to determine the contribution of canonical and non-canonical Notch signaling in regulating the activation and differentiation of peripheral $CD4^+$ T cells.

Finally, while several studies have implied an alternate, RBP-J_K-independent route for Notch to relay its signals, the precise mechanism underlying such non-canonical Notch signaling is obscure. While several studies have

demonstrated a physical interaction of Notch with components of the NF-κB and mTOR pathways, the precise mechanism and functional consequences of these interactions in peripheral T cells remains to be determined. Thus, the Third Aim of this study to address whether non-canonical Notch signaling, involves cross-talk of Notch with the NF-κB and mTOR pathways.

Hence, my hypothesis is that Notch ligands influence the generation of N^{IC} downstream of the TCR. This $N1^{IC}$ regulates T cell activation and differentiation in a manner that likely involves RBP-Jκ- independent, non-canonical Notch signaling. Such non-canonical signaling involves interaction with the NF-κB and mTOR pathways.

I plan to address my hypothesis and fulfill the aims of this study by pursuing the following three specific aims:

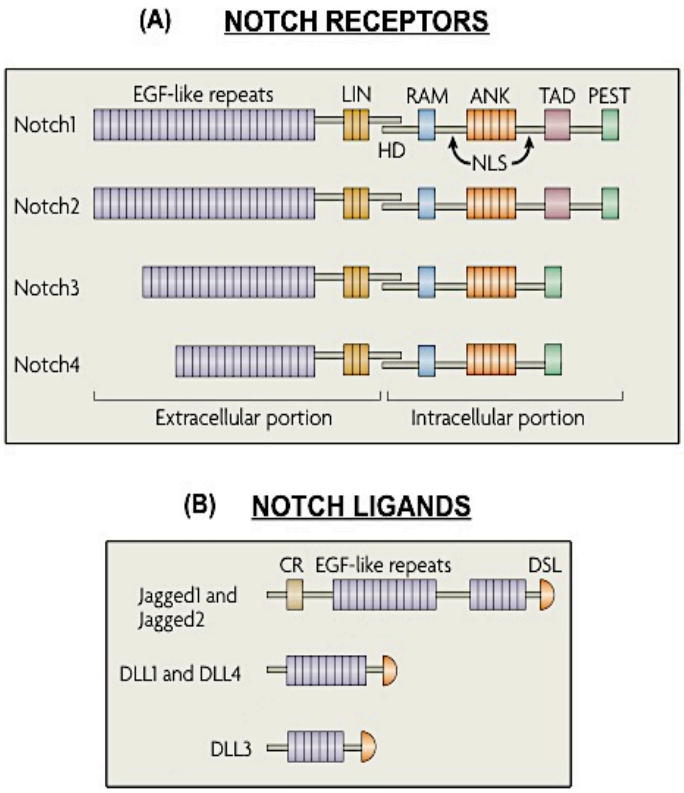
Specific Aim 1: Determine the role of Notch ligands in triggering $N1^{IC}$ downstream of the TCR and influencing peripheral T cell function.

Specific Aim 2: Determine the contribution of canonical and non-canonical Notch signaling in regulating activation and differentiation of peripheral $CD4^+$ T cells.

Specific Aim 3: Determine how Notch interacts with the mTOR pathway

With respect to the expected outcome, the work proposed in Aims 1, 2 and 3 is likely to identify the contribution of Notch ligands in the generation of $N1^{IC}$ downstream of the TCR, whether this $N1^{IC}$ controls peripheral T cell function in an RBP-Jκ-independent function and the precise mechanism underlying such RBP-Jκ-independent function of Notch. This is likely to have an important

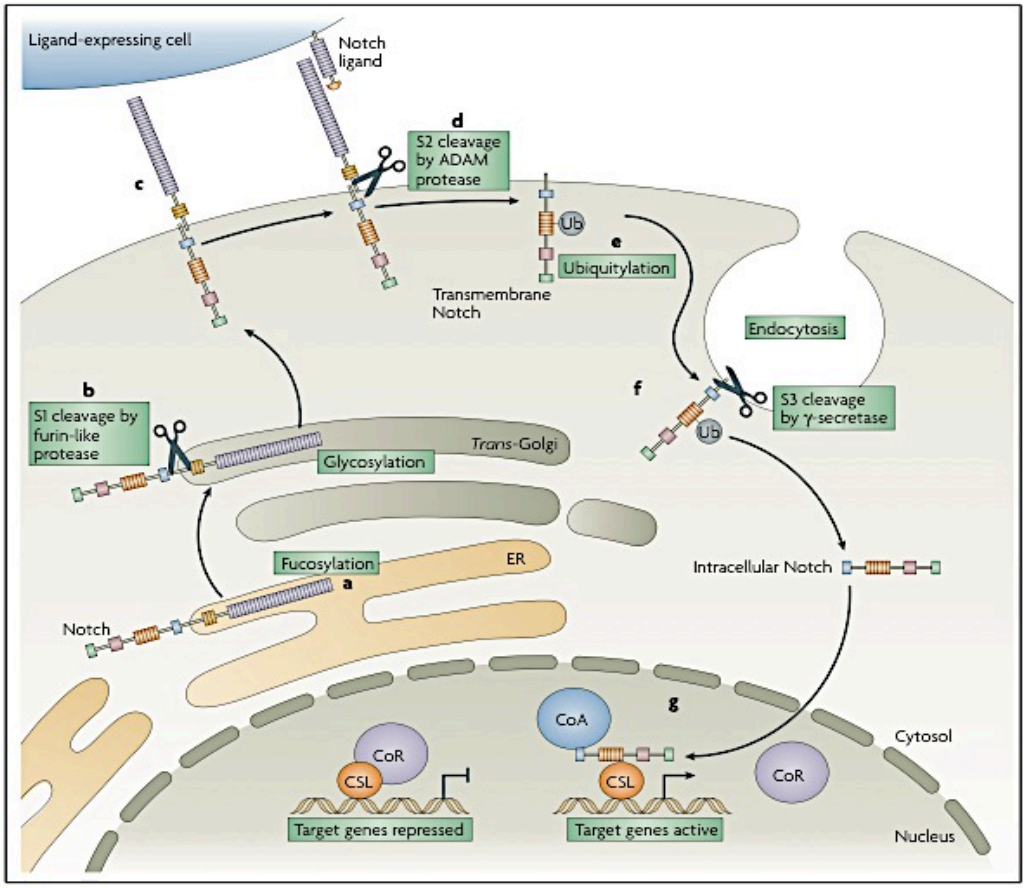
positive impact, because the knowledge gleaned from this study will likely reconcile differences about the precise role of Notch signaling in T cell function by outlining an alternative route for Notch to propagate its signals. Furthermore, this study will also provide an insight into how Notch exerts its pleiotropic effects by cross-talking with other pathways that are also activated downstream of the TCR.



(Adapted from Osborne *et al.,* 2007)

Fig 1.1 The Notch receptor and its ligands

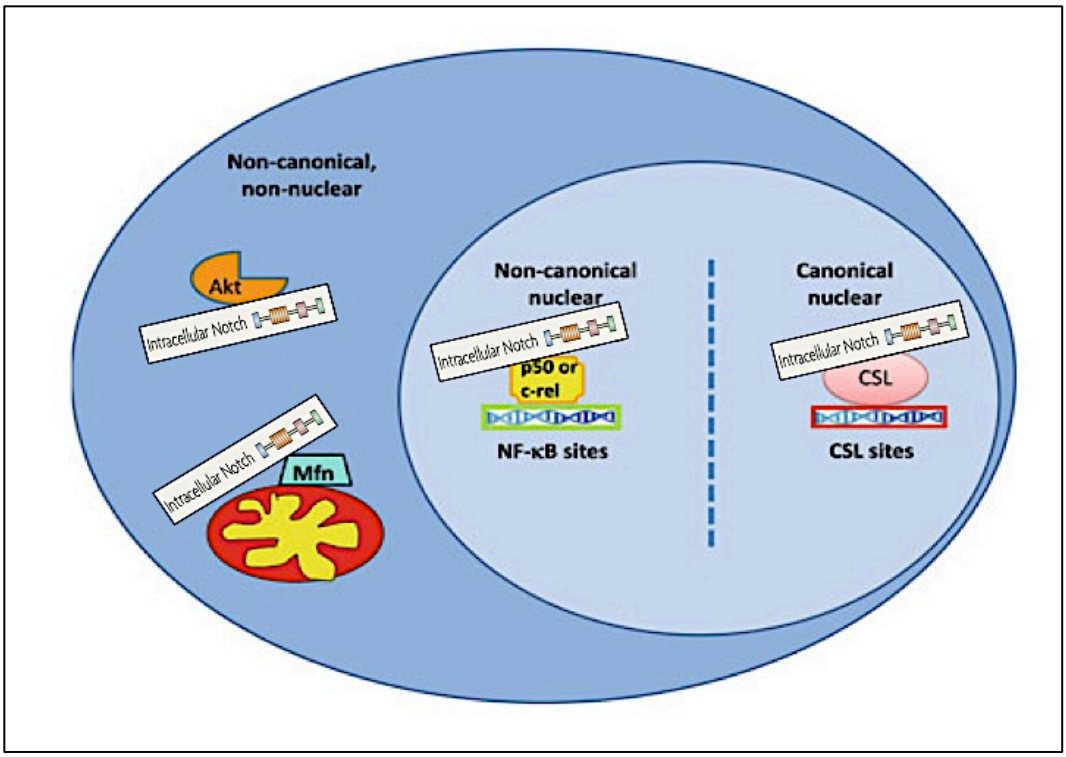
(A) Structural representation of Notch receptors –Notch1-4. All receptors contain an extracellular domain with EGF-like repeats, the LIN domain for heterodimerization, RAM domain and ankyrin repeats for binding proteins and PEST domain for protein degradation. Notch3 and Notch4 lack a transcriptional activation domain (TAD). **(B)** Structural representation of Notch ligands. All ligands contain EGF-like repeats and a conserved DSL requence. Jagged ligands have an additional cysteine rich (CR) domain.



(Adapted from Osborne *et al.,* 2007)

Fig 1.2 The Canonical Notch pathway

After fucosylation in the ER, Notch transits into the golgi where it is cleaved at the S1 site by a furin-like protease. This leads to the expression of a non-covalently linked hetero-dimeric receptor on the cell surface. Following ligand binding, a second cleavage at the S2 site by an ADAM protease leading to the shedding of the extra-cellular domain. Following ubiquitylation, the rest of the receptor is endocytosed and cleavaed at the S3 site by a γ-secretase. This releases the intra-cellular, active domain of Notch, which migrates into the nucleus and interacts with CSL/ RBP-J_K, previously associated with co-repressors (CoR). Recruitment of co-activators (CoA) converts CSL to an activator of transcription leading to transcription of target genes.



(Adapted from Minter *et al.,* 2013)

Fig 1.3 Non-Canonical Notch signaling

Intra-cellular Notch (N^{IC}) can interact with several other proteins besides RBP-J_K in the cytoplasm and nucleus. Non-canonical, non-nuclear binding partners of N^{IC} include Akt and mitofusins (Mfn) located on the mitochondria. Non-canonical, nuclear partners of N^{IC} include the p50 and/or c-Rel subunits of NF-κB.

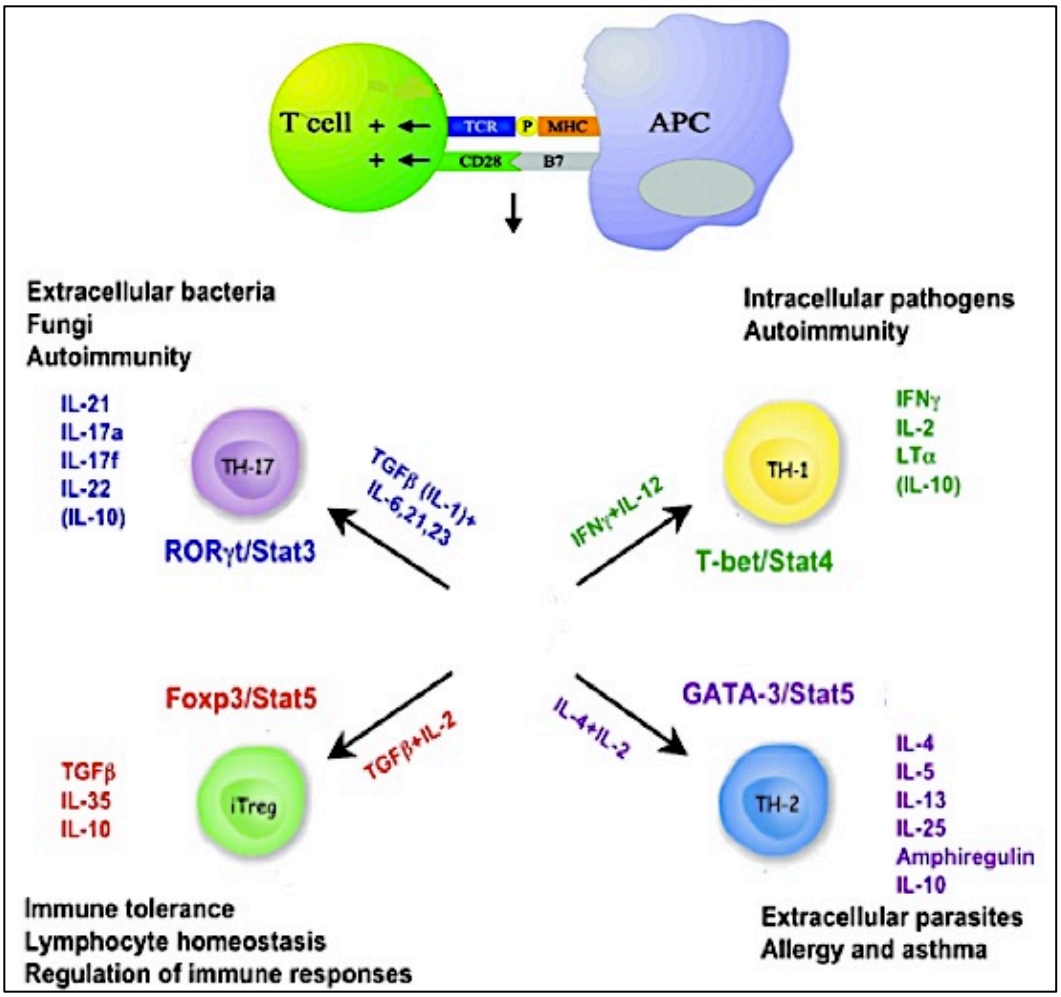
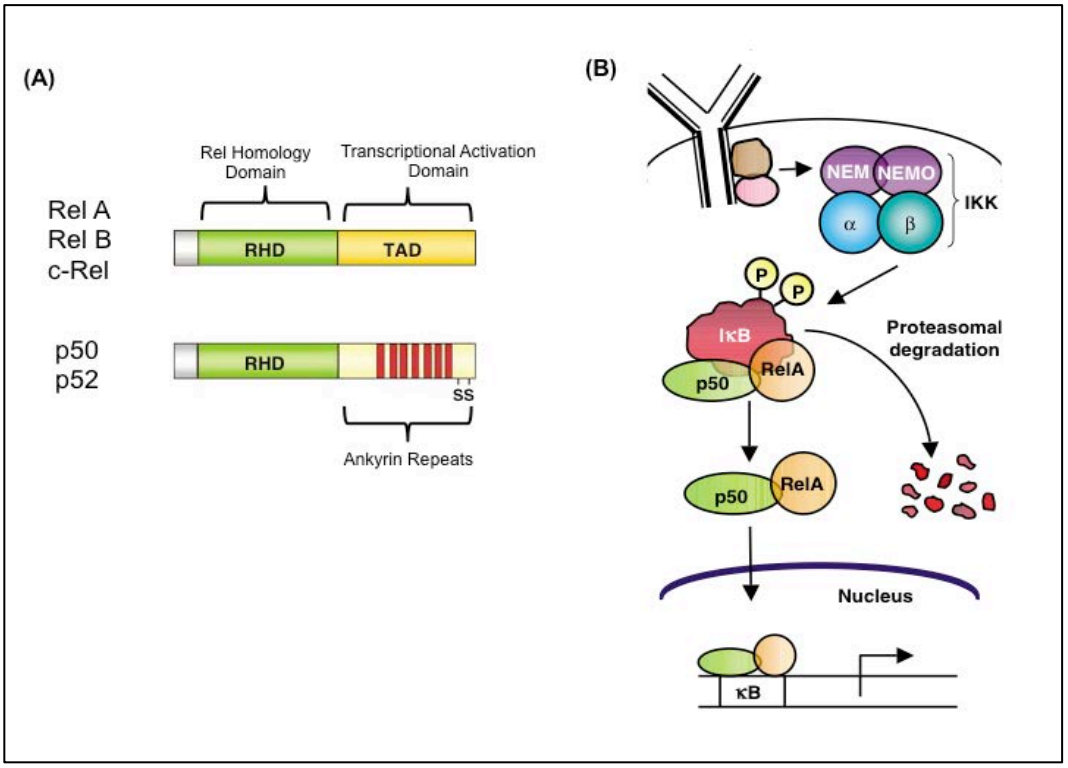


Fig 1.4 Activation and differentiation of helper T cells

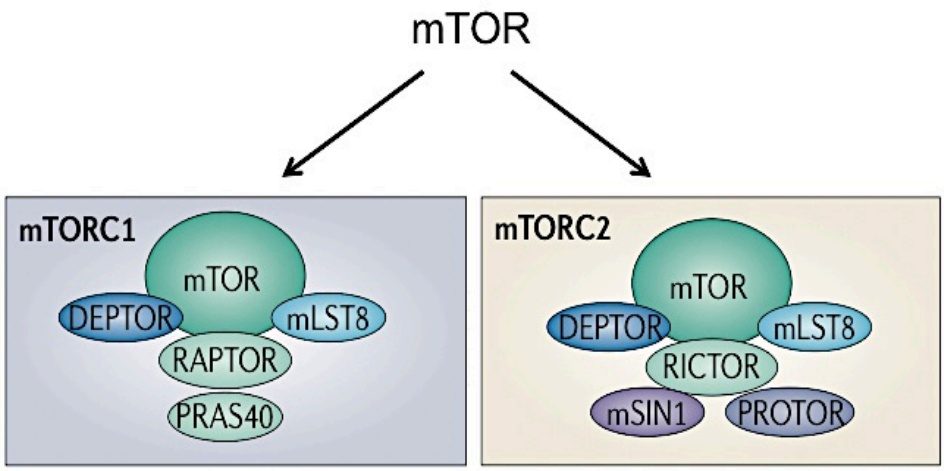
T cells are activated after receiving two signals. Firstly, a peptide (P) presented by an MHC-II molecule by the antigen-presenting cell (APC), binds to the T cell receptor (TCR). The second co-stimulatory signal involves binding of B7 on the APC to CD28 on the T cell. Once activated helper T cells can differentiate into at least four distinct lineages depending on the cytokines present at the time of activation. Each helper T cell expresses a lineage – specific transcription factor and secretes a cohort of signature cytokines.



(Adapted from Gilmore, 2006)

Fig 1.5 The NF-κB pathway

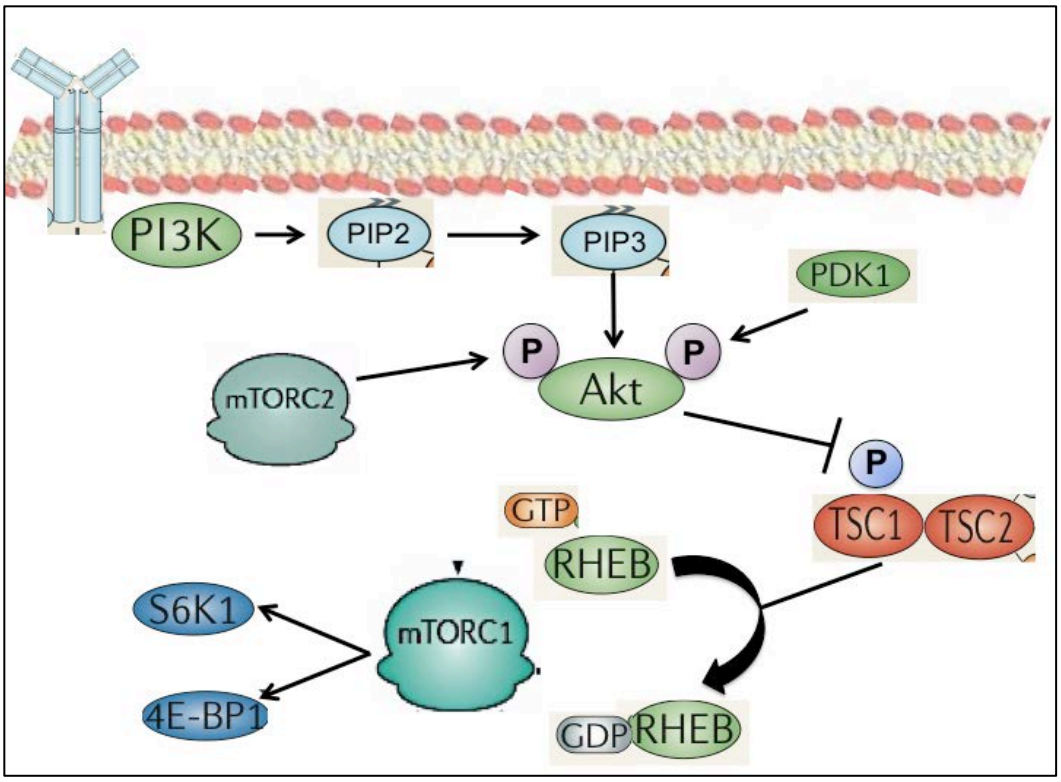
(A) Structural representation of the NF-κB family of transcription factors. All members contain a conserved Rel homology domain. Rel A, Rel B and c-Rel have a transcriptional activation domain. p50 and p52 contain an ankyrin repear rich domain. **(B)** Activation of the NF-κB pathway downstream of the TCR. Activation of T cells by anti-CD3 and anti-CD28 activates IKK, which phosphorylates IκB triggering its degradation by the proteasome. This releases NF-κB, which migrates into the nucleus and mediates transcription.



(Adapted from Zoncu R., *et al* 2012)

Fig 1.6 Composition of mTORC1 and mTORC2

In the mTORC1 complex, mTOR associates with RAPTOR along with DEPTOR and mLST8 which function as negative and positive regulators respectively. PRAS40 also acts as a negative regulator. In the mTORC2 complex, mTOR associates with RICTOR. In addition to DEPTOR and mLST8, mTORC2 also contains mSIN1 and PROTOR which help in complex assembly and targeting mTORC2 to membranes.



(Adapted from Zoncu R., *et al* 2012)

Fig 1.7 The PI3K/mTOR pathway

After activation of T cells via the TCR, the PI3K gets activated. This phosphorylates PIP2 to PIP3. Akt binds to PIP3 via its pleckstrin homology domains. Akt is phosphorylated at residue S473 by mTORC2 and at residue T308 by PDK1. Active Akt phosphorylates multiple downstream substrates, one of which is TSC1/TSC2, which normally converts RHEB-GTP to its inactive GDP bound form. Phosphorylation by Akt inactivates TSC1/TSC2 such that it can no longer inactivate RHEB. GTP-bound RHEB activates mTORC1, which phosphorylates its downstream substrates, S6K1 and 4E-BP1.

CHAPTER 2

THE ROLE OF NOTCH LIGANDS DURING T CELL ACTIVATION AND DIFFERENTIATION

2.1 Introduction

Activation of T cells via the T cell receptor leads to the generation of $N1^{IC}$ in peripheral T cells (Palaga *et al.,* 2003, Adler *et al.,* 2003). Since cross-linking the TCR along with a co-stimulatory signal is sufficient to activate Notch in the absence of Notch ligands, a longstanding question has been how such Notch activation is accomplished. Most importantly, whether such activation is a ligand dependent or independent process is unanswered. One possible explanation for the generation of $N1^{IC}$ in activated T cells is that stimulation via the TCR could also induce the expression of Notch ligands on the surface of T cells. In such a scenario, Notch ligands on one cell could engage the Notch receptor on a neighboring cell triggering activation of the Notch pathway. In fact, Notch ligands are up-regulated on antigen presenting cells (APC) such as dendritic cells following activation (Amsen *et al.,* 2004). In addition, differential up-regulation of Notch ligands is observed after activation of antigen presenting cells with specific helper-T cell inducing stimuli. While activation with T_H1 inducing antigens such as LPS preferentially up-regulates DLL ligands on the surface of APCs, activation with T_H2 inducing stimuli such as cholera toxin and

prostaglandin E2 leads to the expression of Jagged ligands (Amsen *et al.,* 2004; Sun *et al.,* 2008; Worsley *et al.,* 2008). However, whether this trend is also observed in peripheral T cells is unknown.

Not only do T cells express $N1^{IC}$ after activation, but polarizing conditions influence the amount of $N1^{IC}$ that is generated. While T_H1 cells express greater amounts of $N1^{IC}$ in comparison to T_H2 cells (Minter *et al.,* 2005), RBP-Jk expression is thought to be greater in T_H2 cells (Amsen *et al.,* 2004), suggesting a bi-potent ability of Notch in regulating T_H1 and T_H2 lineage commitment. Notch ligands have been thought to influence this ability of Notch to regulate such lineage decisions. Engagement of the Notch receptor with DLL ligands has been shown to induce the expression of T-bet and IFN-γ *in vitro* and *in vivo* (Maekwa *et al.,* 2003, Amsen *et al.,* 2004, Sun *et al.,* 2008). Conversely, some studies have shown that activation of T cells with APCs in the presence of Jagged1 induces the production of IL-4 *in vitro* (Amsen *et al.,* 2004). However, the ability of Jagged ligands to induce Th2 responses *in vivo* has been speculative with some groups suggesting no overt effect in the presence of Jagged (Krawczyk *et al.,* 2008, Worsley *et al.,* 2008) and others suggesting a strong requirement of Jagged 1 for IL-4 production during an allergic airway response (Okamoto *et al.,* 2009). Thus a clear understanding of precisely how different Notch ligands influence T cell differentiation is lacking. Furthermore, whether Notch ligands themselves influence the production of $N1^{IC}$ downstream of the TCR is obscure. We put forth the hypothesis that while DLL ligands may activate Notch, Jagged ligands either do not activate or suppress notch activation (Fig 2.1). This may form the

molecular basis of Notch on T_H1 and T_H2 decisions. Our data demonstrate that Notch ligands are expressed on T cells 12-48 hrs after TCR stimulation irrespective of polarizing conditions. Additionally, DLL1 ligands favor Notch activation and IFN-γ production under pre-existing T_H1 conditions.

2.2 Results

2.2.1 T_H1 conditions favor Notch activation.

Several studies have highlighted the importance of Notch1 in regulating differentiation of helper T cells towards T_H1, T_H2, *i*Treg and T_H17 lineages (Amsen *et al.,* 2004; Keerthivasan *et al.,* 2011; Minter *et al.,* 2005; Samon *et al.,* 2008). However, polarizing conditions themselves can influence the expression of $N1^{IC}$ downstream of the TCR. To confirm whether expression of $N1^{IC}$ is indeed different under T_H1 versus T_H2 conditions, we activated $CD4^+$ T cells *in vitro* under polarizing conditions that favor the development of either T_H1 or T_H2 cells and assayed the expression of $N1^{IC}$ under each of these conditions over time by Western Blotting (Fig 2.2A,B) and flow cytometry (Fig 2.2C,D). While both T_H1 and T_H2 cells expressed $N1^{IC}$, the amount of $N1^{IC}$ was higher under T_H1 polarizing conditions (Fig 2.2A,C). These results concur with our previous observations (Minter *et al.,* 2005) confirming preferential activation of Notch1 under T_H1 polarizing conditions.

2.2.2 Expression of Notch ligands on $CD4^+$ T cells

While it is known that activation of peripheral T cells requires Notch (Palaga *et al.,* 2003, Adler *et al.,* 2003), the mechanism that leads to the activation of Notch in T cells is largely unknown. Whether TCR mediated activation of Notch requires engagement of the Notch receptor by its cognate ligands, or whether such activation is a ligand independent process is obscure. To address whether $CD4^+$ T cells express Notch ligands after stimulation via the TCR in addition to expressing $N1^{IC}$, we determined the expression of DLL1 and Jagged1 on $N1^{IC}$ positive T-cells over time. Furthermore, to determine if polarizing conditions differentially influence the expression of these ligands, we looked at ligand expression in cells polarized to T_H1 and T_H2 cell fates. We observed that a very small percentage of cells expressed both DLL1 and Jagged ligands upto 6 hrs after activation (Fig 2.3A,B). A large proportion of ligand positive cells were observed 12 hrs and 48 hrs after activation (Fig 2.3A,B). Additionally, comparable levels of DLL1 and Jagged1 positive $CD4^+$ T cells were observed under T_H1 and T_H2 conditions. Cytokine levels were determined at each time point to ensure that cells were polarized to their respective fates (Fig 2.3C,D). Thus, these data show that $CD4^+$T cells express Notch ligands maximally at 12 hrs and 48 hrs after activation and polarizing conditions do not influence such ligand expression.

2.2.3 DLL1 favors Notch activation

To determine if DLL and Jagged ligands differ in their ability to generate $N1^{IC}$ post activation via the TCR, we stimulated $CD4^+$ T cells under T_H1 and T_H2 conditions over time as described above. Since we observed maximal expression of DLL1 and Jagged1 on the surface of $CD4^+$ T cells 12-48hrs after activation, we gated on these ligand positive cells at the indicated time points and looked at $N1^{IC}$ expression in these gated populations (Fig 2.4A). We observed that irrespective of the polarizing condition, $CD4^+$ T cells that expressed DLL1 on their surface also expressed higher amounts of $N1^{IC}$ at 12 and 48hrs in comparison to those that expressed Jagged1, suggesting that DLL1 may favor the generation of $N1^{IC}$ in activated T cells. To confirm this observation, we stimulated D0.11.10 TCR transgenic $CD4^+$ T cells with Chinese Hamster Ovary – antigen presenting cells (CHO-APC) expressing either DLL1, Jagged1 or an empty vector (EV) pulsed with ova peptide under non-polarized (NP), T_H1 or T_H2 conditions (Fig 2.5A). We observed $CD4^+$ T cells stimulated with CHO-APCs expressing DLL1 generated the most amount of $N1^{IC}$ in comparison to those stimulated with CHO-APCs that expressed Jagged1 or the empty vector control irrespective of the polarizing condition (Fig 2.5B,C). Additionally, the amount of $N1^{IC}$ expressed by $CD4^+$ T cells was augmented even further under T_H1 conditions (Fig 2.5B,C).

Furthermore, such a DLL1 mediated increase in $N1^{IC}$ was paralleled by a concomitant increase in IFN-γ production under T_H1 conditions (Fig 2,5D). Conversely, no overt differential effect of either ligand was observed on IL-4

production by T_H2 cells (Fig 2.5E). Hence, these results suggest that activation of $CD4^+$ T cells in the presence of DLL1 favors $N1^{IC}$ production in comparison to activation in the presence of Jagged1. Furthermore, such bias towards generation of $N1^{IC}$ is enhanced even further under T_H1 conditions. Thus activation in the presence of DLL1 results in higher levels of $N1^{IC}$ that may eventually push cells towards adopting a T_H1 fate.

2.2.4 Notch ligands do not influence T cell activation

Since we observed increased expression of $N1^{IC}$ and enhanced T_H1 polarization in T cells stimulated in the presence DLL1, we wanted to determine whether various Notch ligands also influence T cell activation differentially. To address this question, we activated $CD4^+$ T cells from D0.11.10 mice with peptide pulsed CHO-APCs expressing the different ligands and determined the expression of T cell activation markers. While $CD4^+$ T cells stimulated in the presence of DLL1 or Jagged1 expressed higher levels of CD25 and CD69 in comparison to those stimulated by CHO-APCs expressing neither of the two (empty vector), both DLL1 and Jagged1 expressing CHO-APCs stimulated T cells comparably (Fig 2.6A,B). Additionally, T-cells stimulated by all CHO-APCs secreted similar levels of IL-2 (Fig 2.6C). Thus while Notch ligands differentially influence the generation of $N1^{IC}$ and acquisition of helper T cell fate, their effect on T cell activation is comparable.

2.3 Discussion

The mechanism of generation of $N1^{IC}$ in peripheral T cells and the eventual consequence of $N1^{IC}$ on regulating helper T cell fate decisions has been obscure. While some studies have implied a requirement of Notch for T_H1 decisions (Minter *et al., 2005*; Maekwa *et al.,* 2003), others have suggested an indispensable role of Notch in regulating T_H2 decisions (Amsen *et al.,* 2004; Amsen *et al.,* 2007; Fang *et al.,* 2007). In this study, we confirm that not only is $N1^{IC}$ generated in activated T cells, but its expression is greater under T_H1 polarizing conditions. These data concur with our previous data, which showed a similar preferential generation of Notch in T_H1 cells and a subsequent requirement of Notch for regulating T_H1 but not T_H2 cell fate by induction of T-bet (Minter *et al.,* 2005).

To probe into the mechanism behind activation of Notch downstream of the TCR, we determined the expression of Notch ligands on the surface of $CD4^+$ T cells under T_H1 and T_H2 conditions. Interestingly, we observed that minimal expression of surface ligands was observed until 12hrs after activation. However, $CD4^+$ T cells start expressing both DLL1 and Jagged1 at approximately the same time as they express $N1^{IC}$. Thus it is unclear whether Notch activation drives the expression of Notch ligands which in turn sustain more Notch activation, or whether basal levels of Notch ligands synergize with TCR signals to activate Notch. Addressing these questions will require a sequential deletion of Notch ligands either individually or in combination. Furthermore, polarizing conditions

did not preferentially induce the expression of either ligand. While T_H1 and T_H2 inducing stimuli have been shown to upregulate the expression of DLL and Jagged ligands respectively on the surface of APCs, our data show that the same cannot be observed in peripheral $CD4^+$ T cells (Amsen *et al.,* 2004; Sun *et al.,* 2008; Krawczyk *et al.,* 2008; Worsley *et al.,* 2008).

In spite of the above, an interesting observation from these experiments was that $CD4^+$ T cells that expressed DLL1 on their surface, also expressed larger amounts of $N1^{IC}$ in comparison to those cell that expressed Jagged1 on their surface. These data suggested that Notch ligands influenced the amount of $N1^{IC}$ generated after TCR stimulation, which could eventually influence lineage decisions. Supporting this notion, several studies have suggested that Notch ligands can in fact regulate the instruction of helper T cell fates. DLL1 and 3 can induce T_H1 polarization at least partially via T-bet (Maekwa *et al*., 2003; Amsen *et al*., 2004; Skokos *et al*., 2007; Sun *et al*., 2008) and Jagged can induce a T_H2 fate (Amsen *et al*., 2004; Okamoto *et al*., 2008). However a recent study by Ong et al showed that Notch ligands cannot instruct helper T cell fate specification on their own but in fact act to augment pre-existing polarizing conditions. We adopted a similar strategy used by Ong and colleagues, to determine if different Notch ligands influence the generation of $N1^{IC}$ and thereby regulate cell fate. We observed that CHO cells expressing DLL1 generated significantly higher levels of $N1^{IC}$ in comparison to those expressing Jagged1 or no ligand at all. In fact, even basal levels of $N1^{IC}$ were augmented in un-stimulated cells in the presence of DLL1. Additionally, DLL1 augmented the production of IFN-γ under pre-existing

T_H1 conditions. Thus our data support a role for DLL1-Notch signaling in augmenting T_H1 polarization.

Since $N1^{IC}$ is crucial for regulating activation and proliferation of $CD4^+$ T cells and we observed greater amounts of $N1^{IC}$ in the presence of DLL1, we determined whether DLL1 could also prime T cells better than Jagged1. We observed that activating T cells in the presence of Notch ligands augmented the expression of CD25 and CD69, however both DLL1 and Jagged1 ligands were just as efficient in activating $CD4^+$ T cells.

In conclusion our data demonstrate that Notch ligands differ in their ability to activate $N1^{IC}$ and instruct specification of helper T cell fates. Further experiments are required to decipher the precise molecular mechanism(s) underlying such differences.

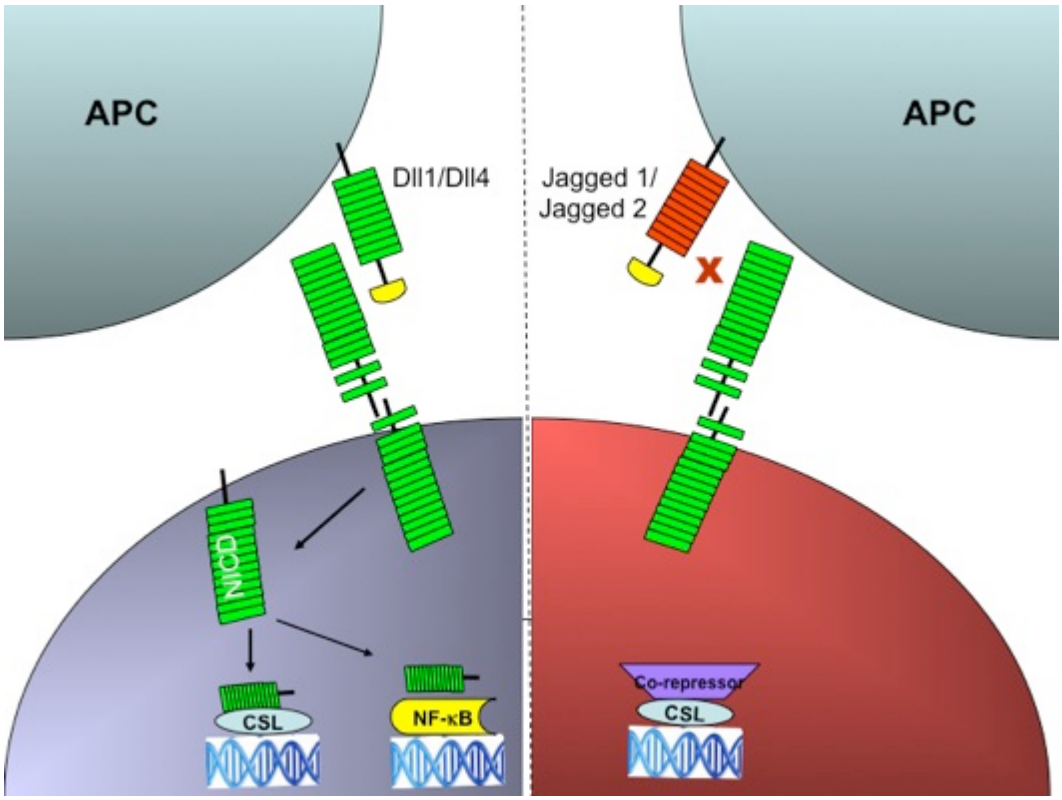


Fig 2.1 A Model suggesting differential activation of Notch by its Ligands

DLL1/4 ligands activate the Notch receptor leading to the generation of $N1^{IC}$ that migrates into the nuclear and interacts with CSL or NF-κB. Jagged ligands do not activate the Notch receptor preventing downstream transcriptional events.

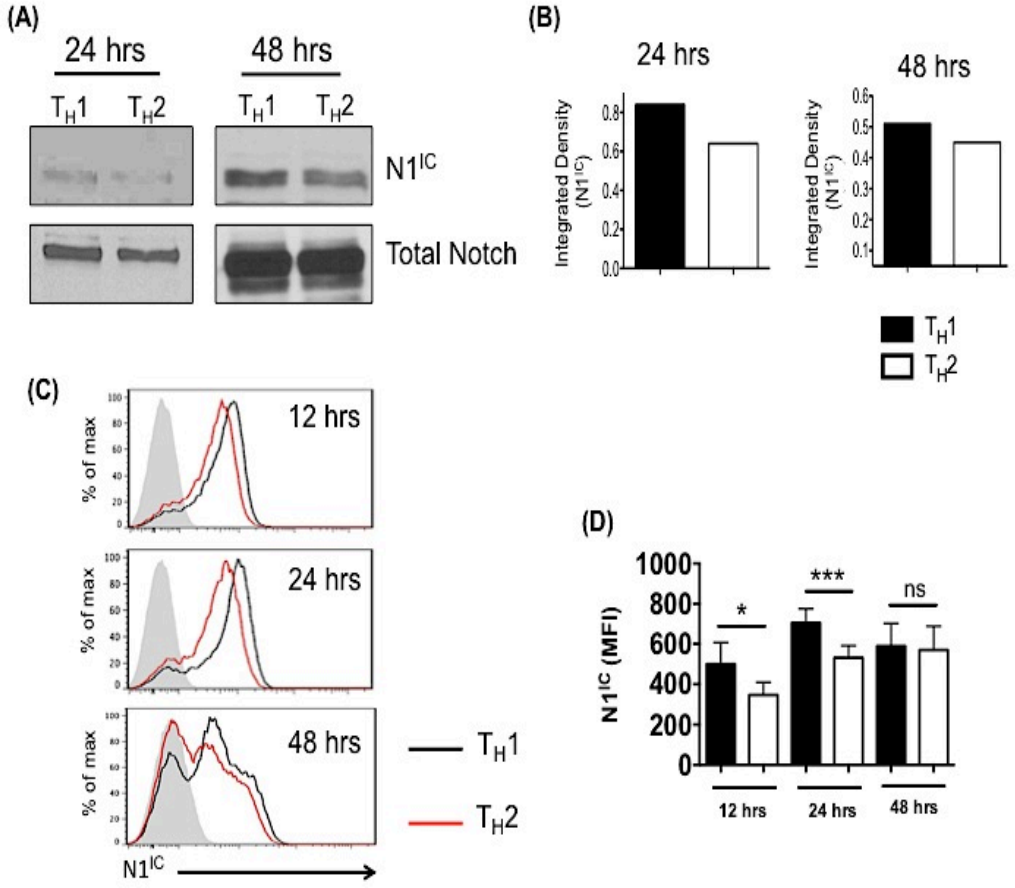


Fig 2.2 T_H1 conditions favor Notch activation.

$CD4^+$ T cells from C57Bl/6 mice were differentiated under T_H1 or T_H2 inducing conditions and activated with plate bound anti-CD3ε and anti-CD28. Cells were harvested at the indicated time points. Some cells were used to make lysates for western blotting. Some cells were used for detecting $N1^{IC}$ by flow cytometry. **(A)** Western Blot for $N1^{IC}$ and Total Notch. Data represent three independent experiments **(B)** Integrated density of $N1^{IC}$ normalized to total Notch obtained from the Western Blot. **(C)** Histogram for $N1^{IC}$. n = 3. **(D)** Mean Fluorescent Intensity (MFI) of $N1^{IC}$ obtained by flow cytometry. n = 3. Data represent the mean ± SEM. * $p < 0.05$, *** $p < 0.001$, ns - not significant.

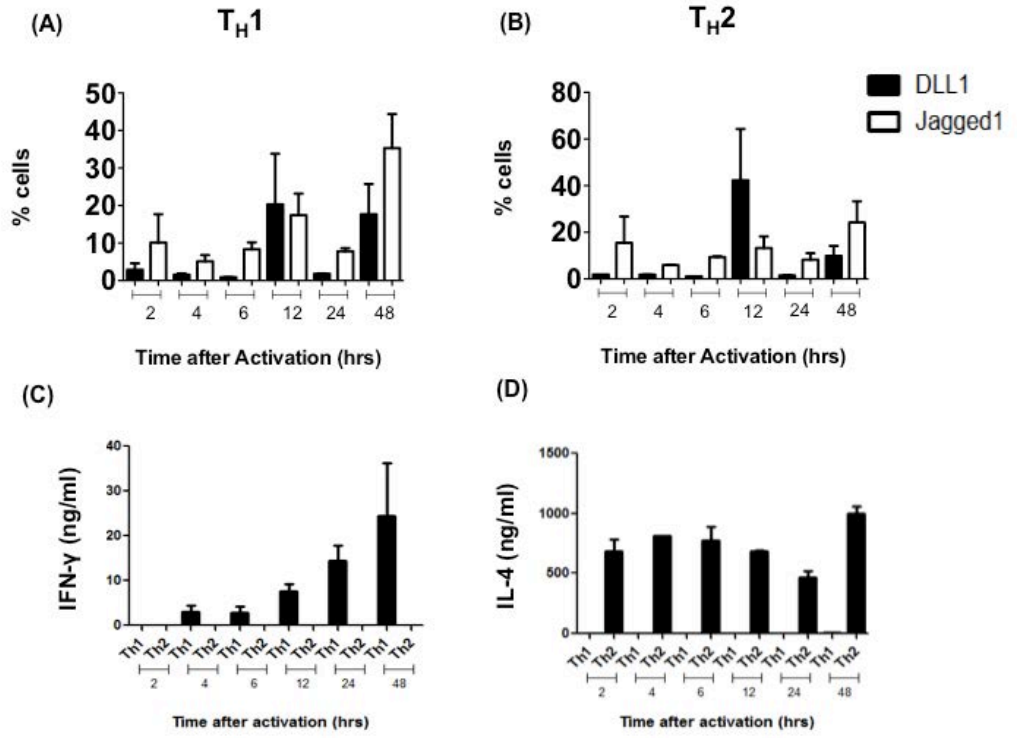


Fig 2.3 Expression of Notch ligands on $CD4^+$ T cells

$CD4^+$ T cells from C57Bl/6 mice were differentiated under T_H1 or T_H2 inducing conditions and activated with plate bound anti-CD3ε and anti-CD28. Cells were harvested at the indicated time points and analyzed by flow cytometry for the surface expression of DLL1 and Jagged1 after gating on $N1^{IC}$ positive cells. Supernatants were used for detecting cytokines by an ELISA. **(A,B)** Percentage of $DLL1^+$ and $Jagged^+$ cells under **(A)** T_H1 and **(B)** T_H2 conditions. **(C)** IFN- γ and **(D)** IL-4 production measured by ELISA. Data represent the mean ± SEM. n = 3-5

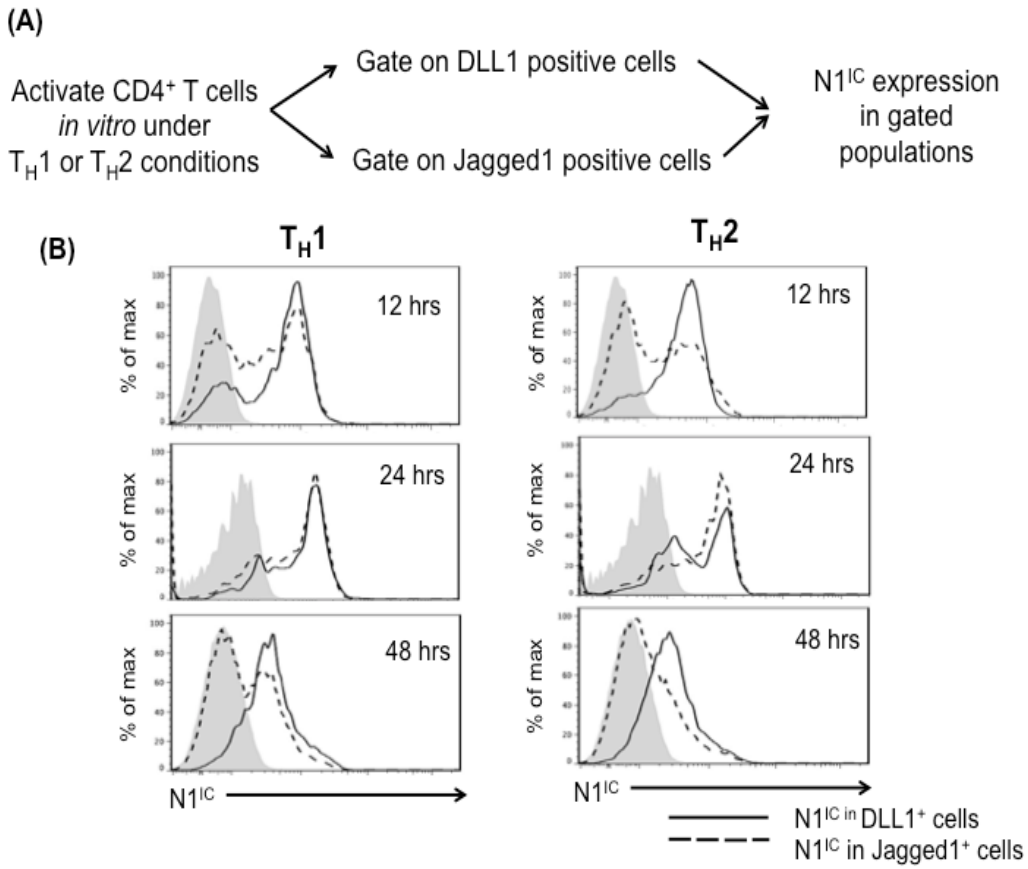


Fig 2.4 Expression of $N1^{IC}$ in ligand positive cells

(A) Schematic representation of experimental design. $CD4^+$ T cells from C57Bl/6 mice were differentiated under T_H1 or T_H2 inducing conditions and activated with plate bound anti-CD3ε and anti-CD28. Cells were harvested at the indicated times and surface stained for DLL1 or Jagged1 and intra-cellular stained for N1IC. $N1^{IC}$ expression was determined after gating on $DLL1^+$ or $Jagged1^+$ cells. **(B)** Histograms for $N1^{IC}$ in gated populations. Data represent three independent experiments.

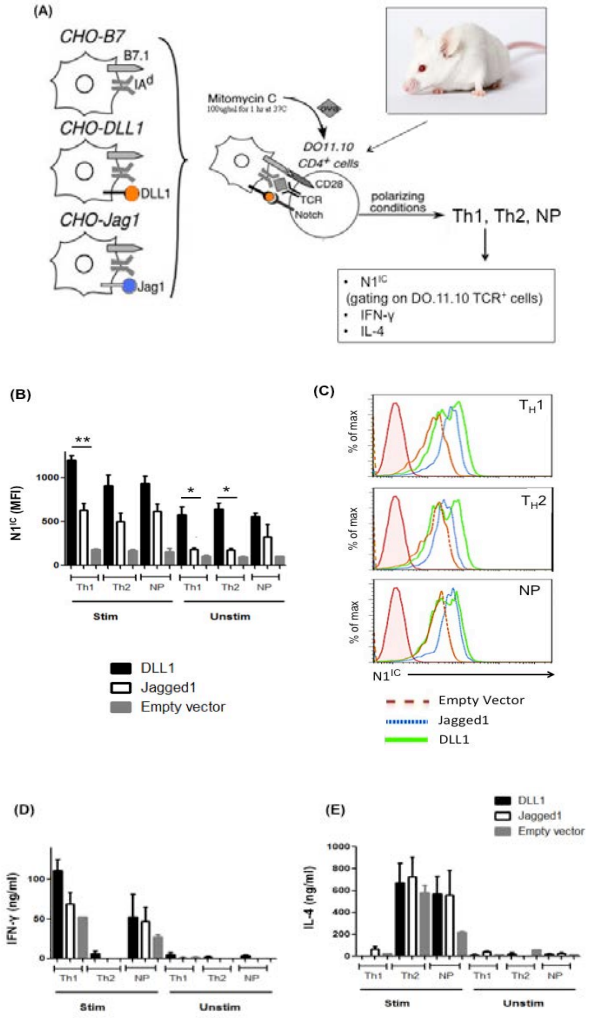


Fig 2.5 DLL1 favors Notch activation and IFN-γ production

(A) Schematic representation of experimental design. Adapted from Ong et al., 2008. CHO-DLL1, CHO-Jag1 and CHO-EV cells were treated with Mitomycin C for 40 mins and mixed with $CD4^+$ T cells isolated from DO.11.10 mice in the presence and absence of ova peptide under T_H1, T_H2 or Non Polarizing (NP) conditions. $CD4^+$ T cells were harvested after 48hrs and analyzed for intra-cellular Notch1 expression after gating on DO.11.10 TCR^+ cells. **(B)** MFI for $N1^{IC}$ obtained by flow cytometry. **(C)** Histogram for $N1^{IC}$. **(D,E)** $CD4^+$ T cells were harvested after 96 hrs and restimulated with plate bound anti-CD3ε. Supernatants were used for detecting **(D)** IFN- γ and **(E)** IL-4 production by an ELISA. Data represent the mean ± SEM. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, ns - not significant.

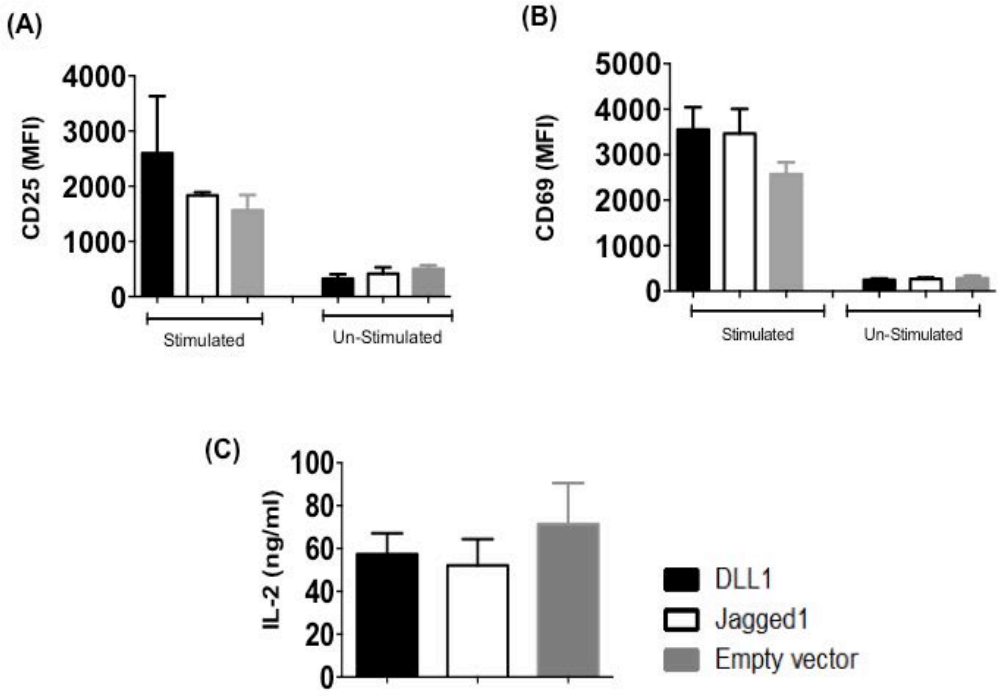


Fig 2.6 Notch ligands do not influence T cell activation

CHO-DLL1, CHO-Jag1 and CHO-EV cells were treated with Mitomycin C for 40 mins and mixed with $CD4^+$ T cells isolated from DO.11.10 mice in the presence and absence of ova peptide. $CD4^+$ T cells were harvested after 48hrs and analyzed for surface expression of CD25 and CD69 after gating on DO.11.10 TCR^+ cells. **(A,B)** MFI of **(A)** CD25 and **(B)** CD69 obtained by flow cytometry. **(C)** Supernatants were used for detecting IL-2 by and ELISA. Data represent the mean ± SEM. n = 3.

CHAPTER 3

NON-CANONICAL NOTCH SIGNALING REGULATES ACTIVATION AND DIFFERENTIATION OF PERIPHERAL CD4$^+$ T CELLS

3.1 Introduction

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 as well as proliferation (Adler *et al., 2003*; Palaga *et al.,* 2003). Since GSIs have multiple substrates and inhibit all Notch receptors (Beel and Sanders, 2008), whether such a decrease in T cell activation is precisely due to Notch1, or a GSI induced effect, requires further investigation. Additionally, precisely where in the TCR signaling cascade Notch exerts influence 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 towards T_H1, T_H2, T_H17 and *i*Treg lineages (Amsen *et al.,* 2004; Keerthivasan *et al.,* 2011; Minter *et al.,* 2005; Samon *et al.,* 2008). Pharmacological inhibition of Notch using GSIs dampens the ability to adopt a T_H1 fate by attenuating the expression of T-bet (Minter *et al.,* 2005). However, inhibition of signaling downstream of RBP-J_K via genetic deletion or by using dominant negative MAML inhibits adoption of a T_H2 fate *in vivo* while

preserving a T_H1 phenotype (Amsen *et al.,* 2007; Fang *et al.,* 2007; Tu *et al.*, 2005). Given the ability of intra-cellular Notch to interact with proteins independently of RBP-J_K, whether such disparate results could be attributed to an RBP-J_K independent function of Notch is unknown. Furthermore, whether canonical and non-canonical signaling affects T cell activation and differentiation processes differently requires further investigation.

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-J_K since conditional deletion of Notch1 impaired these processes while conditional deletion of RBP-J_K had no effect. Such non-canonical, RBP-J_K independent regulation of these processes likely occurs via NF-κB. Conditional deletion of Notch1 also impaired polarization towards T_H1 and induction of regulatory T cells *in vitro*. Nevertheless, RBP-J_K deficiency did not impair T_H1 or *i*Treg cell fate *in vitro* once again supporting a novel role of non-canonical Notch signaling in controlling differentiation towards these lineages. *In vitro* polarization towards T_H2 was not affected in the absence of either Notch1 or RBP-J_K. Our *in vitro* observations demonstrate a cell intrinsic function of RBP-J_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.

3.2 Results

3.2.1 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 ($N1^{IC}$) and its inhibition via γ-secretase inhibitors (GSI), decreases activation and proliferation of T cells (Adler *et al*., 2003; Palaga *et al*., 2003). While Notch has been demonstrated to regulate the expression of T-cell activation, precisely where Notch exerts its influence downstream of the TCR is obscure. Furthermore, whether Notch is required for regulating 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 $N1^{IC}$ in $CD4^+$ T cells activated with plate bound Anti CD3 and Anti CD28 4 hrs. after activation and the amount of $N1^{IC}$ increased over time (Fig 3.1A). This increase was abrogated after GSI treatment (Fig 3.1A). Inhibition of Notch activation did not alter proximal signaling events as evidenced by intact phosphorylation of Zap 70 even in GSI treated cells (Fig 3.1B). 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 3.1C-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.

3.2.2 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 $N1^{IC}$ in DP thymocytes prevented their maturation into single positive $CD4^+$ and $CD8^+$ T cells by interfering with TCR signal strength (Izon *et al.,* 2001). 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 $N1^{IC,}$ we first determined if altering signal strength could also influence the generation of $N1^{IC}$. We stimulated $CD4^+$ T cells with increasing concentrations of Anti-CD3 keeping the amount of Anti-CD28 constant. Increasing signal strength resulted in a corresponding increase in the amount of $N1^{IC}$ expression (Fig 3.2A). This was also accompanied by an increase in the percentage of cells expressing $N1^{IC}$ and this increase could be inhibited after GSI treatment (Fig 3.2B). To determine if Notch is required for regulating signal strength, we stimulated cells with increasing amounts of Anti-CD3 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-CD3 (Fig 3.2C) 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 $N1^{IC}$ as well as the percentage of $N1^{IC}$ positive cells (Fig 3.2D,E). This increase could be blocked after GSI treatment (Fig 3.2E). In addition to $N1^{IC}$, 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 3.2F) These data demonstrate that not only can the levels of $N1^{IC}$ 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 (Guy *et al*., 2013). c-Myc is a downstream target of Notch and is suspected to be important in thymocyte development and T-cell function (Douglas *et al*., 2001; Guy *et al*., 2013; Lindsten *et al*., 1988; Nie *et al*., 2012). 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 (Nie *et al*., 2012). 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 3.2G, 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 3.2I). 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.

3.2.3 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 (Adler *et al*., 2003; Palaga *et al*., 2003). However, GSIs inhibit all isoforms of Notch and have multiple substrates (Beel and Sanders, 2008). 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 (Roderick *et al*., 2013). The Mx-Cre promoter enables acute deletion of Notch1 in peripheral T cells. Since Notch1 is required for development of T cells (Deftos *et al*., 2000; Radtke *et al*., 1999; Wolfer *et al*., 2001), this deletion strategy enables cells to develop normally

in the presence of Notch, before its deletion in the periphery. Both *Notch1* $^{fl/fl}$ Mx Cre $^{+/-}$ mice (abbreviated as cN1KO) and *Notch1* $^{fl/fl}$ Mx Cre $^{-/-}$ mice (abbreviated as control) were injected with equivalent amounts of Poly I: Poly C and rested for 3 weeks before use (Roderick *et al.,* 2013). $CD4^+$ T cells from cN1KO animals showed a significant decrease in Notch1 transcript as well as a marked reduction in the amount of $N1^{IC}$ expression. (Fig 3.3A,D)(Roderick *et al.,* 2013). 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 (Fig 3.3B,C). 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 (Fig 3.3E,F)

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-CD3ε and anti-CD28 and activation markers were observed by flow cytometry. cN1KO animals had a significantly lower percentage of cells expressing CD25 and CD69 (Fig. 3.4A,B,D,E). Furthermore, these cells expressed lower levels of both activation markers (Fig 3.4C,F). This decrease was accompanied by a significant reduction in the levels of IL-2 and IFN-γ secreted post-activation (Fig 3.4G, 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 (Fig 3.4I). 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 (Fig 3.5A,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 3.4J). 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.

3.2.4 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. 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 *i*Treg 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 3.6A, B). Secreted IFN-γ was also reduced significantly in the absence of Notch1 (Fig 3.6C). This decrease was accompanied by a reduction in the amount of the master T_H1 transcription factor T-bet (Fig 3.6D, E, F). In contrast, no marked effect was observed in T_H2 differentiation. Although, the amount of GATA3 was reduced in the absence of Notch1 (Fig 3.6G, H, I), both control as well as cN1KO mice had similar percentages of $CD4^+$ T cells that were positive for intra-cellular IL-4 (Fig 3.6A, B) and expressed comparable levels of secreted IL-4 (Fig 3.6C). 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_H1 and T_H2 conditions. Proliferative capability of $CD4^+$ T cells from cN1KO mice was the same under different polarizing conditions (Fig 3.7), despite of differences in cytokine secretion. In addition to T_H1, 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 3.6J, K). These results show that Notch1 is required for T_H1 and *i*Treg differentiation but is dispensable for T_H2 cell fate acquisition *in vitro*. Furthermore, these data demonstrate an intrinsic role of Notch1 in regulating helper T cell differentiation.

3.2.5. 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 (Auderset *et al.,* 2012; Perumalsamy *et al.,* 2009). This is potentiated by interactions of the intra-cellular domain of Notch with other proteins besides RBP-Jκ in the cytoplasm and nucleus. 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-Jκ-dependent, canonical Notch signaling, we conditionally knocked out RBP-Jκ in peripheral T cells by breeding mice carrying RBP-Jκ *loxp*-flanked sites to mice expressing Cre recombinase under the control of the Mx promoter. CD4$^+$ T cells from *RBP-Jκ*$^{fl/fl}$ Mx Cre $^{+/-}$ mice (abbreviated as cRBP-Jκ -KO) expressed substantially reduced transcript and protein levels of RBP-Jκ in comparison to to CD4$^+$ T cells from *RBP-Jκ*$^{fl/fl}$ Mx Cre $^{-/-}$ mice. (Abbreviated as controls). (Fig 3.8A,B). Additionally, cRBP-Jκ -KO mice had significantly fewer peripheral CD4$^+$ and CD8$^+$ T cells in their spleens (Fig 3.8C,D). 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 intra-cellular Notch1 after TCR stimulation (Fig 3.9A, B, C). In contrast to impaired activation observed in the absence of Notch1, RBP-Jκ

deficiency did not alter the activation of $CD4^+$ T cells. On the contrary, $CD4^+$ T cells from cRBP-Jκ -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 3.9D-I). $CD4^+$ T cells from both cRBP-Jκ – KO mice secreted IL-2 and IFN-γ just as efficiently as Controls. (Fig 3.9K, L). Proliferation of $CD4^+$ T cells was unaffected by the absence of RBP-Jκ irrespective of addition of recombinant IL-2 (Fig 3.9J). These results suggest that activation and proliferation of $CD4^+$ T cells is not impaired in the absence of RBP-Jκ. However, since these processes required Notch1, our data show that activation and proliferation occurs independently of canonical Notch signaling.

3.2.6. 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 3.10A) and significantly reduced the expression of the activation markers CD25 and CD69 (Fig 3.10B,C). This was accompanied by a significant decrease in the cytokines IL-2 and IFN-γ (Fig 3.10D,E). Importantly,

NS-GSI treatment rescued Notch activation as well as CD25, CD69 and IL-2 (Fig 3.10A-D). A partial rescue was observed with IFN-γ (Fig 3.10E). 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 3.10L.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 (Barbarulo *et al*., 2011; Palaga *et al*., 2003; Shin *et al*., 2006; Vacca *et al*., 2006). 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 DHMEQ . Although DHMEQ treatment did not alter the levels of $N1^{IC}$ (Fig 3.10F), 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 3.10G-J). Furthermore, DHMEQ treatment of $CD4^+$ T cells from cRBP-Jκ-KO animals significantly impaired proliferation (Fig 3.10K). 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.

3.2.7. 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 T_H1 and T_H2 cell fates. While the number of cells secreting IFN-γ was reduced in the $CD4^+$ T cells from cRBP-Jκ-KO mice (Fig 3.11A, B), the amount of IFN-γ secreted under T_H1 conditions was unaffected (Fig 3.11C). Levels of T-bet remained unchanged in the absence of RBP-Jκ in comparison to controls (Fig 3.11D, E, F). Similarly, RBP-Jκ -deficiency did not alter polarization towards T_H2. Although $CD4^+$ T cells cells from RBP-Jκ-KO animals had lower amounts of GATA-3 (Fig 3.11G, H, I) this decrease did not influence the number of IL-4 positive cells (Fig 3.11A, B) or the amount of secreted IL-4 under T_H2 conditions *in vitro* (Fig 3.11C). 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 3.11J, K). These data show that differentiation of $CD4^+$ T cells *in vitro* does not require canonical Notch signaling.

3.3 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 (Adler *et al.,* 2003; Palaga *et al.,* 2003). 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-CD3ε 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 (Nie *et al.,* 2012), 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, 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 (Adler *et al.,* 2003; Minter *et al.,* 2005; Palaga *et al.,* 2003). However, the use of GSIs obscures the specific contribution of Notch1 in regulating these processes. 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 T_H1 versus T_H2 lineage decisions remains controversial partially due to the disparate methods used to attenuate Notch signaling. In models of RBP-J_K deletion, the generation of $N1^{IC}$ is preserved (Fig 3.9). This is particularly important since $N1^{IC}$ has also been documented to interact with other proteins besides RBP-J_K (Minter *et al.,* 2005; Perumalsamy *et al.,* 2009; Shin *et al.,* 2006). Therefore we reasoned that since Notch1 is known to interact with proteins other than RBP-J_K, $N1^{IC}$ may be capable of functioning in an RBP-J_K 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-J_K 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 T_H1 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 T_H1 decisions. Another study has shown that deleting Notch1 under the control of a CD4 Cre promoter does not

dampen T_H1 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 T_H1, 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 *i*Treg populations, suggestive of a requirement for Notch1 in regulating these responses. Although we observed a decrease in both T_H1 and *i*Treg populations in the absence of Notch1, we did not see any decrease in T_H2 responses *in vitro*. We have shown previously that inhibition of Notch via GSIs under T_H2 conditions does not alter IL-4 production (Minter *et al.,* 2005). 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* (Ong *et al.,* 2008). 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 $N1^{IC}$ 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 $N1^{IC}$ 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 (Kopan and Ilagan, 2009; Ong *et al.,* 2008; Tanigaki and Honjo, 2007; Tu *et al.,* 2005). However, our study is the first to suggest that this is due to non-canonical Notch signaling as $N1^{IC}$ 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-$_K$B to be the most likely candidate (Barbarulo *et al.,* 2011; Palaga *et al.,* 2003; Shin *et al.,* 2006; Vacca *et al.,* 2006). Thus, to determine if some of the effects of non-canonical Notch signaling require NF-$_K$B, we inhibited NF-$_K$B in the absence of RBP-J_K. DHMEQ treatment reduced nuclear translocation of c-Rel (Fig 3.12A,B). Inhibition of NF-$_K$B 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-$_K$B

inhibition also attenuated proliferation in the absence of RBP-J_K. Furthermore, attenuation of NF-$_K$B in the absence of RBP-J_K attenuated T_H1 response *in vitro* since DHMEQ treatment reduced the expression of T-bet and IFN-γ in cells lacking RBP-J_K (Fig 3.12C, D), suggesting that RBP-J_K independent but Notch1 dependent regulation of these responses may require NF-$_K$B. Additionally, inhibiting NF-$_K$B 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-$_K$B to control these processes. (Fig 3.13). However, deciphering the precise mechanism of such NF-$_K$B 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-J_K independent, but Notch1 dependent regulation of effector T cell responses *in vitro*. A recent study has also suggested such RBP-J_K independent Notch signaling in regulating T_H1 responses *in vivo* by showing that $CD4^+$ T cells lacking RBP-J_K could mount a protective response to *Leishmania major* but those lacking both the Notch1 and Notch2 receptors could not (Auderset *et al*., 2012). In addition to T_H1, we observed an increase in the number of $CD25^+FoxP3^+$ double positive cells in the absence of RBP-J_K 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 Notch. These differences can be reconciled by the fact that cRBP-J_K -KO mice continue to express elevated levels of $N1^{IC}$. Since $N1^{IC}$ is required for T-reg lineage determination, an increase in $N1^{IC}$ expression in the absence of RBP-Jk, could be responsible for a significant increase in the regulatory T cell population. Additionally, since RBP-J_K is a transcriptional repressor, deletion of RBP-J_K 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-J_K deficiency on the secretion of T_H2 cytokines *in vitro*. Our data do not support an intrinsic role for Notch1 signaling in T_H2 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 T_H2 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-J_K independent, non-canonical Notch signaling regulates activation, proliferation and acquisition of T_H1 and *i*Treg fates *in vitro*. Such non-canonical Notch signaling most likely involves NF-$_K$B. Evidence of non-canonical Notch signaling has been observed during axon guidance as well as dorsal closure during embryonic development in *Drosophila* (Crowner *et al.,* 2003; Zecchini *et al.,* 1999). In addition, a cytosolic function of Notch is required for survival of neural stem cells (Androutsellis-

Theotokis *et al*., 2006). 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-$_K$B (Jin *et al*., 2013). Additionally, mammary tumor development has been shown to occur independently of RBP-J_K. 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-$_K$B in mediating non-canonical Notch signaling.

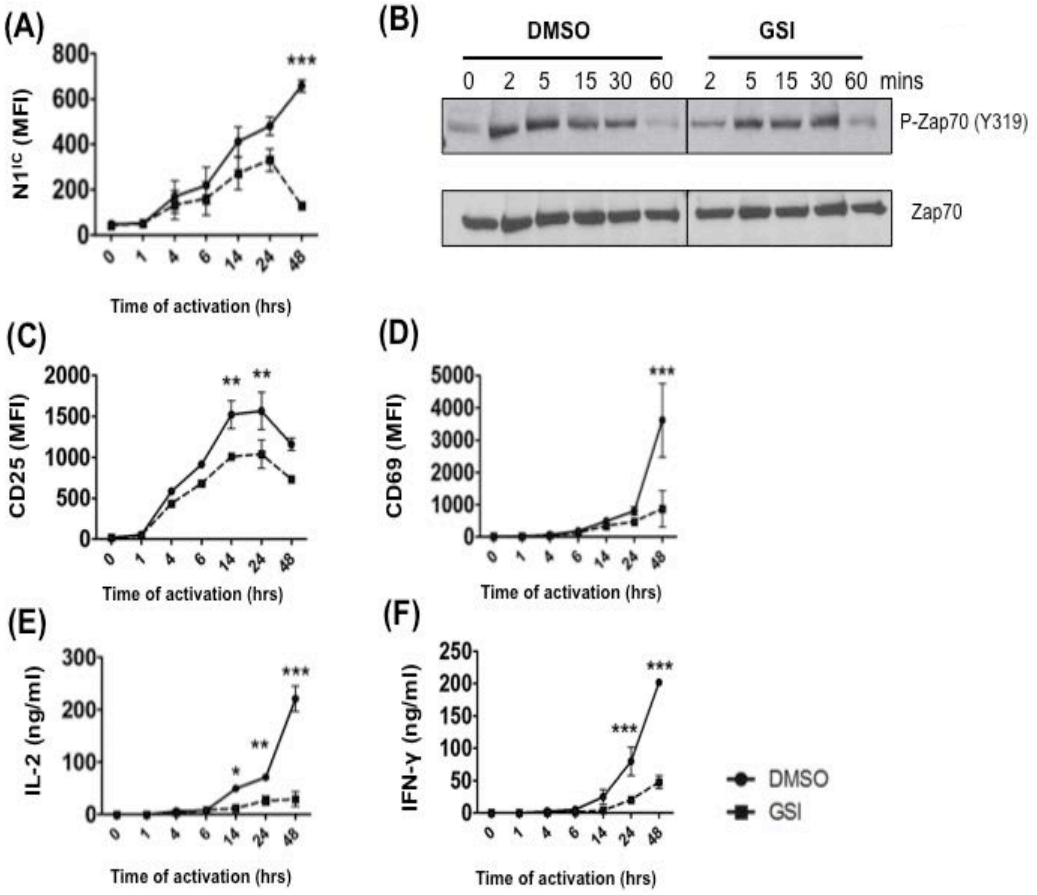


Figure 3.1. **Notch is required for distal TCR signaling events**

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)** $N1^{IC}$ **(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. Data represent the mean ± SEM, n = 3. *, $p < 0.05$, ** $p < 0.005$, ***, $p < 0.001$.

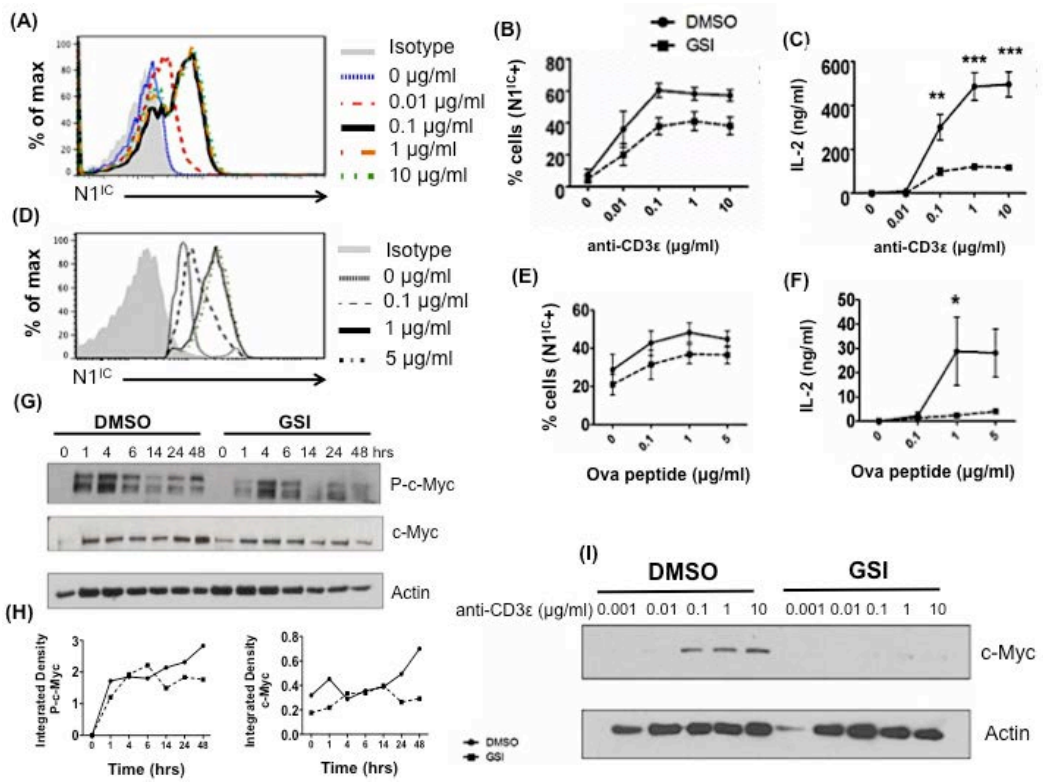


Figure 3.2. Notch as a regulator of signal strength

(A) Histogram for $N1^{IC}$ expressed in $CD4^+$ T cells stimulated with the indicated concentrations of anti-CD3ε and 1 µg/ml of anti-CD28 for 48 hrs. **(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 $N1^{IC}$ 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 $N1^{IC}$. **(E)** Percentage of cells expressing $N1^{IC}$ as determined by flow cytometry. n = 3. **(F)** IL-2 ELISA from supernatants. n = 3. **(G-H)** Western Blot for **(G,H)** 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. **(H)** Integrated density of phosphorylated and total c-Myc after normalizing with 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, *, $p < 0.05$, ** $p < 0.005$, ***, $p < 0.001$.

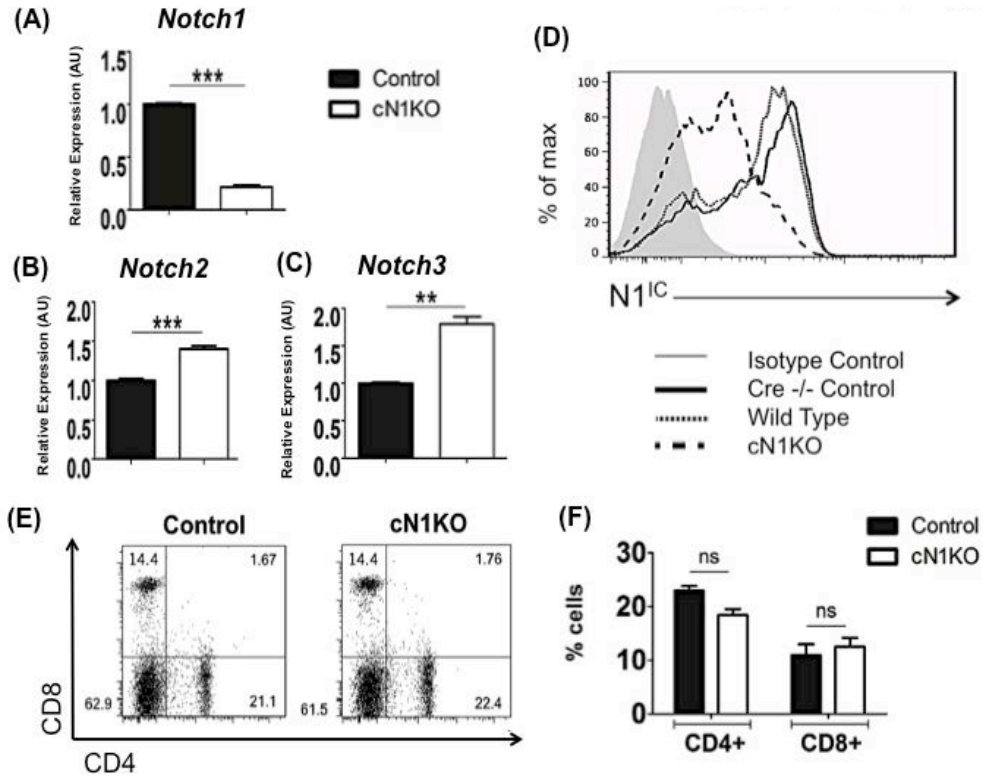


Figure 3.3. Deletion of Notch1 in peripheral CD4⁺ T cells

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. **(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 $2^{-\Delta\Delta CT}$ method. Results are presented as fold change in gene expression after normalization to actin and expressed relative to controls. **(D)** Histogram for $N1^{IC}$ 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.

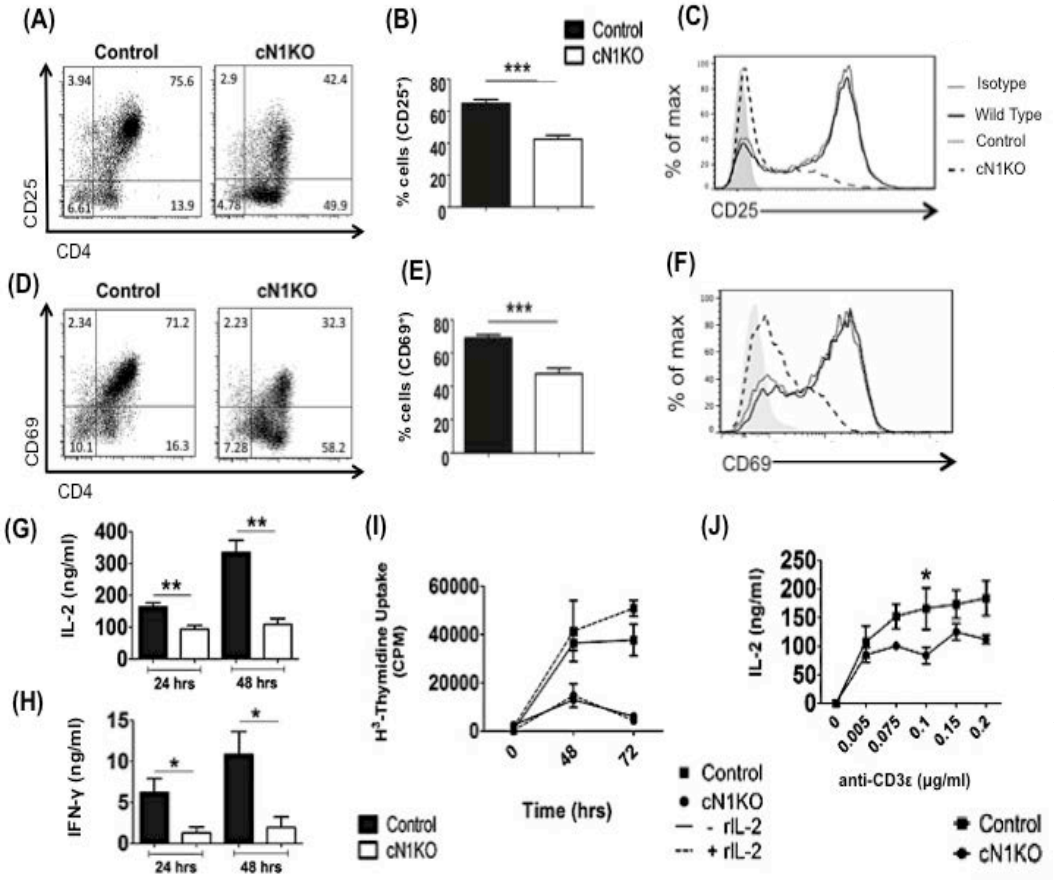


Figure 3.4. Notch1 is required for activation and proliferation of CD4$^+$ T cells

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 N1^{IC} and analyzed by flow cytometry. **(A, D)** Dot plots obtained after flow cytometry. Numbers in each quadrant represent % of cells. **(B)** % of cells positive for CD25 and **(E)** CD69 obtained from dot plots in **(A, D)**. n=4. **(C, F)** Histograms for CD25 and CD69 after gating on Notch negative cells from cN1KO mice. **(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 ^{3}H-thymidine uptake in CD4$^+$ T cells from control and cN1KO mice stimulated as described above with and without rmIL-2 (20ng/ml). **(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. *, $p < 0.05$, ** $p < 0.005$.

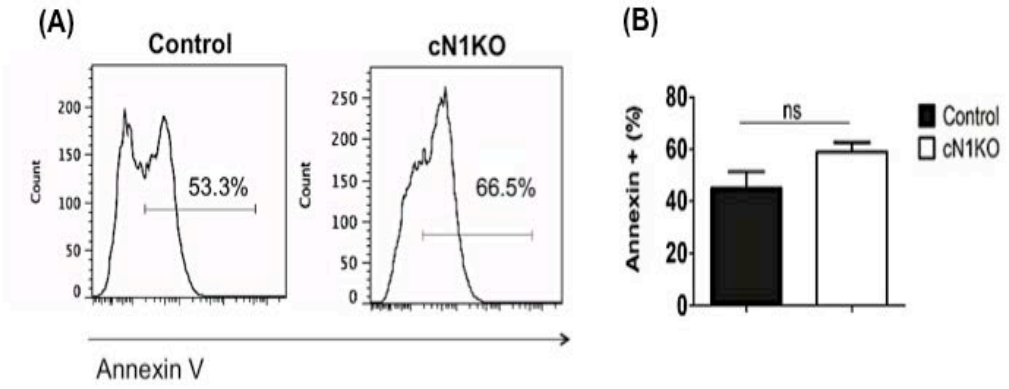


Figure 3.5 Apoptosis in the absence of Notch1

(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. Histograms showing Annexin V expression obtained by flow cytometry, representative of 3 independent experiments. **(B)** % of Annexin V+ cells obtained by flow cytometry. n = 3

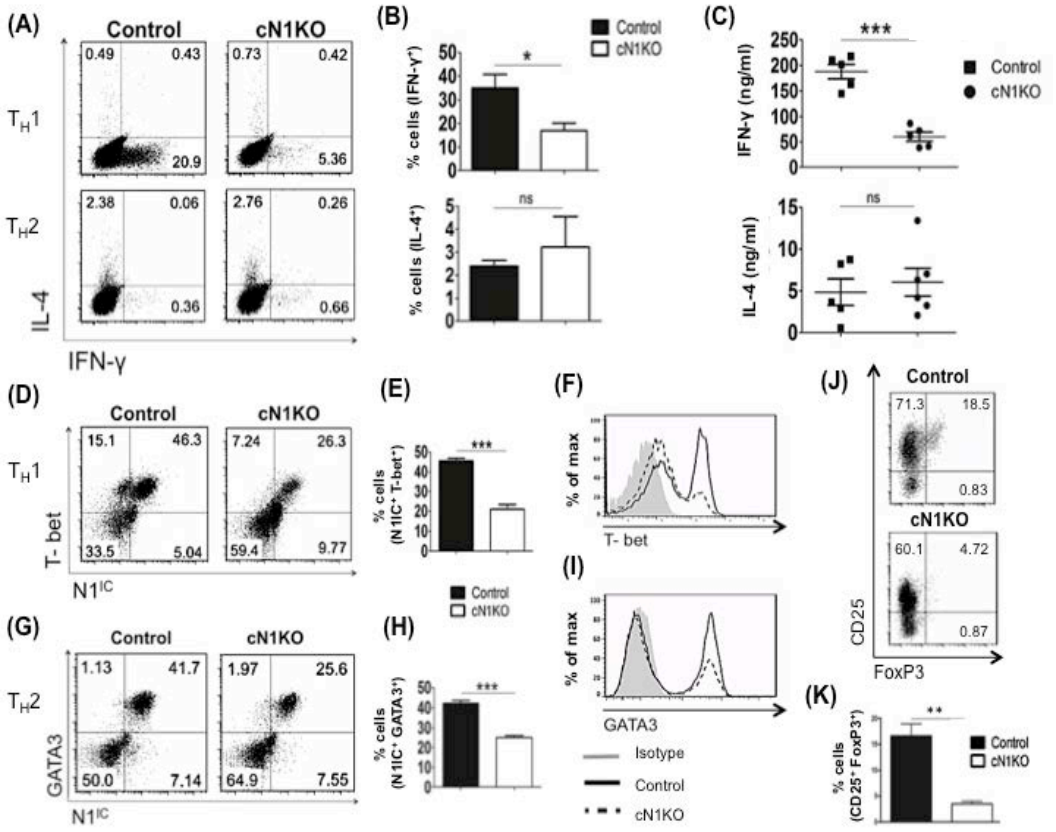


Figure 3.6 Notch1 is required for T_H1 differentiation and production of *i*Tregs *in vitro*

$CD4^+$ T cells from control and cN1KO mice were differentiated under T_H1, T_H2 or *i*Treg 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. **(B)** % 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 $N1^{IC}$ under T_H1 conditions **(G)** or GATA3 and $N1^{IC}$ under T_H2 conditions. **(E, H)** Percentage 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. **(J)** Dot plots for $CD25^+$ and $FoxP3^+$ cells. **(K)** % 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.

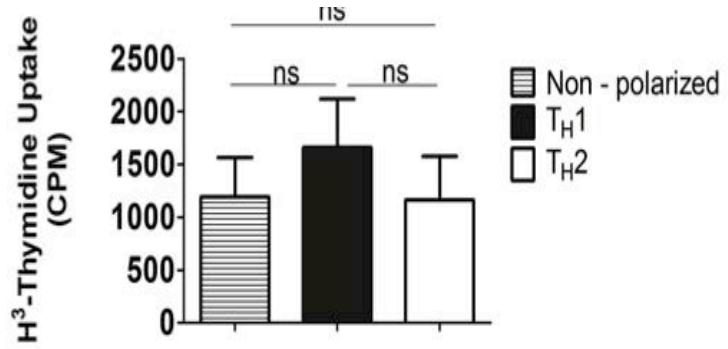


Figure 3.7 Proliferation of CD4+ T cells from cN1KO mouse under different polarizing conditions

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.

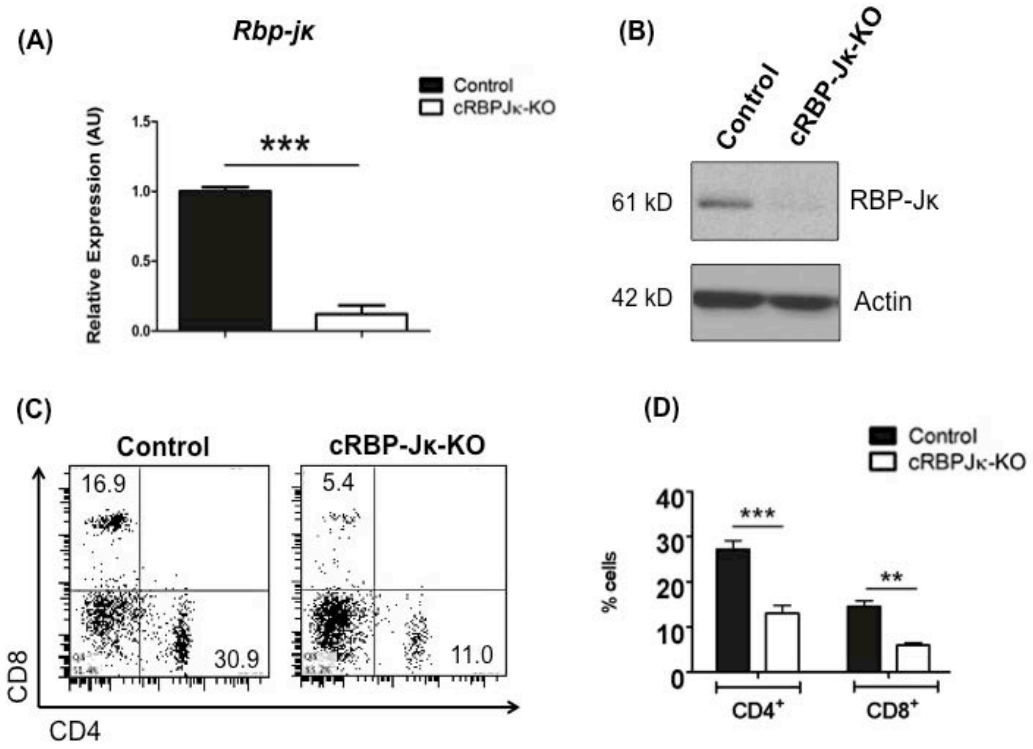


Figure 3.8 Deletion of $RBPJ_K$ in peripheral $CD4^+$ T cells

(A) RNA was isolated from $CD4^+$ T cells from control and $cRBPJ_K$-KO mice and reverse transcribed to cDNA. Expression of $RBPJ_K$ transcript was determined by real time PCR and analyzed by the $2^{-\Delta\Delta CT}$ 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 $cRBPJ_K$-KO mice. **(C)** Splenocytes from control and $cRBPJ_K$-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, *, $p < 0.05$.

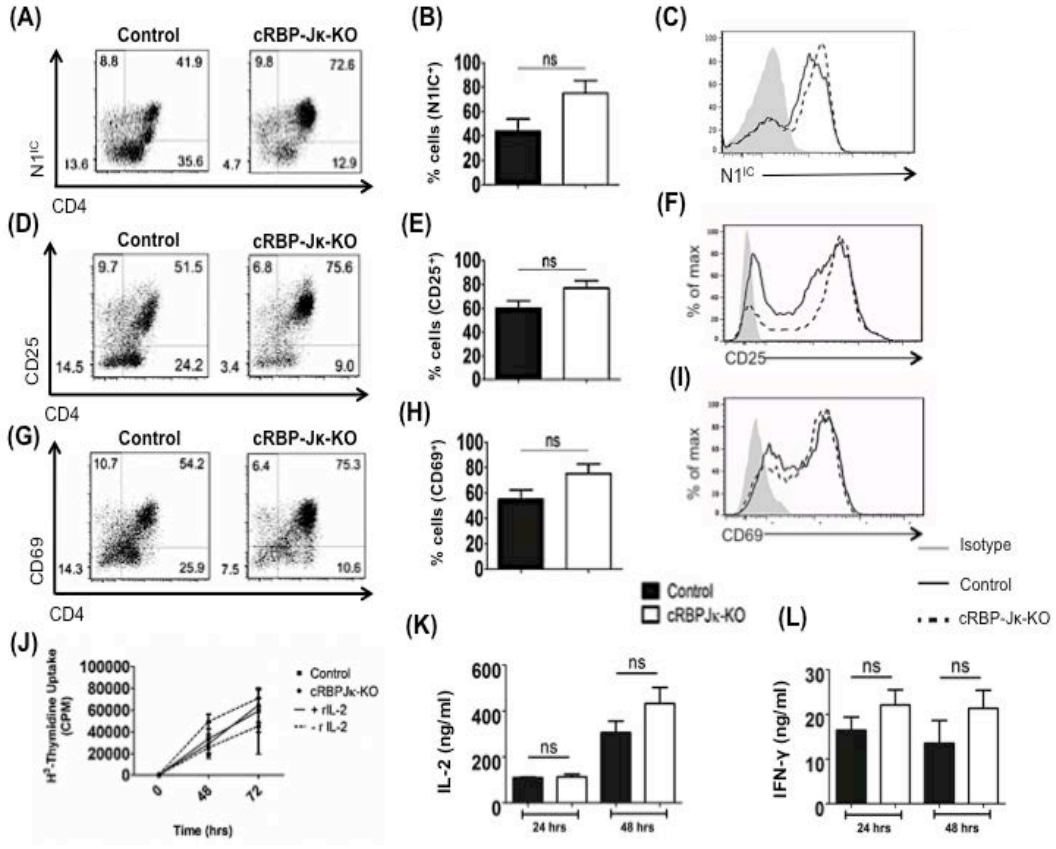


Figure 3.9 Canonical Notch signaling is not required for activation and proliferation of CD4⁺ T cells

$CD4^+$ T cells were isolated from control or $cRBPJ_K$-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 $N1^{IC}$ and analyzed by flow cytometry. **(A, D, G)** Dot plots obtained from flow cytometry showing $CD4^+$ T cells positive for **(A)** $N1^{IC}$ **(D)** CD25 and **(G)** CD69. Numbers in each quadrant represent % of cells. **(B, E, H)** Percentage of cells positive for **(B)** $N1^{IC}$ **(E)** CD25 and **(H)** CD69 obtained from dot plots. n=4. **(C, F, I)** Histograms for **(C)** $N1^{IC}$ **(F)** CD25 and **(I)** CD69. **(J)** Counts per minute (CPM) obtained after ^{3}H-thymidine uptake in $CD4^+$ T cells from control and $cRBPJ_K$-KO mice stimulated as described above with and without rmIL-2 (20ng/ml). **(K)** IL-2 and **(L)** IFN-γ ELISA from supernatants obtained from control and $cRBPJ_K$-KO mice stimulated as described above. n = 6. Data represent mean ± SEM, ns - not significant.

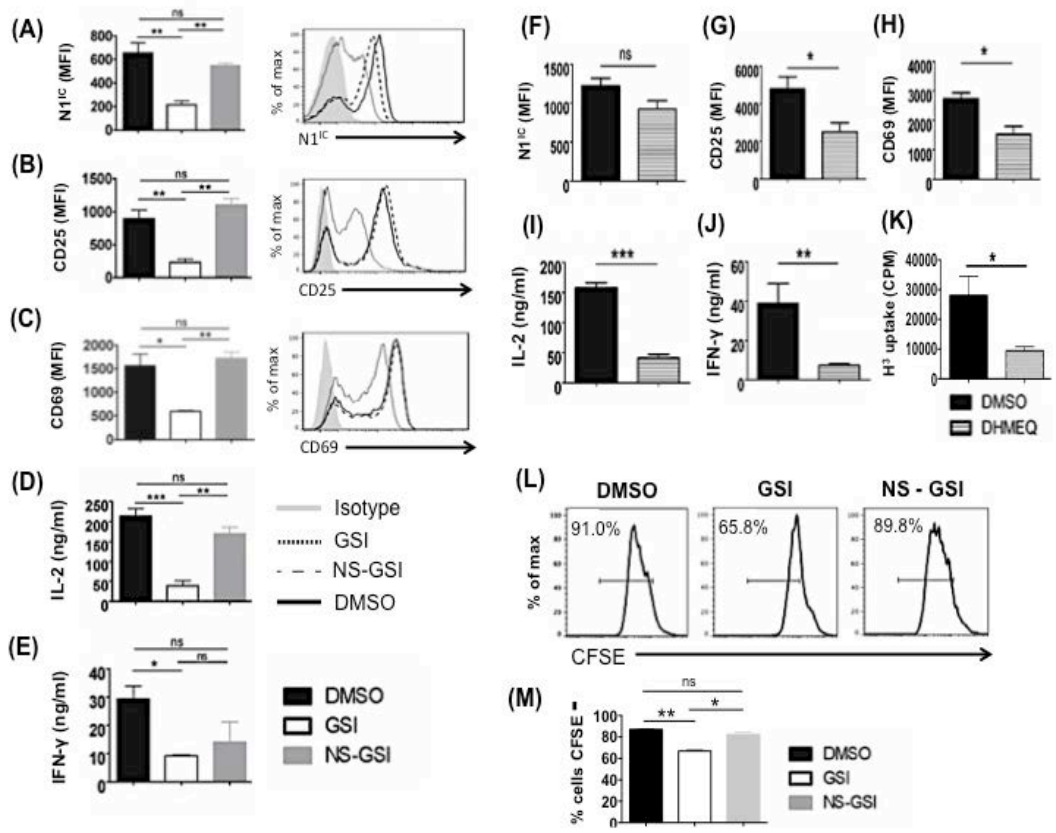


Fig 3.10 Activation and Proliferation of CD4⁺ T cells is $RBPJ_K$ independent but Notch and NF-κB dependent.

$CD4^+$ T cells were isolated from $cRBPJ_K$-KO mice, pretreated with DMSO, GSI or NS-GSI **(A-E, L-M)** or DHMEQ **(F-K)** 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 $N1^{IC}$ 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)** $N1^{IC}$ **(B, G)** CD25 **(C, H)** CD69. n =3-5. Histograms to the right of **(A-C)** show expression of $N1^{IC}$, 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 after tritiated thymidine uptake of cells treated with DMSO or DHMEQ. **(L)** Histograms representing a CFSE Proliferation Assay. $CD4^+$ T cells from $cRBPJ_K$-KO mice pretreated with DMSO, GSI, NS-GSI were labeled with CFSE and activated with plate bound anti-CD3ε and anti-CD28 for 48 hrs followed by flow cytometry analysis. **(M)** Percentage of CFSE negative cells obtained by flow cytometryData represent mean ± SEM * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, ns - not significant.

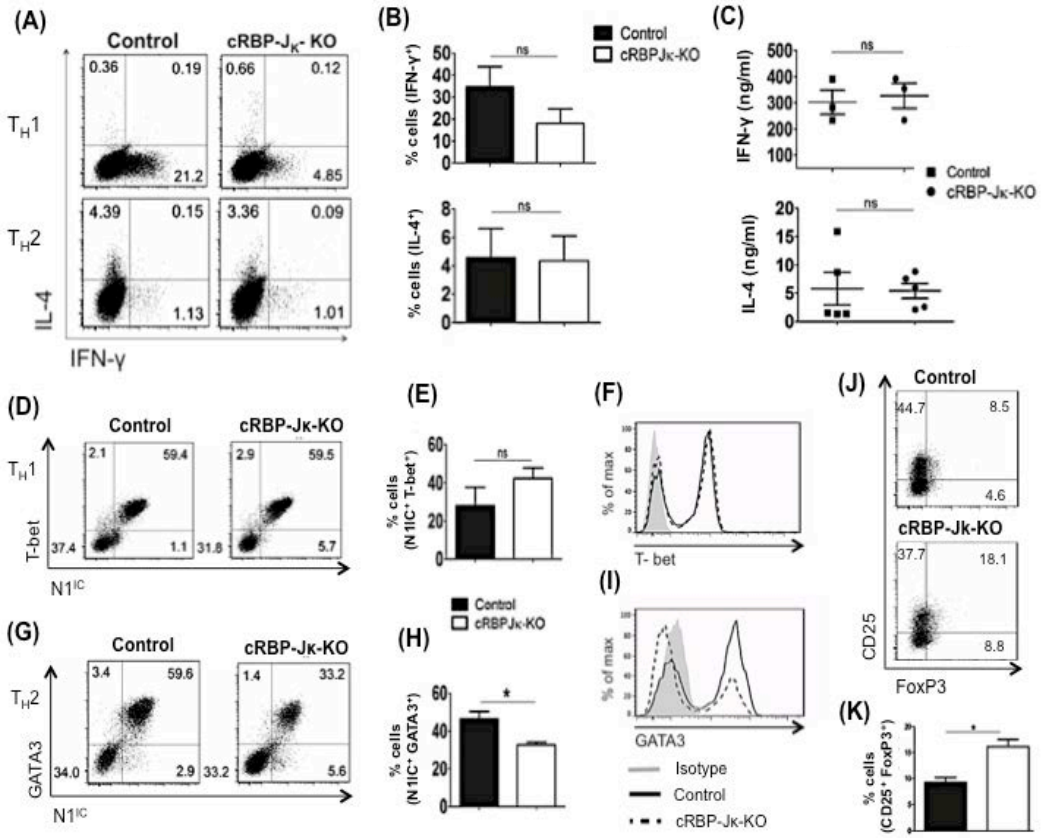


Figure 3.11 RBPJ$_K$ deficiency does not alter CD4$^+$ T cell differentiation *in vitro*

CD4$^+$ T cells from control and cRBPJ$_K$-KO mice were differentiated under T_H1, T_H2 or *i*Treg 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)** % 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_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 N1^{IC} under T_H1 conditions and **(G)** GATA3 and N1^{IC} under T_H2 conditions. **(E, H)** Percentage of double positive cells determined by flow cytometry. n = 5. **(F, I)** Histograms for T-bet and GATA3 expression under T_H1 and T_H2 conditions respectively. **(J)** Flow cytometry plots for CD25$^+$ and FoxP3$^+$ cells. **(K)** Percentage of double positive cells determined by flow cytometry. n= 3. Data represent mean ± SEM. * $p < 0.05$, ns - not significant.

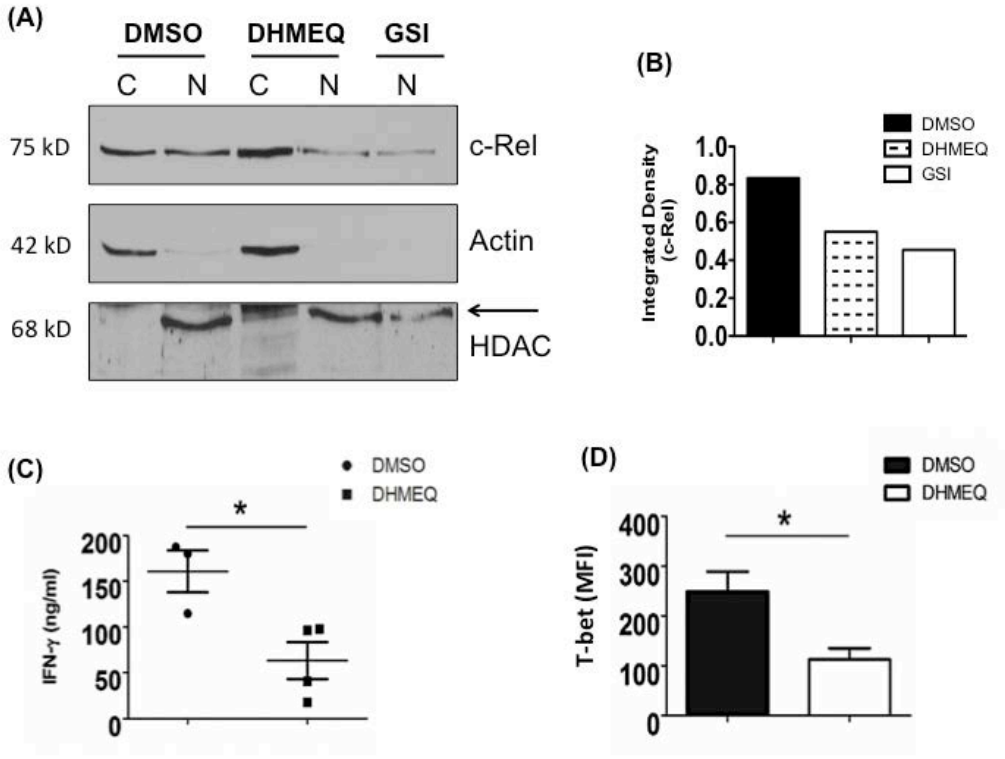


Figure 3.12 T_H1 polarization in the absence of $RBPJ_{K,}$ is NF-$_K$B dependent

(A,B) $CD4^+$ T cells isolated from $cRBPJ_K$-KO mice were pretreated with DMSO DHMEQ or GSI and stimulated in vitro for 48 hrs. Nuclear and cytoplasmic protein fractions were isolated and analyzed by western blotting for translocation of c-Rel. Actin and Histone deacetylase (HDAC) were used as loading controls for cytoplasmic (C) and nuclear (N) extracts respectively. **(B)** Integrated density of c-Rel after normalizing to HDAC. (C,D) $CD4^+$ T cells isolated from $cRBPJ_K$-KO mice were pretreated with DMSO or DHMEQ and differentiated *in vitro* under T_H1 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, *, $p < 0.05$.

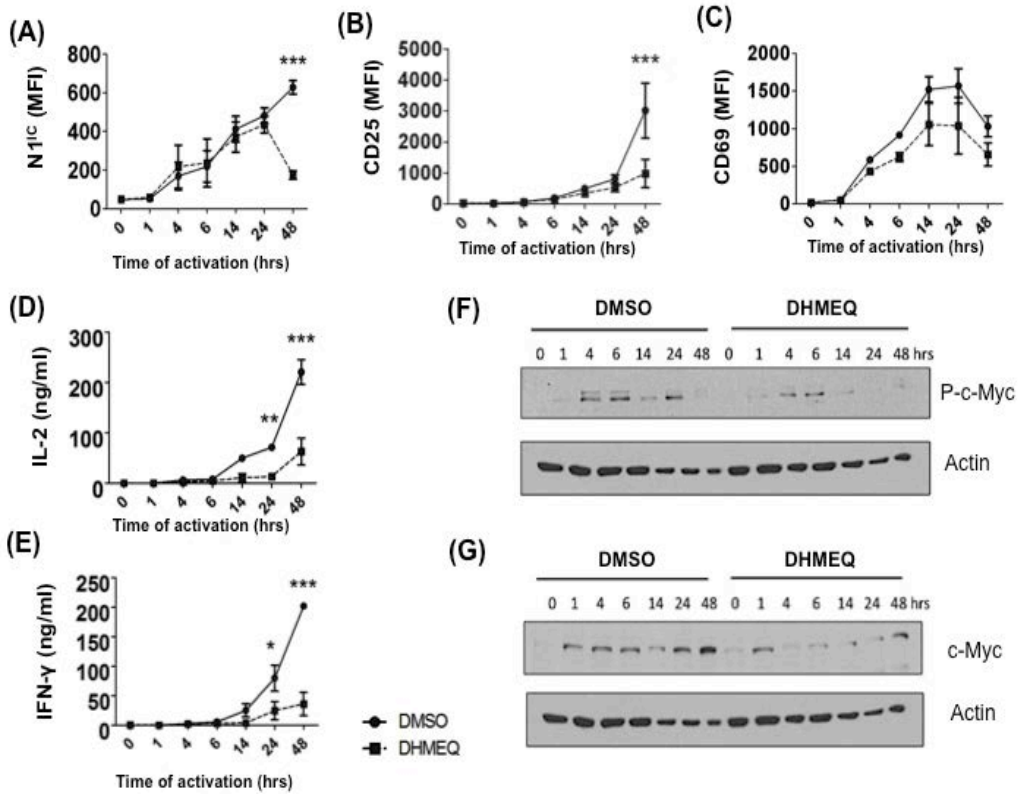


Figure 3.13 Inhibition of NF-$_{\kappa}$B reduces activation and expression of c-Myc

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)** $N1^{IC}$ **(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 the mean ± SEM, *, $p < 0.05$, ** $p < 0.005$, ***, $p < 0.001$.

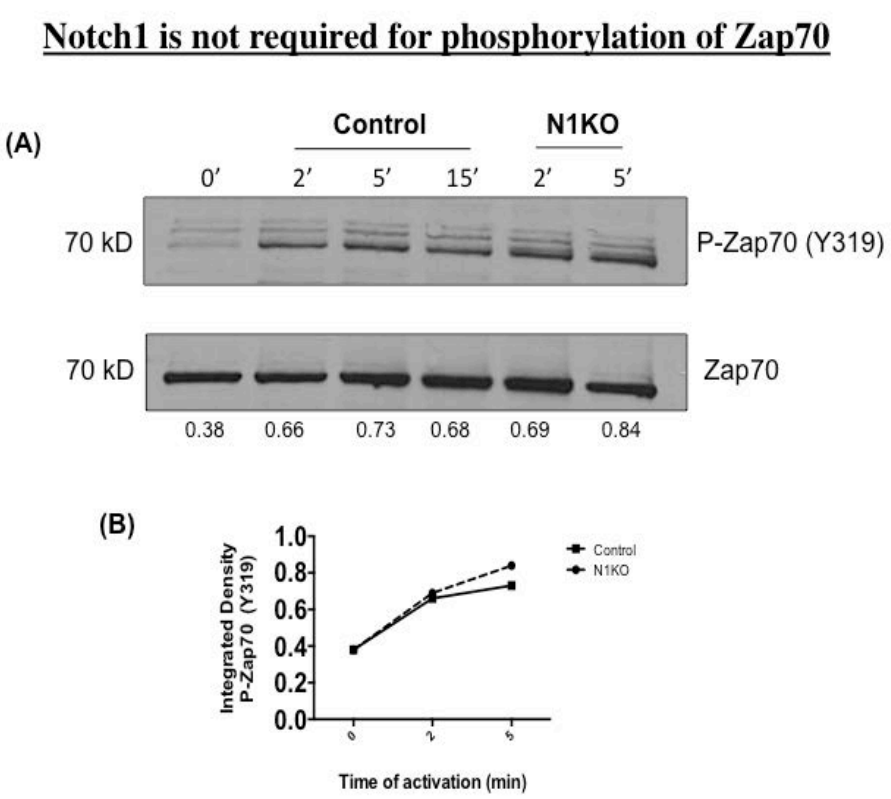


Figure 3.14 Notch1 is not required for phosphorylation of Zap70

While Notch1 was required for regulating signaling events distal to the TCR, it was dispensable for proximal events. Phosphorylation of Zap70 was unaffected in $CD4^+$ T cells conditionally deleted for Notch1.

(A) Western Blot for phosphorylated and total Zap70 **(B)** Integrated Density of Western Blot. Purified $CD4^+$ T cells from Control and cN1KO mice were stimulated with soluble anti-CD3ε and anti-CD28 followed by cross-linking with anti-Hamster IgG for the indicated times. Lysates were made at each time point and used for detecting phospho-Zap70 (Y319) and total Zap70. Data represent two independent experiments.

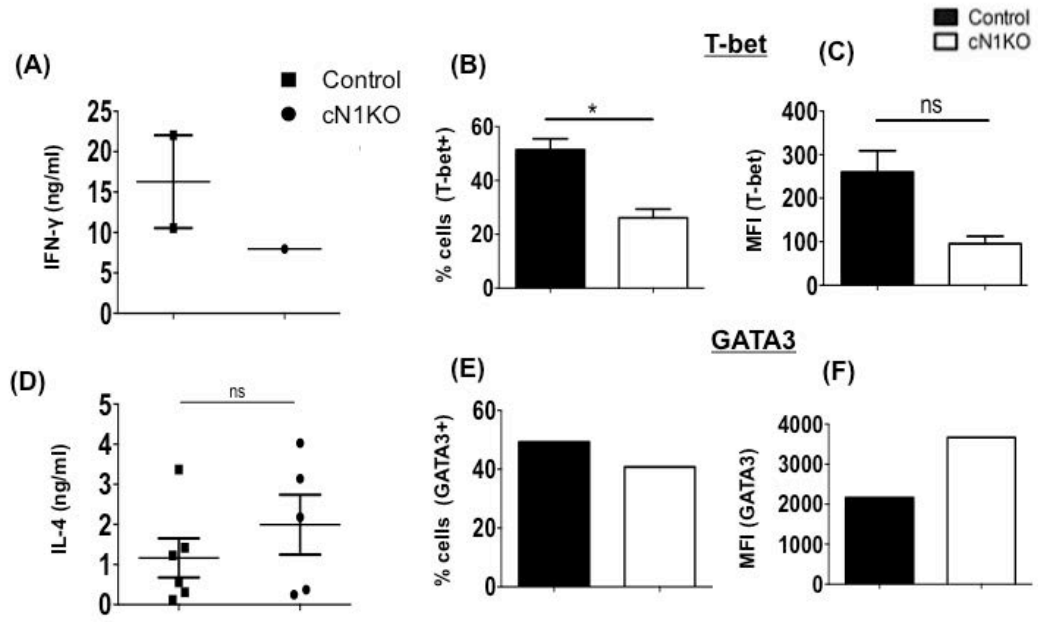


Figure 3.15 Notch1 is required for T-bet and IFN-γ production in Non-polarized cells

To determine if the absence of Notch1 influenced the expression of IFN-γ and IL-4 even in the absence of polarizing cytokines, we determined the expression of each of these cytokines and their associated transcription factors in $CD4^+$ T cells from control and cN1KO mice stimulated under non-polarizing conditions. Absence of Notch1 reduced the expression of IFN-γ and T-bet even in the absence of pre-existing T_H1 conditions (Fig 3.15A-C), but was dispensable for IL-4 and GATA3 (Fig 3.15D-F).

$CD4^+$ T cells from control and cN1KO animals were stimulated with plate bound anti-CD3ε and anti-CD28 for 72 hrs. Cells were used for detecting T-bet and GATA3 by flow cytometry and supernatants were used for detecting cytokines by an ELISA. **(A,D)** IFN-γ and IL-4 detected by ELISA. **(B,E)** Percentage of cells positive for **(B)** T-bet **(E)** GATA3. **(E,F)** Mean Fluorescent Intensity for **(E)** T-bet **(F)** GATA3. n = 1-6. Data represent the mean ± SEM, *, $p < 0.05$. ns- not significant.

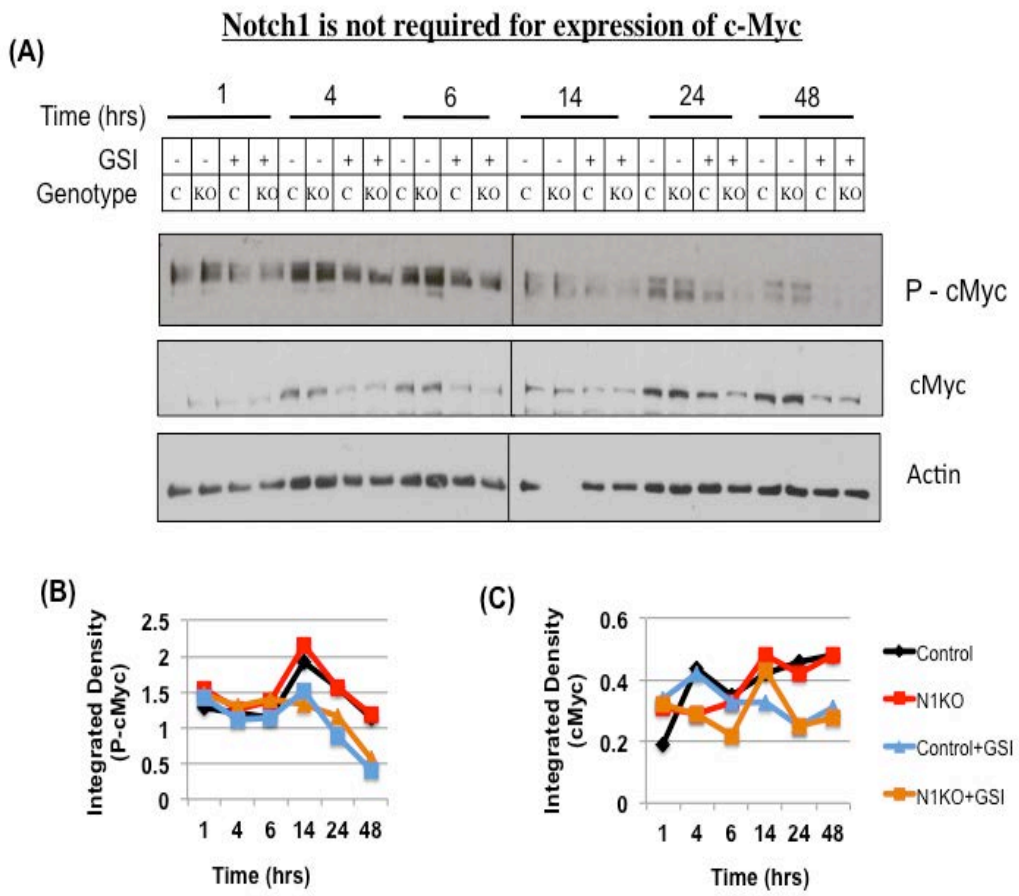


Figure 3.16 Notch1 is not required for expression of c-Myc

Since inhibition of Notch activation via GSI reduced the expression of phosphorylated and total c-Myc, we determined if these effects were Notch1 specific by looking at the c-Myc expression in $CD4^+$ T cells from cN1KO mice over time. We observed that absence of Notch1 did not alter phosphorylated or total-c-Myc. However, GSI treatment of $CD4^+$ T cells lacking Notch1 reduced the expression of both these proteins. Thus, it is likely that c-Myc expression maybe regulated by another Notch family member or could be a GSI mediated effect.

(A) Western Blot for phospho and total c-Myc. Splenocytes from control and cN1KO mice were pre-treated with DMSO or 50μM GSI and stimulated with plate bound anti-CD3ε and anti-CD28 for the indicated time points. Lysates were made at each time point and used for detecting phospho and total c-Myc by Western Blotting. **(B, C)** Integrated density for **(B)** phospho c-Myc **(C)** Total c-Myc

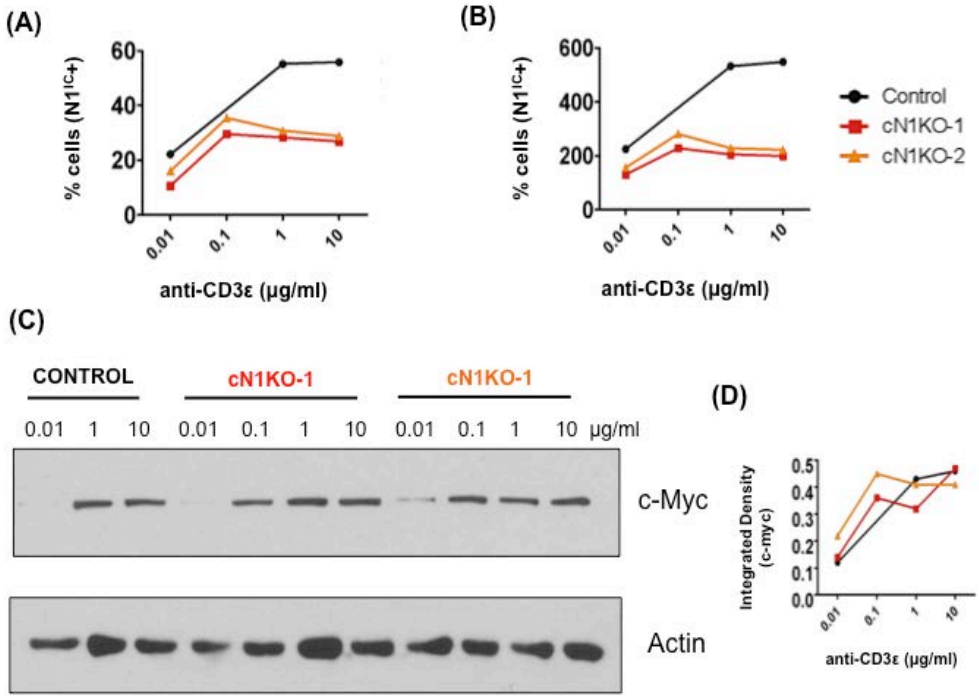


Figure 3.17 Increased expression of c-Myc in response to signal strength is independent of Notch1

To determine if the response of c-Myc to increasing signal strength was Notch1 dependent, we stimulated $CD4^+$ T cells from control and cN1KO mice with increasing signal strength and determined the expression of c-Myc. While $CD4^+$ T cells from control mice showed a dose dependent increase in $N1^{IC}$ with increasing signal strength, this was abolished in mice conditionally deleted for Notch1 (Fig 3.17A,B) However, $CD4^+$ T cells lacking Notch1 continued to express c-Myc in response to increasing signal (Fig 3.17C,D). This suggested that the response of c-Myc to increasing signal strength may require another Notch member or co-operative actions of more than one Notch family members.

$CD4^+$ T cells from control and cN1KO mice were activated with the indicated concentrations of anti-CD3ε and 1μg/ml of anti-CD28 for 48hrs. Cells were harvested and used for detecting intra-cellular Notch1 by flow cytometry. Some cells were used to make lysates to detect total c-Myc by Western blotting. **(A)** Percentage of cells expressing $N1^{IC}$ **(B)** Mean Fluorescent Intensity for $N1^{IC}$ **(C)** Western Blot for c-Myc **(D)** Integrated density for c-Myc.

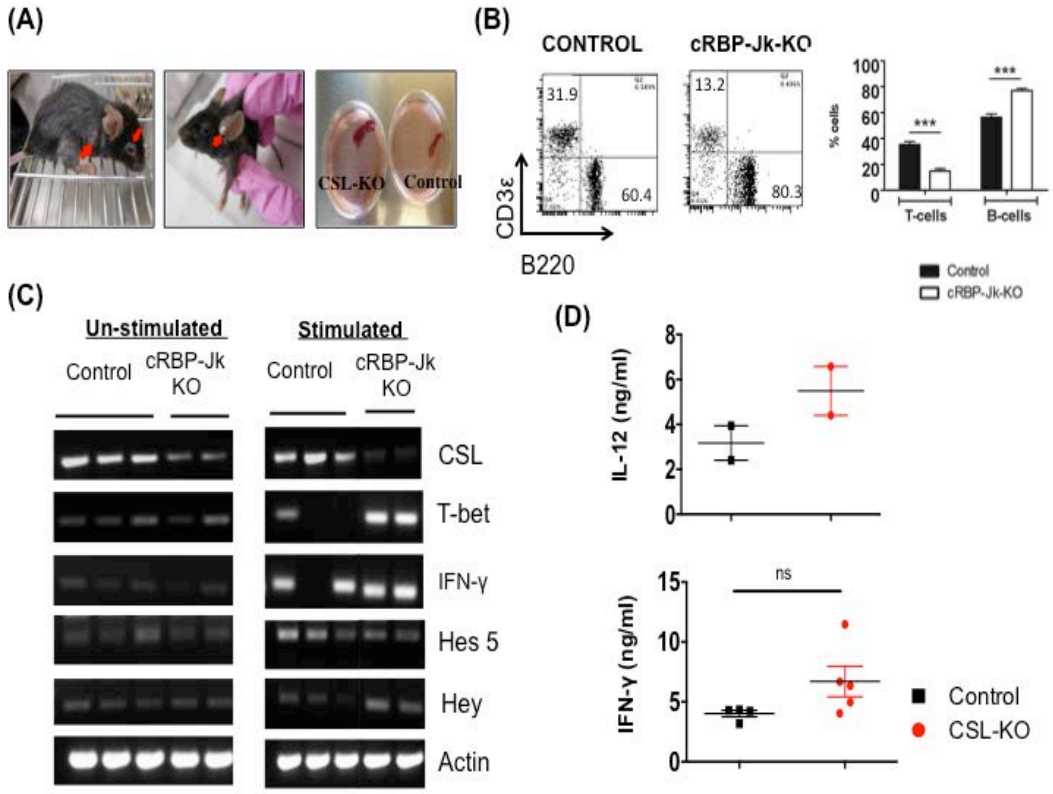


Figure 3.18 Characterization of cRBP-Jk-KO mice

Both control and cRBP-Jk-KO mice were injected with equivalent amounts of Poly (I): Poly (C). However, at the end of the three-week resting period, only cRBP-Jk-KO mice showed phenotypic changes such as hair loss, dry skin, formation of blisters and splenomegaly (Fig 3.18A). Additionally, cRBP-Jk-KO mice had a significantly greater percentage of splenic B-cells at the expense of T-cells compared to control mice (Fig 3.18B). The expression of several downstream target genes was also determined in $CD4^+$ T cells from control and cRBP-Jk-KO animals. A clear increase in the transcript for T-bet and Hey was observed in stimulated cells lacking RBP-Jk compared to control cells, suggesting de-repression of these genes (Fig 3.18C). However, this de-repression was not constitutive since un-stimulated cells lacking RBP-Jk had basal levels of transcripts for all genes. Finally, cRBP-Jk-KO animals also had higher circulating levels of IL-12 and IFN-γ (Fig 3.18D), which may explain the robust T_H1 response observed in the absence of RBP-Jk.

(A) Physical appearance of mice with the red arrows indicating dry skin and blisters. cRBP-Jk-KO mice also have splenomegaly compared to controls. **(B)** Dot plot representing the percentage of T and B cells present in spleens. **(C)** RT-PCR for the indicated target genes in $CD4^+$ T cells from cRBP-Jk-KO and control mice that were either unstimulated or stimulated for 24 hrs with plate bound anti-CD3ε and anti-CD28. **(D)** Serum cytokine levels as detected by an ELISA.

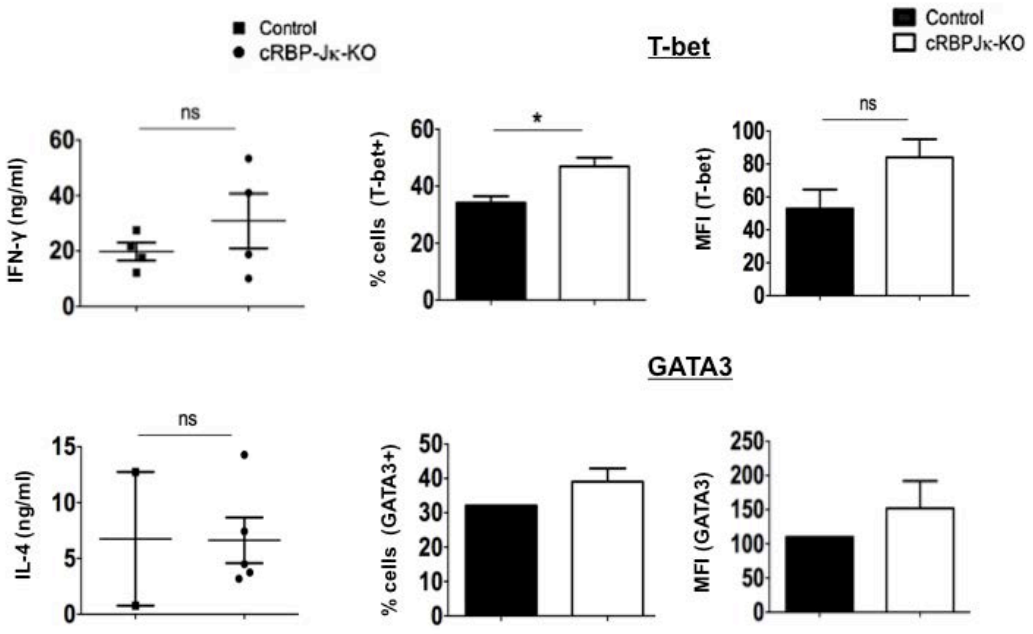


Figure 3.19 Increased expression of IFN-γ and T-bet in the absence of RBP-Jk in non-polarized cells

To determine if the absence of RBP-Jk- influenced the expression of IFN-γ and IL-4 even in the absence of polarizing cytokines, we determined the expression of each of these cytokines and their associated transcription factors in $CD4^+$ T cells from control and cRBP-Jk-KO mice, stimulated under non-polarizing conditions. Absence of RBP-Jk increased the expression of IFN-γ and T-bet even in the absence of pre-existing T_H1 conditions (Fig 3.19A-C).

$CD4^+$ T cells from control and cRBP-Jk-KO animals were stimulated with plate bound anti-CD3ε and anti-CD28 for 72 hrs. Cells were used for detecting T-bet and GATA3 by flow cytometry and supernatants were used for detecting cytokines by an ELISA. **(A,D)** IFN-γ and IL-4 detected by ELISA. **(B,E)** Percentage of cells positive for **(B)** T-bet **(E)** GATA3. **(E,F)** Mean Fluorescent Intensity for **(E)** T-bet **(F)** GATA3. n = 1-6. Data represent the mean ± SEM, *, $p < 0.05$. ns- not significant.

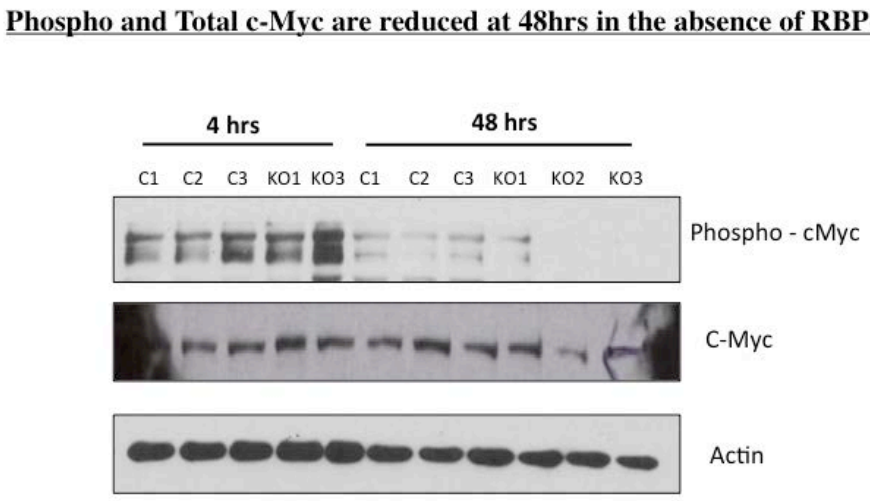


Figure 3.20 RBP-Jk maybe required for expression of c-Myc at later time points

We observed previously that the expression of phosphorylated and total c-Myc did not require Notch1. Hence to determine if c-myc expression required canonical Notch signaling, we determined the levels of phosphorylated and total c-Myc in $CD4^+$ T cells from control and cRBP-Jk-KO at two different time points after stimulation. 48 hrs after activating via the TCR, CD4+ T cells from cRBP-Jk-KO mice had substantially lower levels of both phosphorylated and total c-Myc compared to cells from control mice. However, this difference was not observed 4 hrs after activation. Thus canonical Notch signaling may be dispensable for the first wave of c-Myc expression but maybe required for the second wave of c-Myc, distal to the TCR.

(A) Western Blot for phospho and total c-Myc. Splenocytes from control and cRBP-Jk-KO mice were stimulated with plate bound anti-CD3ε and anti-CD28 for the indicated time points. Lysates were made at each time point and used for detecting phospho and total c-Myc by Western Blotting.

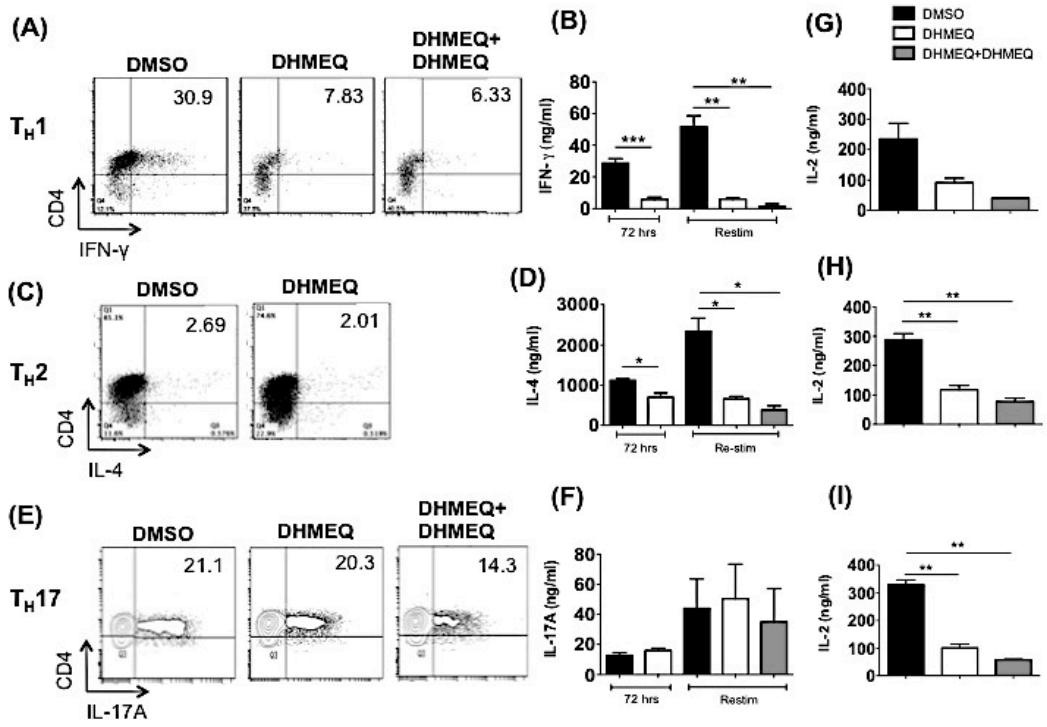


Figure 3.21 Regulation of helper T cell fate by NF-$_K$B

To determine the contribution of NF-$_K$B in regulating T cell fate, we treated CD4$^+$ T cells with DHMEQ and asked how the absence of NF-$_K$B activation affected polarization to T_H1, T_H2 and T_H17 fates. We observed that inhibition of NF-$_K$B significantly decreased the number of IFN-γ positive cells as well as the amount of secreted IFN-γ and IL-4 (Fig 3.21A-D). However, DHMEQ treatment did not significantly alter the percentage of IL-17 positive cells or the amount of secreted IL-17 (Fig 3.21 E,F). Additionally, DHMEQ treatment significantly decreased IL-2 secretion under all polarizing conditions. Thus these data imply that NF-$_K$B is required for T_H1 and T_H2 cell fate specification but maybe dispensable for T_H17.

CD4$^+$ T cells were pre-treated with DMSO or DHMEQ and stimulated with plate bound anti-CD3ε and anti-CD28 for 48 hrs. After harvesting cells were-re-stimulated with or without DHMEQ (DHMEQ+DHMEQ - DHMEQ pre-treated and DHMEQ added during re-stimulation) in the presence and absence of Golgi Plug. Cells treated with Golgi Plug were used for detecting intra-cellular cytokines by flow cytometry. Supernatants from cells that did not receive Golgi Plug were used for detecting cytokine levels by an ELISA. **(A,C,E)** Dot plots representing the percentage of cells positive for the specified cytokines **(B,D,F)** ELISAs for **(B)** IFN-γ **(D)** IL-4 and **(F)** IL-17 **(G-I)** IL-2 ELISAs for cells polarized to **(G)** T_H1 **(H)** T_H17

CHAPTER 4

CROSS-TALK BETWEEN THE NOTCH AND mTOR PATHWAY

4.1 Introduction

Engagement of the T cell receptor stimulates the onset of a multitude of signal transduction pathways that play critical roles in regulating T cell function. Of these, the conserved Serine/Threonine kinase, mTOR (mechanistic target of Rapamycin) plays a central role as an environmental sensor that integrates extrinsic signals to regulate cellular growth and metabolism (Laplante *et al.,* 2009). mTOR exists in two distinct complexes that differ in their composition. In the mTORC1 complex, mTOR associates with RAPTOR (regulatory associated protein of mTOR) along with mLST8, Deptor and Pras40 (Laplante *et al.,* 2009). Association of mTOR with RICTOR (Rapamycin insensitive companion of TOR) along with PROTOR, mSIN1, mLST8 and Deptor yields the mTORC2 complex (Laplante *et al.,* 2009). mTORC1 can be activated by various stimuli such as amino acids, growth factors and nutrients leading to phosphorylation of its downstream substrates - S6 kinase 1 (S6K1) and eLF4e binding protein 1 (4E-BP1), which associate with mRNAs and control the rate of protein synthesis (Ma and Blenis, 2009)In sharp contrast to mTORC1, the precise upstream regulator(s) of mTORC2 are only recently being discovered with one study implying mTORC2 activation by ribosomes (Zinzalla *et al*., 2011). Upon activation,

mTORC2 phosphorylates the AGC family of kinases some of which are controlled by the phosphoinositide 3 – kinase (PI3K) – Akt pathway. These include phosphorylation of the hydrophobic motif of Akt at S473, SGK1 and PKC (Guertin *et al*., 2006). Recent studies have outlined a central role for mTOR during thymocyte differentiation as well as acquisition of helper T cell fate (Ciofani and Zuniga-Pflucker, 2005; Delgoffe *et al*., 2009; Lee *et al*., 2010; Lee *et al*., 2012)

In addition to mTOR, the Notch pathway also plays a crucial role in regulating key processes during thymocyte development as well as peripheral T cell function (Radtke *et al*., 1999; Palaga *et al*., 2003). Notch promotes survival of pre-T cells at the Beta selection checkpoint by enhancing cell size, glucose uptake and metabolism via Akt (Ciofani *et al*, 2005). Furthermore, mTORC2 and PDK1 have been suggested to be important for relaying Notch derived trophic signals for regulating Akt during thymocyte differentiation (Lee *et al*., 2012; Kelly *et al*., 2008). Additionally, Notch interacts with PI3K and exerts anti-apoptotic effects in a manner that requires mTORC2 but is independent of RBP-J_K (Sade *et al*., 2004; Perumalsamy *et al*., 2009). While these observations imply cross-talk between the Notch and mTOR pathways, whether Notch also regulates the activity of mTOR in peripheral T cells is obscure. Most importantly, while the downstream substrates of mTORC2 are well defined, the upstream regulators of mTORC2 in peripheral T cells are unknown. We have previously demonstrated that activation of T cells via anti-CD3ε and anti-CD28 *in vitro* generates $N1^{IC}$ in T cells (Palaga *et al*., 2003; Adler *et al*., 2003). Given the requirement of mTORC2 for relaying

Notch induced signals in thymocytes, we ask whether $N1^{IC}$ generated in T cells is required for regulating the activity of mTOR in peripheral T cells.

In this study, we demonstrate that Notch activation is required for regulating mTORC2 activity but is dispensable for mTORC1 activity since pharmacological inhibition as well as conditional deletion of Notch1 impaired phosphorylation of mTORC2 but not mTORC1 substrates. Such regulation of mTORC2 occurs via non-canonical Notch signaling. Additionally, we provide evidence that Notch1 controls the activity of mTORC2 by regulating the levels of eIF6. This likely involves cross-talk with the NF-κB pathway. Furthermore, Notch relays its signals through mTORC2 and mTOR to control IFN-γ and IL-2 production. Thus, our data show for the first time in peripheral T cells that Notch acts upstream of mTOR. Most importantly, our data outline a mechanism underlying non-canonical function of Notch signaling and its regulation of mTORC2.

4.2 Results

4.2.1. GSI treatment reduces mTORC2 activity

To determine if Notch activation is required for mTORC2 activity, we asked how inhibition of Notch activation in $CD4^+$ T cells affected the phosphorylation of its downstream targets. GSI treatment abrogated phosphorylation of - Akt at the hydrophobic motif S473, 60 mins after stimulating via the TCR (Fig 4.1A,B). A

partial decrease in phosphorylation was observed at residue T450 (Fig 4.1A,B). In addition, GSI treatment also reduced phosphorylation of FOXO3a, a direct downstream target of Akt (Fig 4.1A,C). In sharp contrast, phosphorylation of Akt at residue T308, mediated by the PDK1 kinase, was unaffected after GSI treatment (Fig 4.1A,B). Similarly, GSI treatment did not reduce phosphorylation of the mTORC1 substrate S6K1 (Fig 4.1A,D). Thus, these data suggest that while Notch activation is required for mTORC2 activity, it likely is dispensable for mTORC1 activity. Furthermore, Notch most likely acts upstream of mTORC2.

4.2.2 Notch1 is required for mTORC2 activity

Since GSIs have multiple substrates other than Notch and inhibit all isoforms of the Notch receptor, we asked if reduced phosphorylation of mTORC2 substrates in the absence of Notch activation was uniquely due to Notch1 and not a GSI mediated effect. To address this question, we used to different approaches. Firstly, we treated cells with a NS-GSI. Treatment with NS-GSI did not affect phosphorylation of Akt at residues S473 and T450 (Fig 4.2A-C) suggesting that these phosphorylations were Notch specific. Secondly, to determine the contribution of Notch1 in regulating phosphorylation of mTORC1 and mTORC2 targets, we isolated $CD4^+$ T cells from mice with a conditional deletion in Notch1 (cN1KO). $CD4^+$ T cells from cN1KO mice were smaller in size after activation via the TCR compared to $CD4^+$ T cells from control or wild type mice (Fig 4.3A). Furthermore, $CD4^+$ T cells from cN1KO mice had substantially reduced

phosphorylation of Akt at residue S473. (Fig 4.3B,C). However, deficiency of Notch1 did not affect the phosphorylation of Akt at T450, T308, FOXO3a or the mTORC1 target-S6K1 (Fig 4.3B-E). Collectively, these data show that Notch1 is required for phosphorylation of Akt at S473 but is not needed for phosphorylation of PDK1 or mTORC1 targets. Furthermore, these data also confirm that Notch1 specifically controls mTORC2 mediated phosphorylation of Akt-S473 and this is not simply a GSI mediated effect.

4.2.3. Regulation of mTORC2 by Notch1 occurs independently of RBP-J_K

In addition to interacting with RBP-J_K, $N1^{IC}$ has also been shown to interact with other proteins in the cytoplasm and nucleus, exerting some of its effects in a non-canonical fashion (Minter *et al.,* 2012). Thus we asked whether canonical Notch signaling was required for phosphorylation of mTORC1 and mTORC2 targets by isolating $CD4^+$ T cells from mice with a conditional deletion in RBP-J_K (cRBP-J_K-KO). Absence of RBP-J_K did not alter phosphorylation of any of mTORC2 targets, nor did it alter phosphorylation of the PDK1 target T308 -Akt, or the Akt target FOXO3a (Fig 4.4A). Additionally, phosphorylation of the mTORC1 target - pS6K1 Thr 389 was also unaffected by the absence of RBP-J_K (Fig 4.4A). These results demonstrated that canonical Notch signaling was not required for regulating the activity of either mTORC1 or 2.

To reconcile differences observed between phosphorylation of mTORC2 targets in the absence of Notch1 versus RBP-J_K, we determined whether $CD4^+$ T

cells that lacked RBP-J_K, express $N1^{IC}$. We observed that $CD4^+$ T cells from cRBP-J_K - KO mice expressed $N1^{IC}$ after T cell activation just as efficiently as $CD4^+$ T cells from control mice (Fig 3.9A,B). Thus we reasoned that phosphorylation of mTORC2 targets in the absence of RBP-J_K was Notch dependent but RBP-J_K independent. To confirm this, we inhibited Notch activation in $CD4^+$ T cells lacking RBP-J_K, by GSI treatment. We observed that inhibition of Notch activation in the absence of RBP-J_K, reduced phosphorylation of mTORC2 targets – residues S473-Akt and partially reduced phosphorylation of T450-Akt (Fig 4.4A,B,C), but had no effect on the PDK1 target – T308-Akt or mTORC1 target T389-S6K1 (Fig 4.4F,D). In addition, phosphorylation of the Akt substrate FOXO3a-S253, was also modestly reduced (Fig 4.4A,E). These data confirm that regulation of mTORC2 function requires Notch activation but is independent of RBP-J_K.

4.2.4. Notch1 acts upstream of mTOR

Our data showing a requirement of $N1^{IC}$ for the phosphorylation of mTORC2 targets suggested that Notch likely acts upstream of mTORC2. To confirm this, we determined if the absence of Rictor or Raptor altered the ability of $CD4^+$ T cells to generate $N1^{IC}$ after stimulation via the TCR. We isolated $CD4^+$ T cells from mice in which Rictor or Raptor was deleted under the control of the tamoxifen-inducible UbiquitinC-CreERT2 promoter (Obtained from D. Sabatini and D. Lamming). These have been referred to as Rictor-KO or Raptor –KO respectively. $CD4^+$ T cells from both KO mice had a substantial decrease in Rictor

or Raptor (Fig 4.5A,D). However, upon stimulation via the TCR, Rictor and Raptor-KO mice showed a similar percentage of cells expressing $N1^{IC}$ in comparison to $CD4^+$ T cells obtained from wild type (WT) mice (Fig 4.5B,E). Additionally, deletion of either Rictor or Raptor did not influence the amount of $N1^{IC}$ generated post stimulation. These data suggested that Notch acts upstream of both mTORC1 and mTORC2 since deletion of either of these complexes did not influence Notch activation.

Since the association of the central mTOR protein with either Rictor or Raptor forms mTORC1 or 2, we asked whether inhibition of mTOR activity itself could alter the generation of $N1^{IC}$. We addressed this question by employing two frequently used pharmacological inhibitors of mTOR function – Pp242 and Ku-0063794. Treatment of $CD4^+$ T cells with 80 nm of Pp242 effectively abolished phosphorylation of Akt-S473 and reduced phosphorylation of pS6K1-Thr389 while treatment with 1μM Ku-0063794 abrogated phosphorylation of both these residues confirming inhibition of mTOR activity (Fig 4.5G). However, despite of mTOR inhibition, $CD4^+$ T cells continued to express $N1^{IC}$ after stimulation via the TCR (Fig 4.5H-K). Collectively, these results show that $CD4^+$ T cells deficient for mTORC1, mTORC2 or functional mTOR, continue to express $N1^{IC}$ after stimulation via the TCR. Thus Notch acts upstream of the mTOR pathway.

4.2.5. Notch1 may regulate IFN-γ and IL-2 production via mTOR

$N1^{IC}$ generated following activation via the TCR, plays a crucial role in regulating downstream signaling events such as the activation and proliferation of peripheral T cells. These include secretion of cytokines such as IL-2 and IFN-γ. Additionally, both Notch and mTORC2 have been demonstrated to be required for specification of T_H1 fate and secretion of IFN-γ (Lee *et al*., 2012; Maekawa *et al*., 2003; Minter *et al*., 2005) by peripheral $CD4^+$ T cells. However, whether Notch mediates control of peripheral T cell activation events via mTORC2 is unknown. Although mTORC2 is thought to be necessary for relaying Notch derived trophic signals in DN3 thymocytes, (Ciofani *et al*., 2005; Lee *et al*., 2012) whether mTORC2 is also required for propagating signaling events involving $N1^{IC}$ downstream of the TCR is unknown. Furthermore, the contribution of mTORC1 and the mTOR complex itself in relaying Notch induced signaling events remains obscure. We observed that $CD4^+$ T cells from cN1KO mice that have reduced mTORC2 function are also deficient in secreting IFN-γ and IL-2 after activation (Fig 4.6A,B). Furthermore, $CD4^+$ T cells that lack Rictor, Raptor or mTOR continue to express $N1^{IC}$ after activation (Fig 4.5). Since $N1^{IC}$ is required for the production of IFN-γ and IL-2, we asked whether these $CD4^+$ T cells, which expressed $N1^{IC}$ but lacked functional Rictor, Raptor or mTOR, could also secrete IFN-γ and IL-2 after activation. We observed that $CD4^+$ T cells from Rictor KO mice were significantly impaired in their ability to secrete IFN-γ and IL-2 compared to $CD4^+$ T cells from control mice (Fig 4.6B,E). On the contrary,

cells from Raptor KO mice had a partial reduction in IFN-γ and no significant difference in IL-2 compared cells from control mice (Fig 4.6B,E). Additionally, inhibition of mTOR function by two different inhibitors –Pp242 and Ku-0063794 significantly impaired the ability of $CD4^+$ T cells to secrete both these cytokines (Fig 4.6B,E). Therefore these results showed that despite the expression of $N1^{IC}$ after activation, $CD4^+$ T cells that are deficient for either Rictor or functional mTOR are unable to secrete significant levels of IFN-γ and IL-2. Thus we propose that Notch regulates expression of these cytokines via mTORC2 and mTOR but does not require mTORC1.

4.2.6. Notch1 is required for the expression of eIF6

The precise upstream regulators of mTORC2 are obscure. A recent study suggested that mTORC2 is activated by association with the ribosome (Zinzalla *et al*., 2011). In fact, the ribosome anti-association factor eIF6 is required for ribosome assembly (Ceci *et al*., 2003). Importantly, eIF6 is a transcriptional target of Notch and is required for invasion and migration of ovarian cancer cell lines (Benelli *et al*., 2012). Since a direct relationship between Notch and mTOR has never been demonstrated in peripheral $CD4^+$ T cells, we reasoned that Notch may control mTORC2 activity by regulating the levels of eIF6. Thus we determined whether inhibition of Notch activation also reduced the levels of eIF6. GSI treated $CD4^+$ T cells as well as $CD4^+$ T cells obtained from cN1KO mice showed a substantial decrease in eIF6 (Fig 4.7A,B,C). In contrast, $CD4^+$ T cells from

cRBP-J_K - KO mice expressed marginally increased levels of eIF6 in comparison to controls. However, this increase was abolished after GSI treatment (Fig 4.7A,D). These data demonstrate that RBP-J_K independent Notch signaling may likely regulate mTORC2 via eIF6.

4.2.7 Notch1 is required for the expression of Rictor and Raptor

Since eIF6 is thought to be required for the assembly of mTORC2 with the ribosome and since the expression of eIF6 itself is reduced in the absence of Notch activation, we asked whether Notch signaling was also required for the expression of total Rictor and Raptor. We observed that $CD4^+$ T cells in which Notch activation was inhibited via GSI treatment or $CD4^+$ T cells from cN1KO animals had substantially low levels of Rictor and modestly low levels of Raptor compared to DMSO treated cells or cells from control mice (Fig 4.8A,B,C,E,F). On the contrary $CD4^+$ T cells from cRBP-J_K-KO mice were unimpaired in their ability to express Rictor and Raptor (Fig 4.8A,D,G). These data suggest that $N1^{IC}$ is required for the expression of Rictor and at least partially required for raptor expression. Thus, Notch may regulate the activity of mTORC2 by regulating the total levels of eIF6 and Rictor.

4.2.8 NF-κB is required for the expression of eIF6, Rictor and Raptor

Notch has been shown to transcriptionally regulate the expression of eIF6 (Benelli *et al*., 2012). However although we observed a decrease in eIF6

expression in the absence of Notch1, such a decrease was independent of RBP-J_K. Since Notch has been demonstrated to interact with other transcription factors besides RBP-J_K, we reasoned that interaction of Notch with these alternate transcription factors could control eIF6 expression. One of these alternate transcription factors that Notch has been shown to interact with is NF-κB (Shin *et al.,* 2006). Thus we asked whether inhibiting the activity of NF-κB using DHMEQ influenced the expression of eIF6. We observed that DHMEQ treated $CD4^+$ T cells had a substantial decrease in eIF6 expression (Fig 4.9A,D). This was accompanied by a decrease in total levels of Rictor and Raptor suggesting that NF-κB is required for the expression of all these proteins (Fig 4.9A-C).

Discussion

The mTORC2 kinase is the only known kinase to phosphorylate Akt at residue S473. Activated Akt executes several downstream functions, some of which involve cell proliferation, metabolism and apoptosis, making it a critical regulator of cell growth and survival. Despite this mTORC2-mediated regulation of Akt, the upstream regulator(s) of mTORC2 itself are largely unknown. In this study we show for the first time that Notch controls the expression and activity of mTORC2 (as determined by the ability of mTORC2 to phosphorylate its substrates) in peripheral $CD4^+$ T cells. We show that pharmacological inhibition of Notch activation abrogates phosphorylation of two different mTORC2 substrates – Akt-S473 and Akt-T450 as well as the direct downstream substrate of

Akt, FOXO3a. We confirmed these observations in $CD4^+$ T cells conditionally deleted for Notch1. Although $CD4^+$ T cells from cN1KO mice had almost no phosphorylation of Akt at S473, unlike GSI treatment, phosphorylation of Akt-T450 and FOXO3a was unimpaired. Since GSIs inhibit all isoforms of the Notch receptor and have multiple substrates besides Notch, this discrepancy could be explained by the fact that mTORC2 -mediated phosphorylation of Akt at T450 may require another Notch receptor or maybe a GSI mediated effect. However, treatment with a Notch sparing GSI did not alter phosphorylation of Akt T450 (Fig 4.8) suggesting that another Notch receptor maybe involved in regulating phosphorylation of this residue.

Additionally, we also observed that canonical Notch signaling did not alter phosphorylation of mTORC2 targets. This could be explained by the fact that $CD4^+$ T cells from cRBP-J_K - KO animals continue to express $N1^{IC}$ after activation suggesting a Notch dependent but RBP-J_K independent regulation of mTORC2. Supporting this notion, inhibition of Notch activation in $CD4^+$ T cells deficient for RBP-J_K reduced phosphorylation of Akt-S473, T450 and FOXO3a. We did not see any effect of Notch inhibition on mTORC1 targets suggesting that Notch maybe more important for regulation of mTORC2 but not mTORC1 function. Thus our data show for the first time that Notch regulates the activity of mTORC2 in peripheral $CD4^+$ T cells. Although a similar Notch-mTORC2 association has been implied by several studies in DN3 thymocytes (Ciofani *et al.,* 2005; Lee *et al.,* 2012), ours is the first to show cross-talk between the two pathways in peripheral $CD4^+$ T cells. Furthermore, our data also demonstrate for

the first time an RBP-J_K independent role of Notch-mediated regulation of mTORC2. Given the dispensability of RBP-J_K in regulating mTORC2, it is possible that membrane bound or activated Notch may act as a scaffolding-like protein that stabilizes the mTORC2 complex, facilitating its function. Indeed, intra-cellular Notch has been shown to physically associate with PI3K and mTORC2 in cell lines (Perumalsamy *et al.,* 2009; Sade *et al.,* 2004). However, further experiments are required to address this possibility and determine whether Notch does in fact co-localize with mTORC2 and/or Akt in peripheral $CD4^+$ T cells.

To confirm whether Notch acts upstream of the mTOR pathway, we determined whether absence of either Rictor, Raptor or inhibition of mTOR activity influenced the ability of the TCR to trigger the activation of Notch upon stimulation. Interestingly, deletion of neither of the aforementioned proteins affected Notch activation suggesting that Notch does in fact act upstream of the mTOR pathway. Lee et al. have demonstrated a similar observation in a study where DN3 thymocytes lacking Rictor expressed Notch after co-culture with OP9 cells expressing DLL1. They also went on to show that not only does Notch act upstream of mTORC2, but also mediates some of its functions through mTORC2 since these Rictor deficient thymocytes were small, had impaired proliferative capacity and were also unable to differentiate into DP thymocytes inspite of ongoing DLL1-Notch signaling (Lee *et al.,* 2012). Thus, we determined whether Notch exerts some of its effects via mTORC2 in peripheral $CD4^+$ T cells. We observed that Notch required mTORC2 and mTOR but not mTORC1 to regulate

the production of IFN-γ and IL-2. Although our data imply that mTORC2 and mTOR relay some of the effects of Notch, additional experiments are required to determine if addition of active mTORC2 or Akt into $CD4^+$ T cells from Rictor KO mice or in $CD4^+$ T cells lacking Notch1 can rescue the production of these cytokines.

The upstream regulators of mTORC2 are not defined. A recent study demonstrated that activation of mTORC2 can be accomplished by its association with the ribosome (Benelli *et al*., 2012). Interestingly, the ribosome anti-association factor- eIF6, has been demonstrated to be a transcriptional Notch target in cancer cells. Supporting this observation, inhibition of Notch activation substantially decreased the levels of eIF6 suggesting that Notch may regulate the activity of mTORC2 through eIF6. While we observed a decrease in eIF6 in the absence of Notch1, we did not observe any difference in eIF6 expression after deletion of RBP-J_K. Thus it is likely that Notch may exert transcriptional control over eIF6 by its association with transcription factors besides RBP-J_K, such as NF-κB. Indeed, inhibition of NF-κB reduced the levels of eIF6 suggesting that transcriptional regulation of eIF6 may require both Notch and NF-κB. However, further experiments are required to confirm precisely how Notch co-operates with NF-κB to control the expression of eIF6 and whether NF-κB binding sites exist upstream of the eIF6 promoter.

In addition to eIF6, inhibition of Notch activation also impaired the expression of total Rictor and Raptor, although no change was observed in phosphorylation of mTORC1 targets. Thus, it maybe possible that a small amount

of Raptor may be sufficient for phosphorylation of downstream targets. In addition, inhibition of NF-κB activity also reduced the levels of Rictor and Raptor suggesting that Notch and NF-κB may co-operate to control the expression of both these proteins. However the precise molecular mechanism underlying such regulation requires further experimentation.

Thus our data show for the first time that $N1^{IC}$ generated downstream of the TCR controls the activity of mTORC2 by regulating the expression of eIF6. Such regulation likely occurs via NF-κB. Additionally, our data also demonstrate that Notch may directly control the expression of both Rictor and Raptor via NF-κB (Fig 4.10). In conclusion, our data identify Notch to be a novel regulator of mTORC2. Further experiments are required to elucidate the role of NF-κB in Notch mediated control of mTORC2.

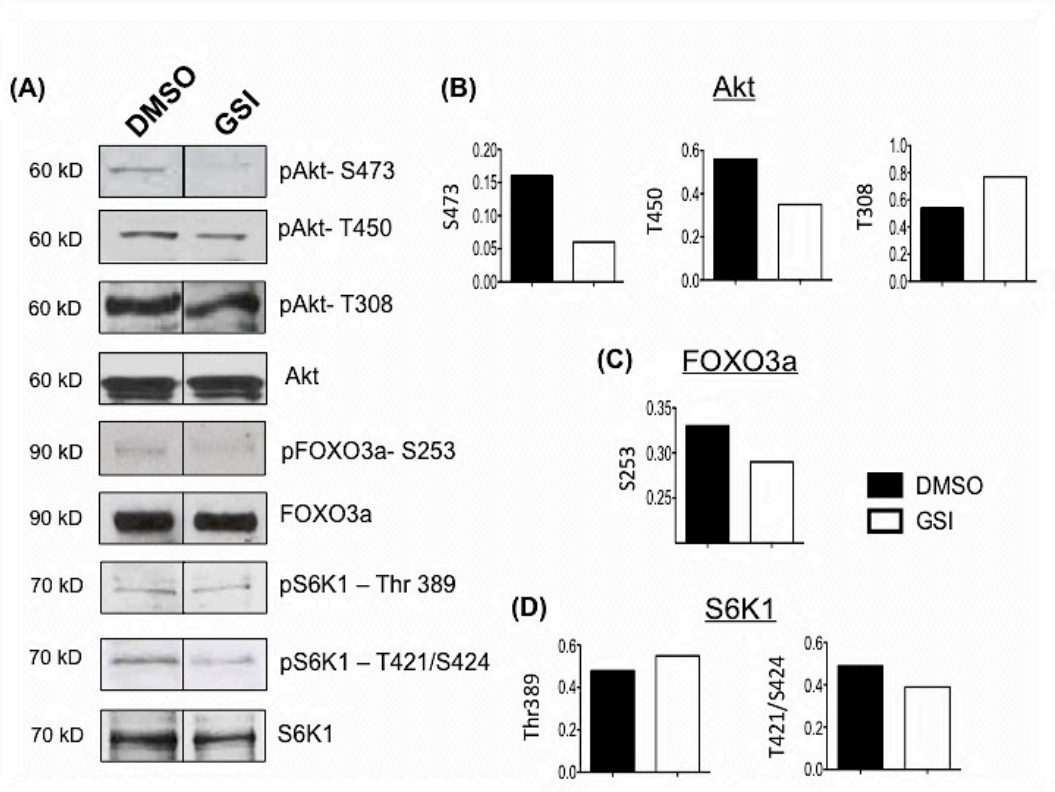


Fig 4.1 GSI treatment reduces mTORC2 activity

(A) Western Blot for mTORC1 and mTORC2 targets. $CD4^+$ T cells were isolated from C57Bl/6 mice and pre-treated with DMSO or 50μM GSI and activated with 1μg/ml of soluble anti-CD3ε and anti-CD28 cross-linked with anti-Hamster IgG. Cells lysates were made after 60 mins of stimulation and analyzed for the expression of the indicated phosphorylated proteins by Western blot. Integrated density values were obtained after normalizing each phosphorylated protein to the corresponding total protein. **(B-D)**Bar graph showing integrated density values for **(B)** Akt phosphorylated at S473, T450, T308 **(C)** Phospho FOXO3a – S253 **(D)** Phospho –S6K1 at Thr389 and T421/S424. Data represent three independent experiments.

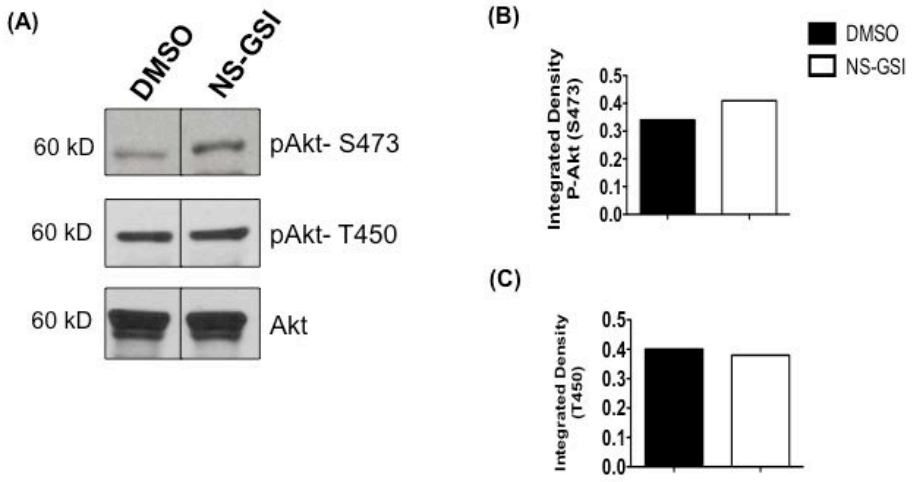


Fig 4.2 Treatment with a NS-GSI does not alter phosphorylation of Akt

Western Blot for mTORC2 targets. $CD4^+$ T cells were isolated from C57Bl/6 mice and pre-treated with DMSO or 1 μM NS-GSI and activated with 1μg/ml of soluble anti-CD3ε and anti-CD28 cross-linked with anti-Hamster IgG. Cells lysates were made after 60 mins of stimulation and analyzed for the expression of the indicated phosphorylated proteins by Western blot. Integrated density values were obtained after normalizing **(B)** S473 and **(C)** T450 to total Akt.

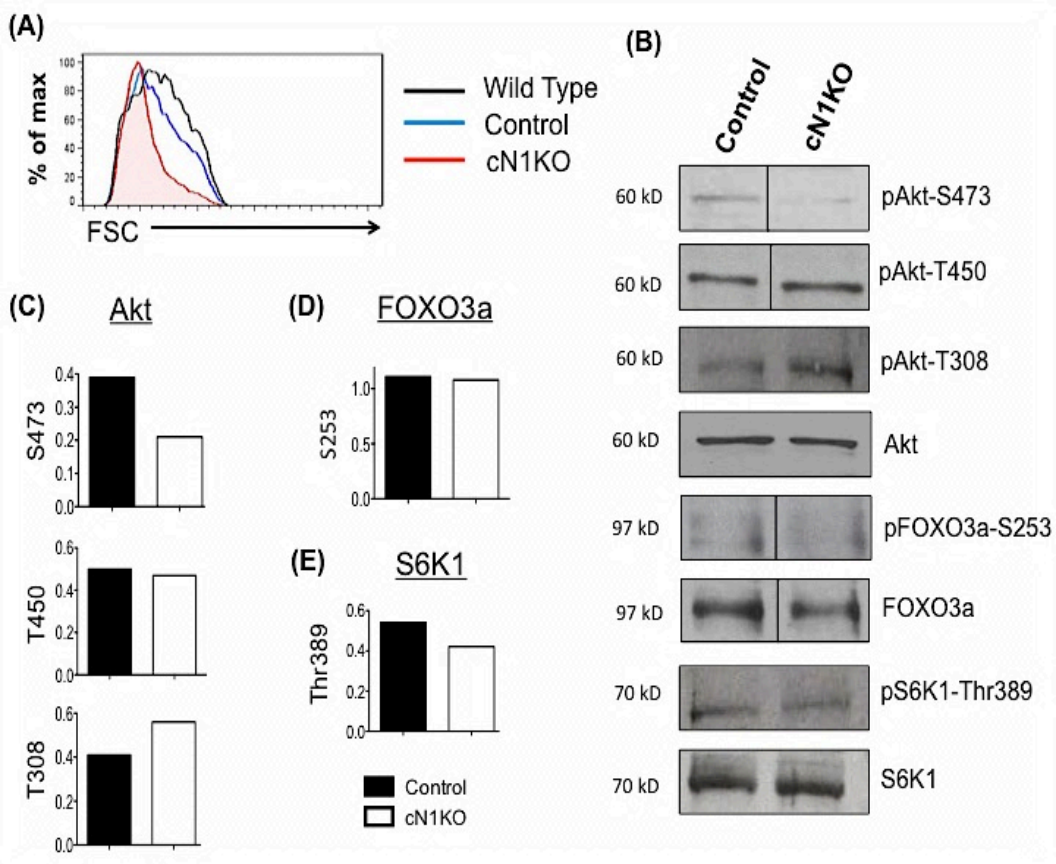


Fig 4.3 Notch1 is required for mTORC2 activity

(A) Histogram showing cell size as a measure of Forward Scatter (FSC). $CD4^+$ T cells were isolated from wild type C57Bl/6, control and cN1KO mice and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48 hrs and analyzed by flow cytometry. **(B)**Western Blot for mTORC1 and mTORC2 targets. $CD4^+$ T cells were isolated from control or cN1KO mice were activated with 1μg/ml of soluble anti-CD3ε and anti-CD28 cross-linked with anti-Hamster IgG. Cells lysates were made after 60 mins of stimulation and analyzed for the expression of the indicated phosphorylated proteins by Western blot. Integrated density values were obtained after normalizing each phosphorylated protein to the corresponding total protein. **(C-E)** Bar graph showing integrated density values for **(C)** Akt phosphorylated at S473, T450, T308 **(D)** Phospho FOXO3a – S253 **(E)** Phospho –S6K1 at Thr389. Data represent three independent experiments.

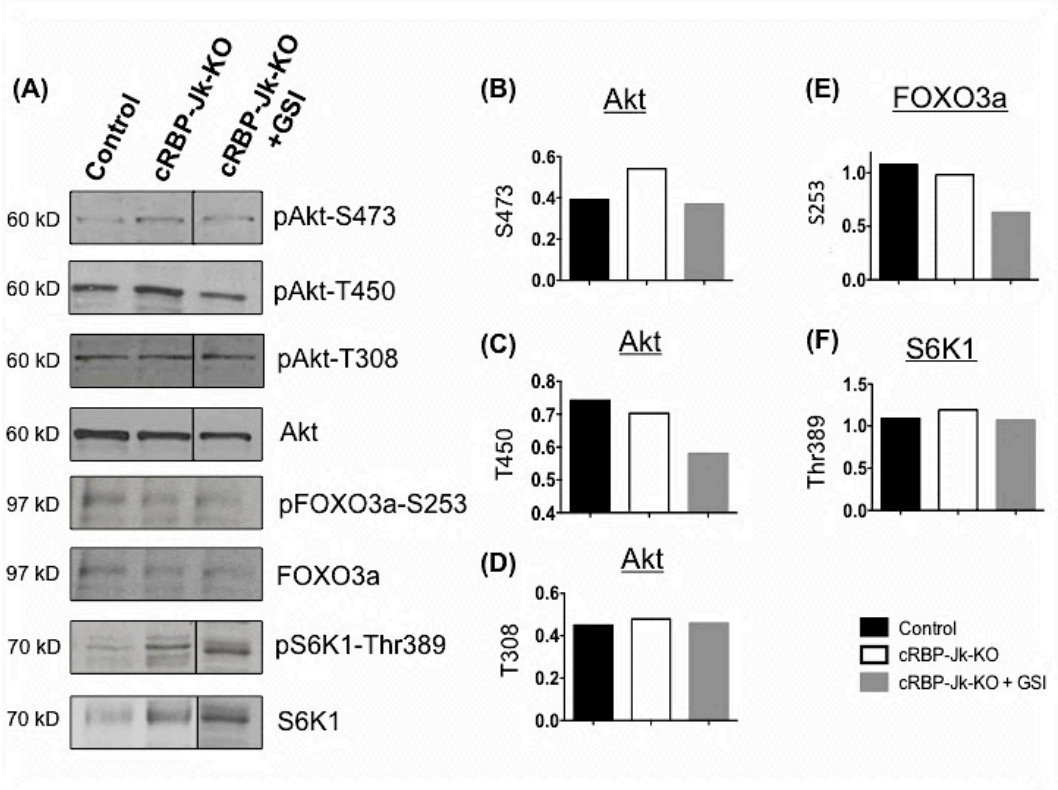


Fig 4.4 Regulation of mTORC2 by Notch1 occurs independently of RBP-J_K

(A) Western Blot for mTORC1 and mTORC2 targets. $CD4^+$ T cells were isolated from control or cRBP-J_K-KO and pre-treated with DMSO or 50μM GSI and activated with 1μg/ml of soluble anti-CD3ε and anti-CD28 cross-linked with anti-Hamster IgG. Cells lysates were made after 60 mins of stimulation and analyzed for the expression of the indicated phosphorylated proteins by Western blot. Integrated density values were obtained after normalizing each phosphorylated protein to the corresponding total protein. **(B-F)** Bar graph showing integrated density values for Akt phosphorylated at **(B)** S473, **(C)** T450, **(D)** T308 **(E)** Phospho FOXO3a – S253 **(F)** Phospho –S6K1 at Thr389. Data represent three independent experiments.

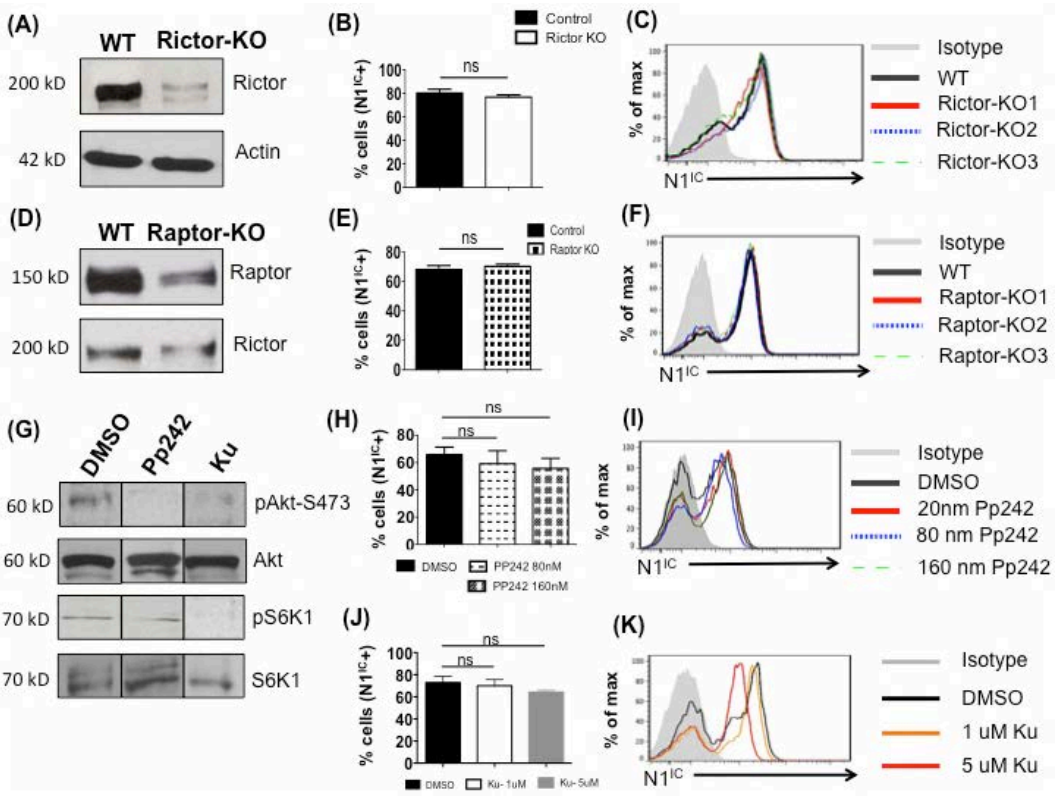


Fig 4.5 Notch1 acts upstream of mTOR

(A-D) Expression of $N1^{IC}$ in the absence of Rictor or Raptor. Spleens from wild type, Rictor-KO or Raptor-KO animals were obtained from the laboratory of Dr. David Sabatini, Whitehead Institute for Biomedical Research. $CD4^+$ T cells were isolated from these spleens and activated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48 hrs and split into two samples. One sample was used for detecting $N1^{IC}$ by flow cytometry. The other sample was used for making lysates for detecting Rictor or Raptor by Western blot. **(A)**Western Blot for **(B)** Rictor and **(D)** Raptor. **(B-E)** Percentage of cells positive for $N1^{IC}$. n = 3. **(C-F)** Histograms for $N1^{IC}$ expression. (G) $CD4^+$ T cells from C57Bl/6 mice were pretreated with DMSO, or 80 nm Pp242 or 1 μM Ku-0063764 and activated with 1μg/ml of soluble anti-CD3ε and anti-CD28 cross-linked with anti-Hamster IgG. Cells lysates were made after 60 mins of stimulation and analyzed for the expression of the indicated phosphorylated proteins by Western blot. (H-K) $CD4^+$ T cells from C57Bl/6 mice were pretreated with DMSO, or the indicated concentrations of Pp242 or Ku-0063764 and stimulated as described in (A). **(H-J)** Percentage of cells positive for $N1^{IC}$. n = 3-5. **(I-K)** Histograms for $N1^{IC}$ expression. Data represent three independent experiments.

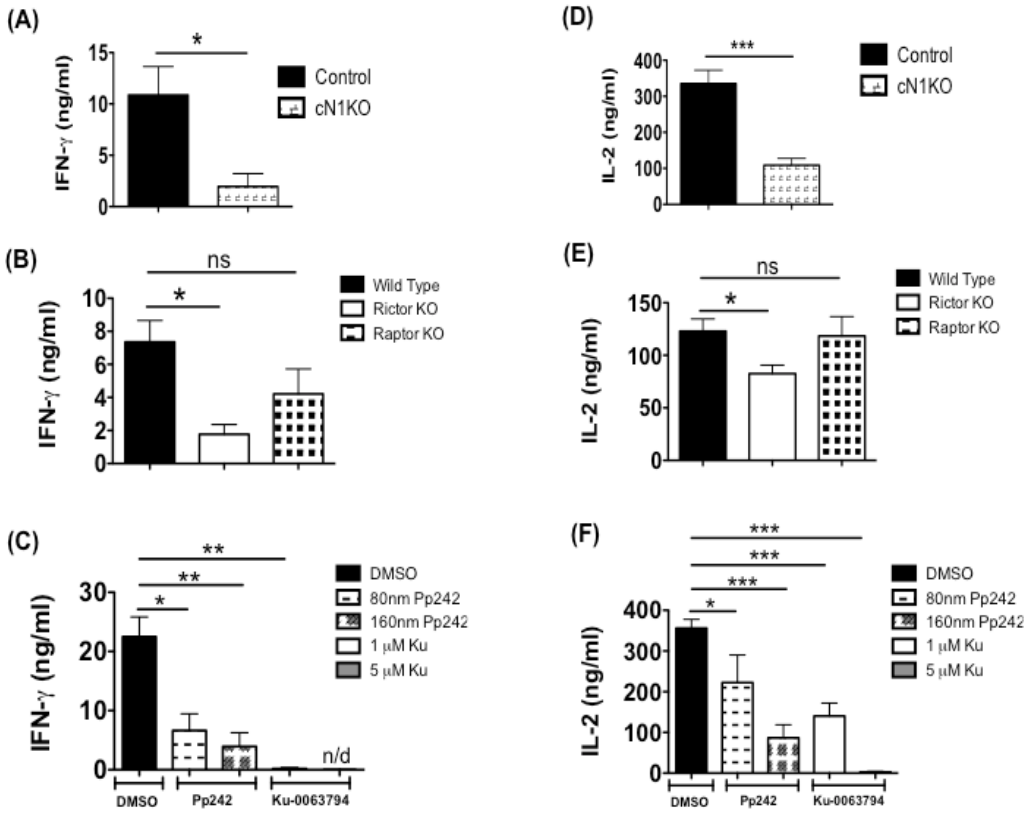


Fig 4.6 Notch may regulate IFN-γ and IL-2 production via mTOR

$CD4^+$ T cells were isolated from the spleens of **(A,D)** control and cN1KO mice **(B,E)** Wild type, Rictor-KO and Raptor-KO mice **(C,F)** C57Bl/6 mice pretreated with DMSO or the indicated concentrations of Pp242 or Ku, and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48 hrs and supernatants were used to detect **(A-C)** IFN-γ or **(D-F)** IL-2 by an ELISA. n= 3-7. Data represent the mean ± SEM, *, $p < 0.05$, ** $p < 0.005$, ***, $p < 0.001$, ns- not significant.

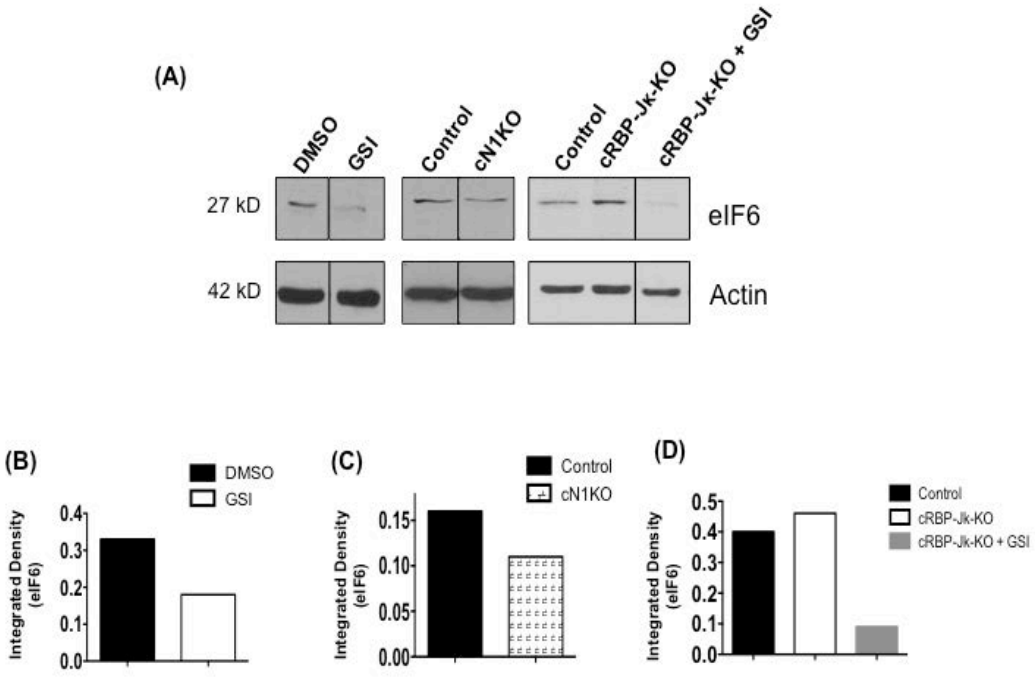


Fig 4.7 Notch1 is required for the expression of eIF6

(A) Western Blot for eIF6. $CD4^+$ T cells were isolated from the spleens of C57Bl/6 mice and pretreated with DMSO or GSI or from control, cN1KO and cRBP-J_K-KO mice and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48 hrs and used for making lysates for detecting eIF6 by western blot. **(B-D)** Integrated density plots for eIF6 after normalizing with actin. Data represent three independent experiments.

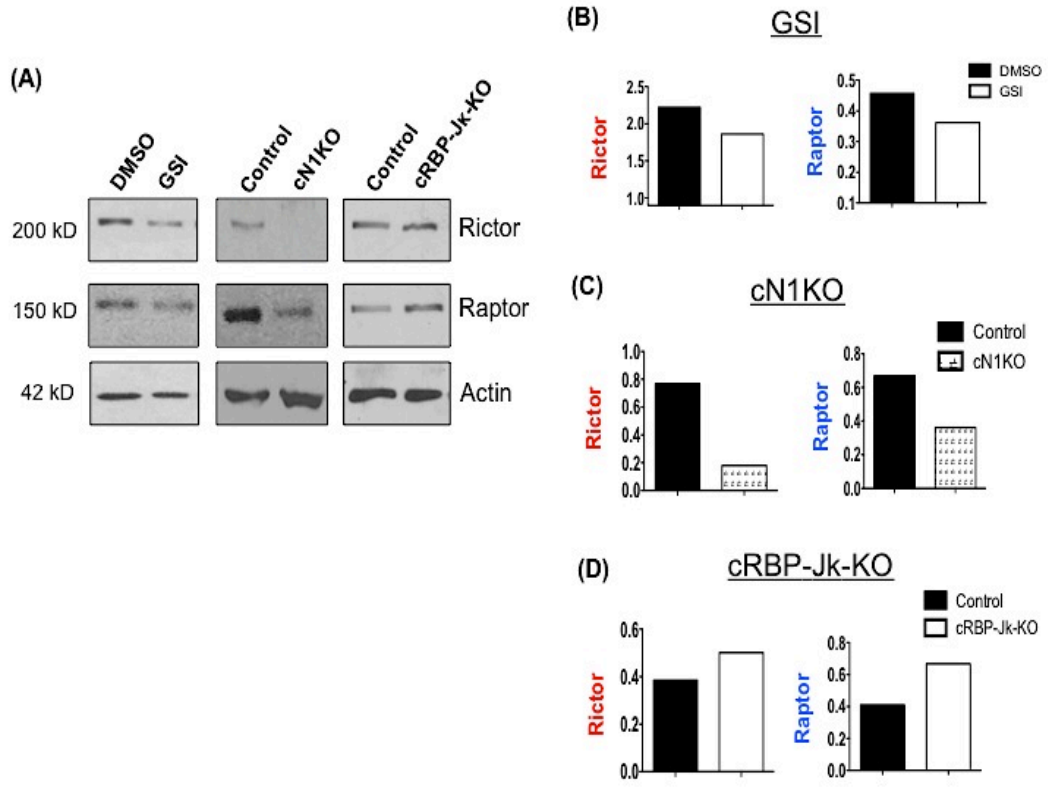


Fig 4.8 Notch1 is required for the expression of Rictor

(A) Western Blot for Rictor and Raptor. $CD4^+$ T cells were isolated from the spleens of C57Bl/6 mice and pretreated with DMSO or GSI or from control, cN1KO and cRBP-J_K-KO mice and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48 hrs and used for making lysates for detecting Rictor and Raptor by western blot. **(B-D)** Integrated density plots for Rictor after normalizing with actin. **(E-G)** Integrated density plots for Raptor after normalizing with actin. Data represent three independent experiments.

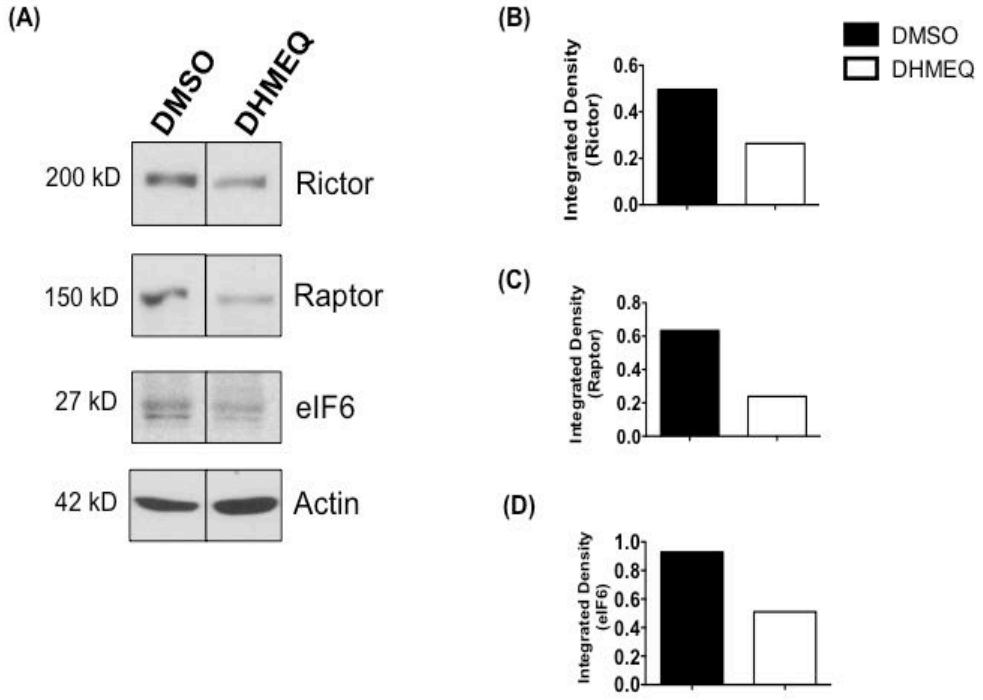


Fig 4.9 NF-κB is required for the expression of Rictor, Raptor and eIF6

(A) Western Blot for eIF6. $CD4^+$ T cells were isolated from the spleens of C57Bl/6 mice and pretreated with DMSO or DHMEQ and stimulated with plate bound anti-CD3ε and anti-CD28. Cells were harvested after 48 hrs and used for making lysates for detecting Rictor, Raptor and eIF6 by western blot. **(B-D)** Integrated density plots for **(B)** Rictor **(C)** Raptor **(D)** eIF6 after normalizing with actin. Data represent two independent experiments.

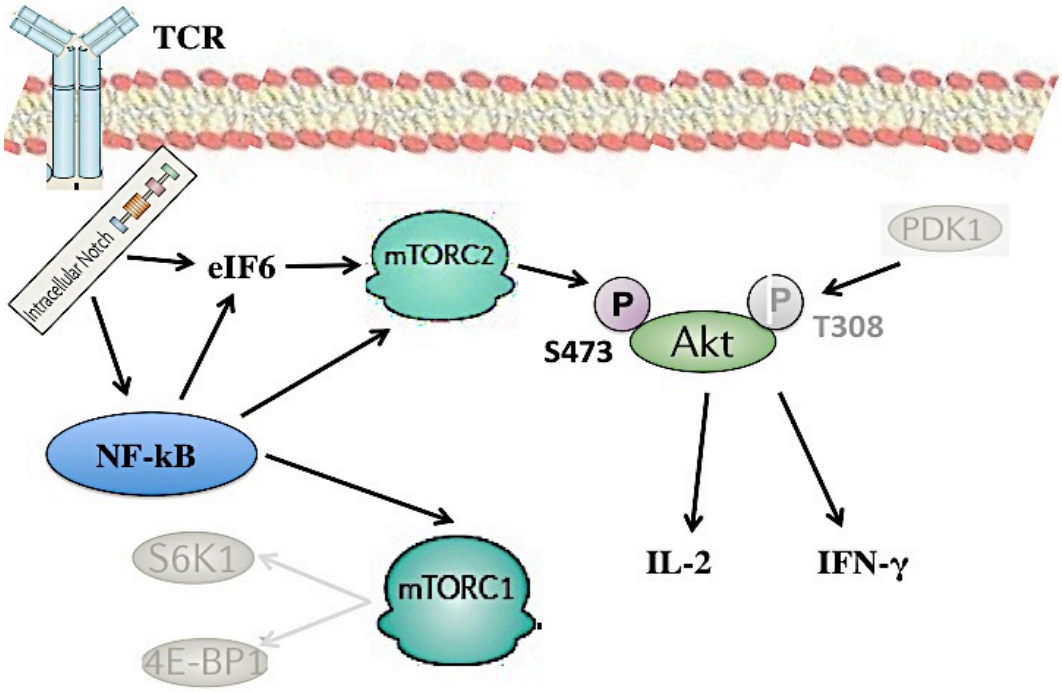


Fig 4.10 Model proposing Notch mediated regulation of mTORC2

Activation of T cells via the T cell receptor activates PI3K, which converts PIP2 to PIP3. Akt and PDK1 are recruited to the cell membrane. Akt gets phosphorylated at T308 by PDK1. Cross linking the TCR also activates $N1^{IC}$, which may control the expression of eIF6 which can in turn facilitate assembly of mTORC2 with the ribosome and its subsequence activation leading to phosphorylation of Akt at S473. Additionally, $N1^{IC}$ may also regulate the levels of eIF6, Rictor and Raptor via NF-κB.

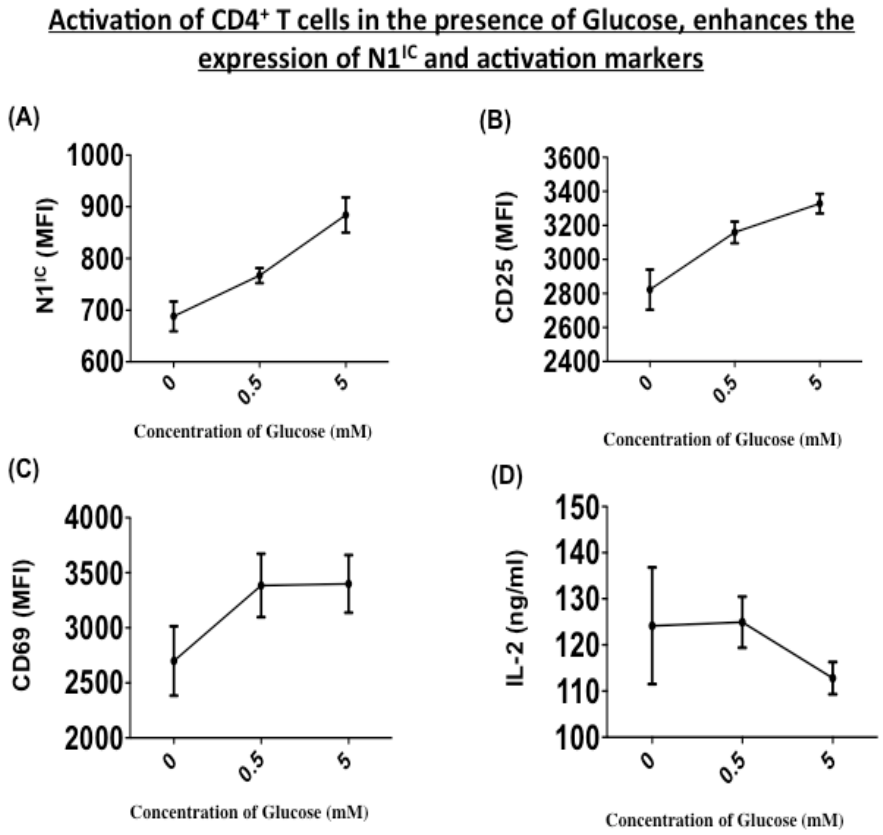


Fig 4.11 Glucose enhances $N1^{IC}$ production and T cell activation

Glucose has been shown to regulate T cell activation and proliferation. Since these processes are Notch dependent, we determined whether glucose could also regulate the levels of $N1^{IC}$ generated after TCR activation. We observed that increasing glucose concentrations did in fact increase the amount of $N1^{IC}$ (Fig 4.10A) as well as the amount of activation markers expressed by $CD4^+$ T cells (Fig 4.10C-D).

$CD4^+$ T cells from wild type C57Bl/6 mice were stimulated with plate bound α-CD3ε and α-CD28 in glucose free RPMI containing increasing concentrations of glucose. Cells were harvested after 24 hrs and stained by flow cytometry. Mean Fluorescent Intensity (MFI) values were plotted for **(A)** Intra-cellular Notch **(B)** CD25 **(C)** CD69 **(D)** Supernatants were used for an IL-2 ELISA.

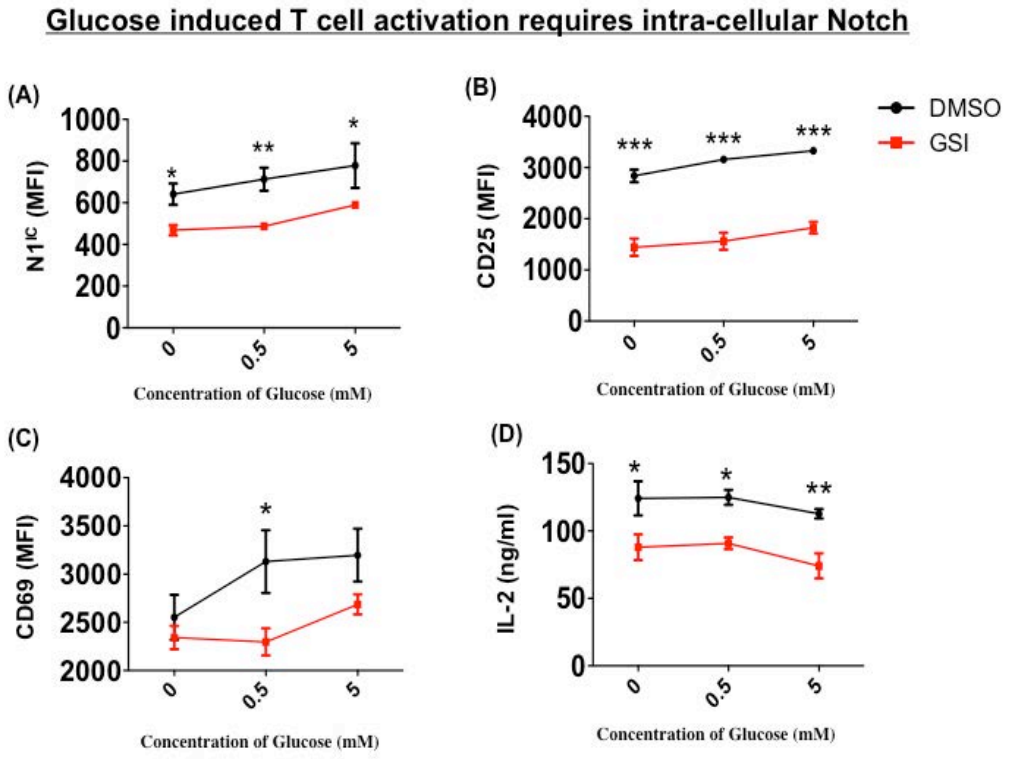


Fig 4.12 Enhanced T cell activation in the presence of Glucose requires activated Notch

To determine whether glucose induced activation of T cells was Notch dependent, we inhibited Notch activation via GSI treatment and asked whether T cells could respond to increasing concentrations of Glucose in the absence of Notch activation. We observed that GSI treatment significantly reduced the amount of $N1^{IC}$ (Fig 4.11A). Additionally, GSI treatment significantly reduced the expression of CD25, CD69 as well as levels of IL-2 even in the presence of glucose (Fig 4.11B-D). Thus glucose-induced activation of T cells requires Notch activation, as high concentrations of glucose cannot rescue activation in the absence of Notch signaling.

$CD4^+$ T cells from wild type C57Bl/6 mice were pretreated with DMSO or GSI and stimulated with plate bound α-CD3ε and α-CD28 in glucose free RPMI containing increasing concentrations of glucose. Cells were harvested after 24 hrs and stained by flow cytometry. Mean Fluorescent Intensity (MFI) values were plotted for **(A)** Intra-cellular Notch **(B)** CD25 **(C)** CD69 **(D)** Supernatants were used for an IL-2 ELISA.

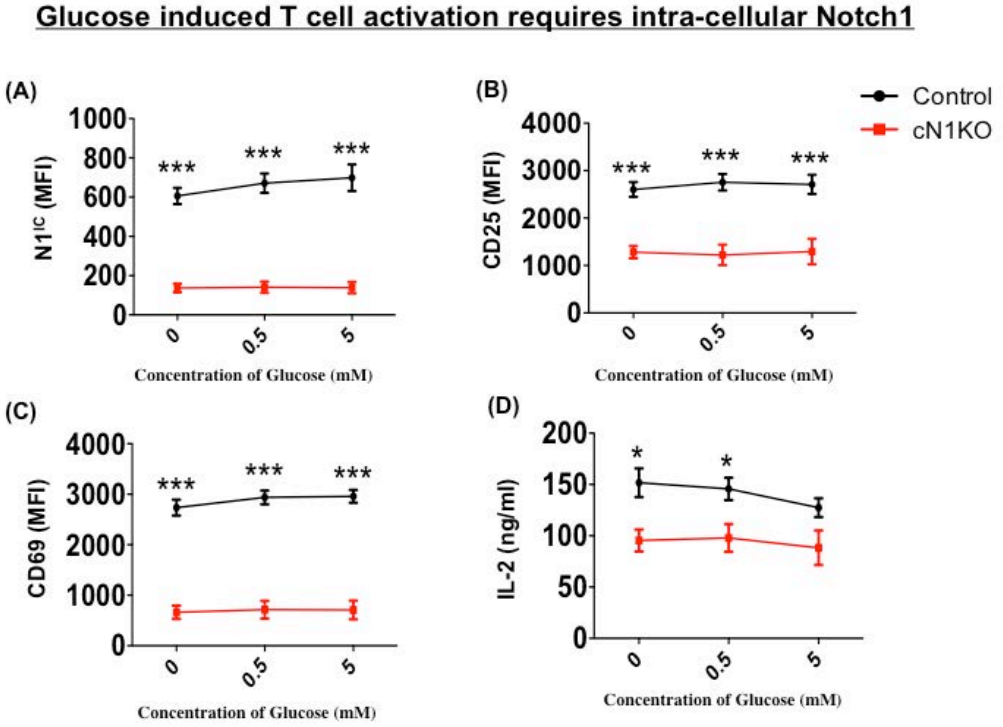


Fig 4.13 Enhanced T cell activation in the presence of Glucose requires activated Notch1

To determine whether glucose induced activation of T cells was Notch1 dependent and not a GSI effect, we activated T cells from control and cN1KO animals with increasing concentrations of Glucose. We observed that CD4+ T cells from cN1KO animals expressed significantly lower levels of $N1^{IC}$ compared to cells from control mice (Fig 4.12A). Additionally, these cells also expressed significantly lower levels of CD25, CD69 and IL-2 even in the presence of glucose (Fig 4.12B-D). Thus glucose-induced activation of T cells specifically requires Notch1.

$CD4^+$ T cells from control and cN1KO animals were stimulated with plate bound α-CD3ε and α-CD28 in glucose free RPMI containing increasing concentrations of glucose. Cells were harvested after 24 hrs and stained by flow cytometry. Mean Fluorescent Intensity (MFI) values were plotted for **(A)** Intra-cellular Notch **(B)** CD25 **(C)** CD69 **(D)** Supernatants were used for an IL-2 ELISA.

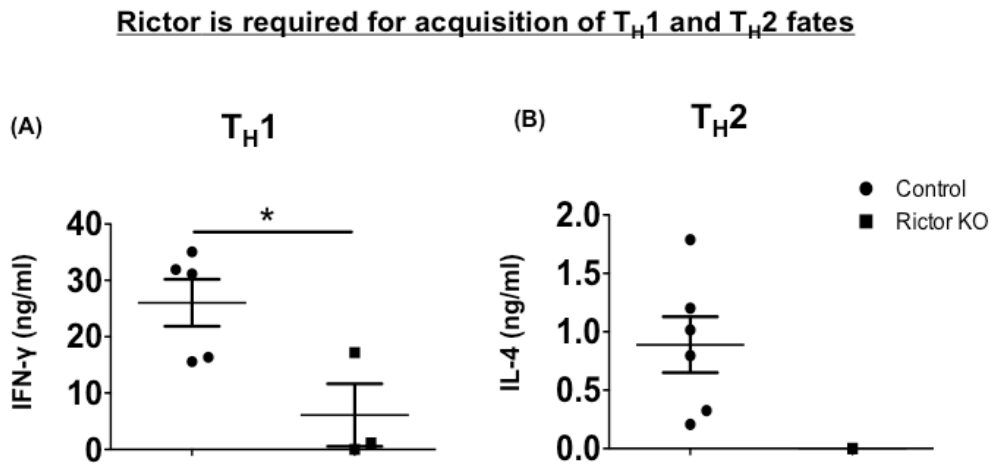


Fig 4.14 Rictor is required for acquisition of T_H1 and T_H2 fate

To confirm the contribution of mTORC2 in regulating helper T cell differentiation, we determined whether $CD4^+$ T cells that lacked Rictor could differentiate to the T_H1 and T_H2 fate. We observed that in the absence of Rictor, polarization to both these lineages was abrogated confirming that Rictor does indeed control specification of helper T cells to both these lineages.

$CD4^+$ T cells from control or Rictor-KO mice were stimulated with plate bound α-CD3ε and α-CD28 under T_H1 or T_H2 conditions. Cells were harvested after 72 hrs and cytokines in the supernatants were used to detect **(A)** IFN-γ and **(B)** IL-4 by an ELISA.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The function of Notch in regulating activation and differentiation of peripheral $CD4^+$ T cells has been well defined (Radtke *et al.,* 2010). In this study we specifically demonstrate the requirement of non-canonical Notch signaling for regulating activation of $CD4^+$ T cells as well as their differentiation to the T_H1 and *i*Treg lineages. We also underline the mechanism of such non-canonical Notch signaling by showing cross-talk with the NF-κB and mTOR pathways. Although it was known that ligation of the TCR triggers generation of $N1^{IC}$ in peripheral T cells, the mechanism of such Notch activation remained obscure. In this study we demonstrate that Notch ligands are expressed on $CD4^+$ T cells only 12 hrs after activation of the TCR, which is also the same time at which $N1^{IC}$ is detected. Thus it is likely that Notch generated downstream of the TCR may control the expression of Notch ligands, which could in turn support more Notch activation. It may also be possible that that activation of Notch downstream of the TCR, could be ligand in-dependent. Determining the precise role of Notch ligands on triggering Notch activation would require a sequential deletion of each ligand either individually or in combination. While our data do not support a role for ligand-dependent Notch activation, our data show that Notch ligands can indeed influence the amount of $N1^{IC}$ generated downstream of the TCR. DLL1 ligands enhanced $N1^{IC}$ production. Furthermore this increase was paralleled by a

concomitant increase in the production of IFN-γ under T_H1 conditions. Thus our data suggest that Notch ligands could be one way in which Notch dictates T_H1 versus T_H2 fates.

Another mechanism by which Notch exerts differential effects on helper T cell differentiation involves the use of an alternate, RBP-J_K independent pathway. Our data suggest that NF-κB and mTOR relay non-canonical, cytosolic, Notch signals. We show that inhibition of NF-κB via DHMEQ treatment can dampen activation as well as differentiation to T_H1 fate in the absence of RBP-J_K. A more specific approach to determine the contribution of NF-κB to these processes would be to breed cN1KO animals to animals over-expressing IKK and determining if constitutive activation of NF-κB can rescue the defect in activation and differentiation seen in the absence of Notch1. Furthermore, to determine if decreased activation and differentiation seen in the absence of Notch1 is directly dependent on Akt/mTORC2, one could determine if constitutively active Akt can rescue the small cell size, dampened activation and differentiation observed in the absence of Notch1.

To determine the precise interplay between Notch, NF-κB and components of the mTOR pathway, it would be useful to determine if NF-κB and/or RBP-J_K binding sites can be found upstream of the eIF6, Rictor and Raptor promoters. More importantly, it would also be insightful to see if complexes of $N1^{IC}$ and NF-κB can be co-immunoprecipitated from these promoters. If these complexes can be found even in the absence of RBP-J_K binding sites, then such

observations would strengthen the role of NF-κB – dependent, non-canonical Notch signaling.

In conclusion, our data suggest that the pleiotropic effects of Notch are due its ability to propagate its signals by interaction with several other RBP-J_K independent pathways. These observations have an important positive impact for therapeutic targeting of the pathway, because manipulation of different components of the Notch pathway can have radically different outcomes

CHAPTER 6

MATERIALS AND METHODS

6.1 Materials

6.1.1 Mice

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-J_K-KO mice were generated by breeding *Notch1*$^{fl/fl}$ (*notch1*$^{tm2Rko/GridJ}$) or *Rbp-j*$_k^{fl/fl}$ (*Rbpj* ^{tm1Hon}) 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 (cN1KO), *Notch1* $^{fl/fl}$ x Mx Cre $^{-/-}$ (Control), *RBP-Jκ* $^{fl/fl}$ x Mx Cre $^{+/-}$ (cRBP-Jκ -KO) and *RBP-Jκ* $^{fl/fl}$ 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. Spleens from Rictor – KO, Raptor – KO and their corresponding control mice were obtained from Dudley Lamming and David Sabatini, Whitehead Institute for Biomedical Research, Cambridge, MA. Mice aged 7-12 weeks were used for all experiments

6.1.2 Media

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).

6.1.3 Antibodies

The following antibodies were used for Western blots. Primary Antibodies: anti-RBPSUH, anti-phospho Zap 70 (Y319), anti-Zap70, anti-HDAC, anti-phospho Akt (S473), anti-phospho Akt (T450), anti-phospho Akt (T308), anti-Akt, anti- phospho FOXO3a (S253), anti-FOXO3a, anti- phospho p70S6K1 (Thr389), anti phospho p70S6K1 (T421/S424), anti p70S6K1, anti-eIF6, anti-Rictor, anti-Raptor (Cell signaling), anti-cRel (Santa Cruz Biotechnology) anti-Actin (Sigma), anti-cMyc (9E10) was obtained from Dr. Dominique Alfandari. Secondary Antibodies: anti-Rabbit-HRP, anti-Mouse-HRP (Amersham). The following antibodies were used for flow cytometry. CD25-APC, CD69-FITC, 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, anti-Mouse DLL1 Alexa Fluor 488 (eBioscience), CD4-PerCP, Streptavidin PerCP, anti-Mouse IFN-γ FITC, anti-Mouse IL-4 PE, anti-CD3ε, anti-CD28 (BD Pharmingen), Rat Jagged1 Antibody, Donkey anti-goat IgG-APC (R&D systems), purified anti-mouse DLL4 (BioLegend), Jagged1 antibody (GeneTex, Inc.). PE

Annexin V Apoptosis Detection Kit I was from BD Pharmingen. The following antibodies were used for the ELISA assay. Capture antibodies purified rat anti-mouse IL-2, purified rat anti-mouse IFN-γ, purified rat anti-mouse IL-4, as well as detection antibodies biotin rat anti-mouse IL-2, biotin rat anti-mouse IFN-γ, biotin rat anti-mouse IL-4, Streptavidin HRP.

6.1.4 Chemical Reagents and Commercial kits

The γ-secretase inhibitor z-IL-CHO and DHMEQ were synthesized by Abdul H. Fauq. The Notch sparing GSI JLK-6 was from Tocris Bioscience. Recombinant IL-2, IFN-γ and IL-4 standards and the TMB Substrate reagent were from BD Pharmingen. Nucleur and cytoplasmic protein extraction kit, BCA assay reagents and Halt ™ Phosphatase Inhibitor Cocktail were from Thermo Scientific. 30% Acrylamide was from BioRad. ECL reagents were from Amersham. The FoxP3 kit for intra-cellular staining was from eBioscience. Intra-cellular cytokines were detected by using the kit for intra-cellular cytokine staining from BD Pharmingen. The Cell Trace CFSE staining kit was from Life Technologies. Mitomycin C was from Sigma. Ova peptide 323-339 was from GenScript. Reagents for polarization - recombinant IL-12, recombinant IL-4, anti-mouse NA/LE IFN-γ and anti-mouse NA/LE IL-4 were from BD Pharmingen. Poly (I):Poly (C) was from Amersham, Imgenex and Invivogen. Inhibitors for mTOR – Pp242 and Ku-0063794 were from Selleckchem. Tritiated thymidine used for proliferation assays was from Perkin Elmer.

6.2 Methods

6.2.1 T cell isolation and *in vitro* polarization 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 10^6 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. T_H1: 10 μg/ml anti-IL-4 and 1 ng/ml recombinant mouse IL-12 (BD Pharmingen). T_H2: 10 μg/ml anti-IFN-γ and 1 ng/ml of recombinant mouse IL-4 (BD Pharmingen). Cells were harvested after 72 hrs of stimulation and re-stimulated with plate bound anti-CD3ε in the presence or absence of Golgi Plug (IFN-γ) or Golgi Stop (IL-4). Cells were harvested after 5hrs and used for detection of intra-cellular cytokines by flow cytometery. Supernatants from cells stimulated without Golgi Plug or Stop were used for detecting cytokine levels by ELISA.

6.2.2 Proliferation Assays

For thymidine uptake assays, 3 x 10^5 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 ^{3}H-thymidine (Perkin Elmer) at a final concentration of 1μc*i*/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. Cells were stimulated for 48 hrs before analysis by flow cytometry.

6.2.3 Drug Treatments

For drug treatments, $CD4^+$ T cells were pretreated with 50 μM of the GSI z-IL-CHO, 5 μM NS-GSI JLK-6, 1.5 μM of DHMEQ, 80nm and 160nm Pp242, 1 μM and 5 μM of Ku-0063794 at 37ºC for 30 minutes before activation.

6.2.4 Co-culture experiments using CHO-APCs

Chinese Hamster Ovary - Antigen presenting cells (CHO-APCs) expressing DLL1, Jagged1 or Empty vector were fixed by treating with Mitomycin C at 37ºC for 45 mins. 6 x 10^5 cells/ml of CHO-APCs were pulsed with the indicated concentrations of Ova peptide 323-339 for strength of signal

assays, or 2.5 μg/ml ova-peptide for activation and differentiation experiments. $CD4^+$ T cells were isolated from spleens of BALB/c-Tg(DO11.10)10Loh/J and mixed with peptide pulsed APCs at 2.5 x 10^6 cells/ml in each well of a 12 well plate. For polarization experiments, polarizing reagents were added as described in section 6.2.1. Cells were harvested after 48 hrs for strength of signal assays and for detection of activation markers. Cells were harvested after 96 hrs for differentiation assays and re-stimulated with plate-bound anti- anti-CD3ε for 5 hrs. Cells were analyzed by flow cytometry after gating on $CD4^+$ T cells expressing the DO.11.10 TCR. Supernatants were used for detection of cytokines by an ELISA

6.2.5 Strength of signal assays

$CD4^+$ T cells were isolated from the spleens of C57BL/6J as described and stimulated with the indicated concentrations of commercial anti-CD3ε and 1 μg/ml of commercial anti-CD28 for 48 hrs. Cells were analyzed by flow cytometry and IL-2 was detected in the supernatants by an ELISA. For strength of signal experiments using CHO-APCs, co-culture experiments were performed. Empty vector Chinese Hamster Ovary – Antigen presenting cells (CHO-APCs) were fixed by treating with Mitomycin C at 37ºC for 45 mins. 6 x 10^5 cells/ml of CHO-APCs were pulsed with the indicated concentrations of Ova peptide 323-339. $CD4^+$ T cells were isolated from spleens of BALB/c-Tg(DO11.10)10Loh/J and mixed with peptide pulsed APCs at 2.5 x 10^6 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

6.2.6 Stimulating T cells for detection of phosphorylated proteins

$CD4^+$ T cells were adjusted to a final concentration of 3 x 10^6 cells/ml and aliquoted into 1ml eppendorf tubes. (2-3 tubes were used for each condition. Example: for lysates made from control and KO mice, 3 individual tubes of cells were used for control and KO mice respectively). 1 μg/ml of commercial anti-CD3ε and 1 μg/ml of commercial anti-CD28 were added to each tube containing 1 ml of cells. Cells were mixed with the antibody by end over end rotation for a few minutes and incubated on ice for 15 mins. 4.5 μg/ml of cross-linking anti-Hamster IgG was added to each tube. Once again, this was suspended by end over end rotation followed by incubation on ice for 15 mins. Cells were stimulated in a water bath at 37C for 45 mins. Cells were immediately transferred on ice at the end of 45 mins and centrifuged at 3000rpm for 5 mins. All cells for the same condition were then pooled into a single tube, washed with PBS and re-suspended in 40 μl of RIPA buffer containing Pefabloc and 1X phosphatase inhibitor cocktail.

6.2.7 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 nucleur 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.

6.2.8 Flow cytometry

Surface staining was performed in PBS with 1% BSA. Intra-cellular staining was performed for detection of intra-cellular Notch1, T-bet, GATA3 and FoxP3 using the FoxP3 staining buffer set. 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. 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.

6.2.9 Statistical Analysis

All data are represented as mean +/- SEM. Statistical Analysis was performed using GraphPad Prism 5 software. P values were calculated using an unpaired two-tailed student's *t* test.

Table 6.1 Primers used for qRT-PCR

SYMBOL	5′ PRIMER SEQUENCE	3′ PRIMER SEQUENCE
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Rbpj	ATGCCCTCCGGTTTTCCTC	GGACAAGCCCTCCGAGTAGT
Notch1	CCCTTGCTCTGCCTAACGC	GGAGTCCTGGCATCGTTGG
Notch2	ATGTGGACGAGTGTCTGTTGC	GGAAGCATAGGCACAGTCATC
Notch3	TGCCAGAGTTCAGTGGTGG	CACAGGCAAATCGGCCATC
Ifng	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Tbox21	CCAGGAAGTTTCATTTGGGAAGC	ACGTGTGTGTTAGAAGCACTG
Hes5	AGTCCCAAGGAGAAAAACCGA	GCTGTGTTTCAGGTAGCTGAC
c-Myc	GGACAGTGTTCTCTGCC	CGTCGCAGATGAAATAGG

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